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Contents

Research Papers

Methods and Techniques

Journal of Life Sciences 5 (2011) 1-6

The Human P_{5B}-ATPase ATP13A2 Is Not a Ca^{2+} Transporting Pump

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Abstract: The human gene ATP13A2 has been proposed to code for an ATP powered ion transporter of the P_{5B} subfamily. Mutations of the human gene ATP13A2 were found to underlie an autosomal recessive form of early-onset parkinsonism (PD) with pyramidal degeneration and dementia. The ion transported by the ATP13A2 pump is not known, but several studies have shown that the P_5-ATPases influence the homeostasis of intracellular Ca^{2+}, and thus it has been suggested that they transport Ca^{2+}. In order to evaluate this possibility Chinese hamster ovary (CHO) cells stably expressing the human ATP13A2 protein have been obtained and the Ca^{2+} transport activity of ATP13A2 was assessed by measuring the ATP-dependent uptake of Ca^{2+} into microsomal vesicles. As a positive control vesicles containing the human plasma membrane Ca^{2+} pump (PMCA) were used. No significant differences were found between vesicles containing the ATP13A2 protein and the control. Moreover, Ca^{2+} was unable to induce the formation of the P-ATPase acylphosphate intermediate in vesicles containing the expressed ATP13A2. These results favor the idea that the ATP13A2 does not transport Ca^{2+}.

Key words: P_{5B}-ATP13A2, calcium uptake, CHO cells overexpression.

Abbreviations: PMCA: plasma membrane Ca^{2+} pump; CHO cells: Chinese hamster ovary cells.

1. Introduction

The P-type superfamily of ion pumps includes transporters energized by the hydrolysis of ATP that transport inorganic cations and other substrates across cell membranes. These P-type ATPases are characterized by the formation of a phosphorylated intermediate during their reaction cycle. They are present in prokaryotes and eukaryotes, and in the basis of their conserved core sequences they have been classified into five subfamilies termed P_1-P_5 or type I-V. The most poorly understood P-type ATPases are those of the P_5 subfamily, which are expressed only in eukaryotes. The P_5-ATPases have been divided in two subfamilies termed P_{5A} and P_{5B} based on protein alignments [1]. Both subfamilies harbor the phosphorylation site (DKTGTLT) found in all P-type ATPases, and the main difference between them resides in the predicted sixth transmembrane segment (M6) which is presumably involved in forming the ion binding site. The predominant M6 sequence motif of the P_{5A} pumps is PP(D/E)LPxE, while the members of the P_{5B} subfamily have a conserved PP(A/V)LPAx sequence motif. Five genes named ATP13A1-ATP13A5 that belong to this group of P_5-ATPases have been identified in humans. Only ATP13A1 belongs to the subgroup A and the others to the subgroup B [2]. The study of these P_5-ATPases has a great interest since they were linked with several neurologic disorders. Loss of function mutations of the human gene ATP13A2 was found to underlie an autosomal recessive form of early-onset parkinsonism with pyramidal degeneration and dementia (Kufor-Rakeb syndrome) [3]. Furthermore the interruption by inversion of the long arm of

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chromosome 3 in the human gene ATP13A4 was found in patients with autism spectrum disorder (ASD) and specific language impairment (SLI) [4].

The ion specificity of the P_5-ATPases is unknown, and recent publications suggest that they affect the intracellular level of different cations [5-8]. The yeast P_{5A}-ATPase Cod1p, in collaboration with the Golgi Ca^{2+} pump Pmr1p, was suggested to supply calcium to the yeast ER. However, the function of Cod1p in cellular Ca^{2+} homeostasis is not equivalent to, nor redundant with that of Pmr1p. Moreover, it was shown that the phenotype of mutant yeast cells lacking Cod1p (cod1Δ) could be partially suppressed by exogenous calcium [9]. Deletion of Cod1p alone did not affect cellular calcium level, but deletion of both Cod1p and Pmr1p produced a synergistic increase in the intracellular calcium level compared with pmr1Δ alone [5]. In addition Cod1p has been shown to be involved in mechanisms that depend on the ER Ca^{2+} concentration like glycosylation of proteins in the secretory pathway, protein insertion orientation, and regulation of HMG-CoA reductase degradation [5, 9-11]. The idea of Ca^{2+} pumping P_5-ATPases is favored by a recently publication which showed that the over-expression of human ATP13A4 in COS-7 cells increased the intracellular calcium level [8].

Here, the ability of the human ATP13A2 to transport Ca^{2+} has been examined under conditions which are optimal for the function of other well known Ca^{2+} pumps. For this purpose CHO cells were stably transfected with the ATP13A2 cDNA, and clones expressing the ATP13A2 protein were isolated. It was found that the expressed ATP13A2 not only lacks any Ca^{2+} transport activity but also unable to promote the formation of the catalytic phosphoenzyme from ATP.

2. Material and Methods

2.1 Materials

Reagents were purchased from the following companies: ^{45}Ca and [γ-^{32}P]ATP, PerkinElmer Life Sciences; Immobilon transfer membranes and nitrocellulose filters, Millipore; immunochemicals, Invitrogen, Molecular Probes, Vector Laboratories and Amersham Biosciences; and reagents for cell culture, thapsigargin, and other chemicals, Sigma. The expression vector pcDNA3.1 carrying the V5-tagged human ATP13A2 cDNA was a generous gift of Drs. Alfredo Ramirez and Christian Kubisch, Institute of Human Genetics, University of Bonn, Germany.

2.2 Protein Expression and Isolation of Cellular Membranes

Stable CHO cell lines expressing the recombinant PMCA were described previously [12]. CHO cells were lipofected with the expression vector pcDNA3.1 carrying the V5-tagged human ATP13A2 cDNA using lipoafectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. To express in a stable form the recombinant ATP13A2, the transfected CHO cells were split into dishes of 10 cm in diameter 24 h post-transfection. 24 hours later the transfected cells were cultured in a selective DMEM medium supplemented with antibiotics and 10% of dialyzed fetal calf serum containing the antibiotic G418 at a final concentration of 600 μg/ml. After 3 weeks about 6-8 of the resulting colonies were cloned and expanded, and the expression of the pump was investigated by immunoblotting. The crude microsomal membrane fractions and the erythrocyte inside-out membranes were prepared by the procedure of Enyedi et al. [13] and Sarkadi et al. [14], respectively. Protein concentration was estimated by means of the Bio-Rad protein assay, with bovine serum albumin as a standard. The amount of the expressed protein was estimated by quantitation of the band intensity using the Gel Pro Analyzer 3.0 program (version 3.1 for Windows™, Media Cybernetics).

2.3 Detection of the Human ATP13A2 Protein

For immunofluorescence, the stable transfected cells were cultured on glass multiwell plates for 24-48 h. The cells were then washed twice with PBS and fixed in 4% formaldehyde in PBS for 15 min on ice.

After three washes with PBS the cells were permeabilized with 0.15% Tween 20 in PBS (PBST) for 5 min on ice. The recombinant ATP13A2 was detected with antibody to V5 (Invitrogen) at a dilution of 1:500 in PBST by incubation over night at 4 ℃. After washing the cells three times with PBST, the anti-V5 was labeled by Zenon Alexa Fluor 568 mouse IgG2a (Molecular Probes) according to the manufacturer's instructions for two hours at room temperature. Then the cells were washed three times with PBST and after a single wash with PBS the cells were covered by 50 μl of PBS and observed in a confocal microscope FluoView 1000 (Olympus, Japan) with an appropriate Alexa Fluor 568 filter.

SDS-PAGE and immunoblotting were carried out as described previously [15]. Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli [16] and subsequently transferred to Millipore Immobilon membranes. The membranes were incubated over night at 4 ℃ with V5 monoclonal antibody (Invitrogen) according to the manufacturer's protocol. For staining, biotinylated anti-mouse immunoglobulin G and avidin-streptoavidin peroxidase conjugate were used.

2.4 Ca^{2+} Transport Assay

Ca^{2+} uptake assays were performed as described previously [12]. The reaction mixture contained 100 mM KCl, 50 mM Tris-HCl (pH 7.3 at 37 ℃), 5 mM NaN_3, 0.1 μM thapsigargin, 4 μg/ml oligomycin, 20 mM sodium phosphate, 1.5 mM ATP, 95 μM EGTA, 2.5 mM $MgCl_2$ and $CaCl_2$ (labeled with ^{45}Ca) to give the desired concentration of free Ca^{2+}. The free concentrations of Ca^{2+} were calculated using the program of Fabiato and Fabiato [17]. Vesicles (10 μg of protein) were preincubated at 37 ℃ for 5 min, and the reaction was initiated by the addition of ATP. The reaction was finished after 5 min by filtering the samples through a 0.45 μm filter. The ^{45}Ca taken up by the vesicles was determined by counting in a scintillation counter.

2.5 Detection of the Phosphorylated Intermediate

The phosphorylation reaction was carried out at 4 ℃ in a medium containing 30 g of microsomal protein, 160 mM KCl, 25 mM Tris-HCl (pH 7.0 at 4 ℃) and 4 μM thapsigargin to obtain a full inhibition of SERCA pump in a reaction volume of 0.25 ml; 0.15 mM $CaCl_2$ was added when indicated. The reaction was initiated by the addition of 1 μM [γ-^{32}P]ATP and terminated after 1 min with 15 μl of a solution containing 100% trichloroacetic acid. The precipitated proteins were dissolved in sample buffer and separated by SDS-PAGE in a 7% acrylamide gel according to Sarkadi et al. [14]. After drying the gel, they were exposed to a storage phosphor screen for 1 day and imaged using a *Storm 840* Optical Scanner.

3. Results

3.1 Expression of the Recombinant ATP13A2 Protein

CHO cells were transfected with the pcDNA3.1 expression vector carrying the human ATP13A2 cDNA and stable clones were selected by their resistance to the antibiotic G418. Immunofluorescence experiments showed that the isolated clones successfully expressed the ATP13A2 protein (Fig. 1a). Microsomal membranes from transfected cells were isolated and submitted to SDS-PAGE and immunobloting. The expressed ATP13A2 had the expected migration according to its predicted size of 129 kDa, and judged by the intensity of the bands, it accounted for about 1% of the microsomal protein (Fig. 1b). Thus, the expression level of ATP13A2 was similar to that reached by other P-type ATPases in the same expression system [18].

3.2 Ca^{2+} Transport Assays

In order to assess the Ca^{2+} transport activity of the expressed ATP13A2 the ATP dependent $^{45}Ca^{2+}$ transport into microsomal vesicles was measured as a function of increasing Ca^{2+} concentrations. As a positive control, the Ca^{2+} transport activity of the plasma membrane Ca^{2+} ATPase (PMCA) was simultaneously

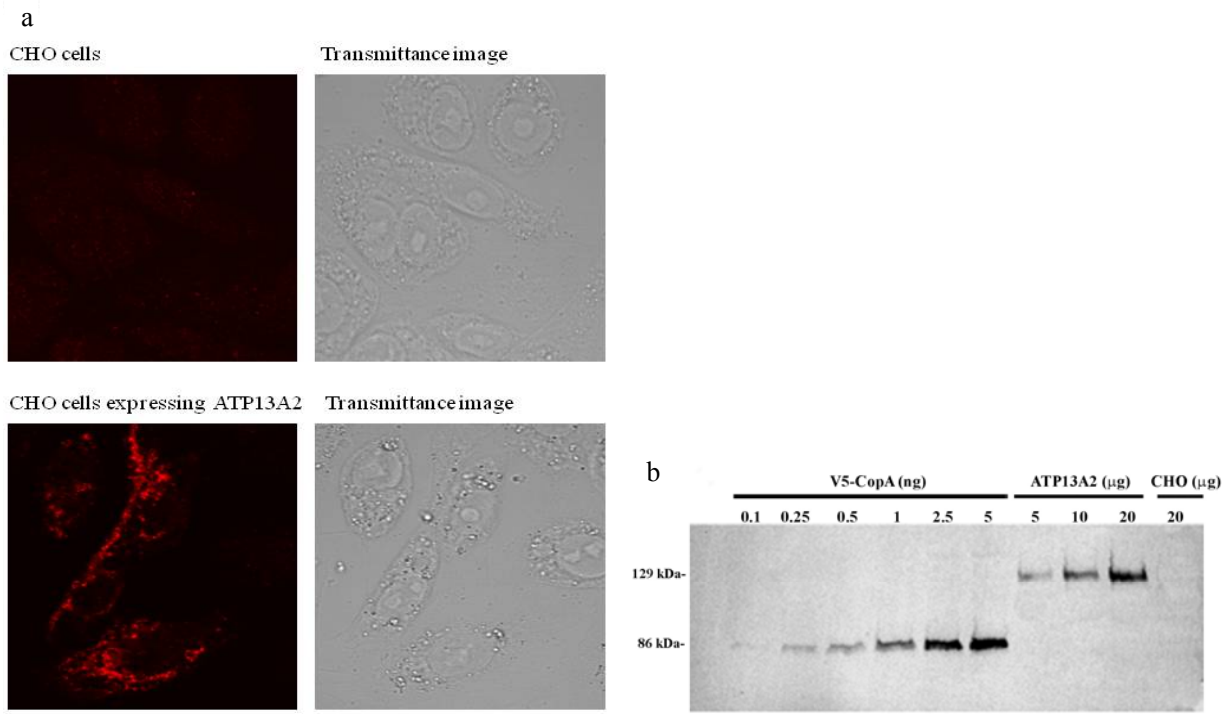

Fig. 1 Expression of the recombinant ATP13A2 pump.

(a) Fluorescence microscopy to visualize ATP13A2 expression in transfected CHO cells. The left panels show CHO cells transfected with the empty vector pcDNA3.1 (upper panels) or with the expression vector pcDNA3.1 carrying the V5-tagged human ATP13A2 cDNA (bottom panels) incubated with an antibody to V5 revealed with Zenon Alexa Fluor 568 mouse IgG2a secondary antibody. The transmittance image of each one is shown in the right panels. (b) Immunoblot of microsomes from CHO cells transfected with cDNA encoding the V5-tagged human ATP13A2. For protein estimation, a V5-tagged Ag^{+}/Cu^{+}ATPase (V5-CopA) of 86 kDa purified from the extremophile organism Archeaglobus fulgidus was used. The amount of protein loaded is indicated at the top of each lane. The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes; finally they were detected with the V5 antibody as described under "Material and Methods".

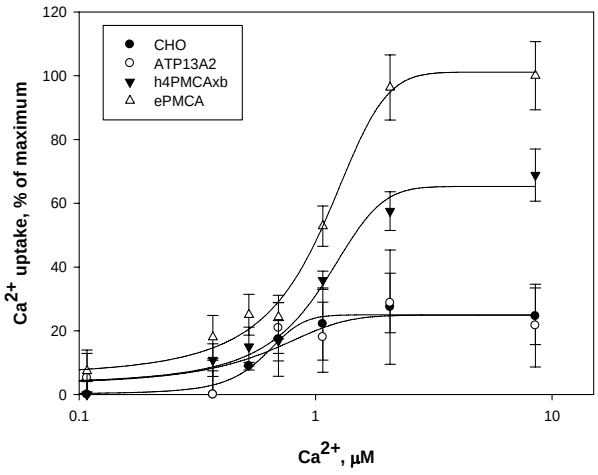

Fig. 2 Ca^{2+} dependence of the Ca^{2+} transport of recombinant ATP13A2 as compared with that of PMCA.

Ca^{2+} uptake by microsomal vesicles was measured at different free Ca^{2+} concentrations for 5 min at 37 ℃ as described under "Material and Methods". The kinetic behavior of the recombinant PMCA (h4PMCAxb, filled triangles) is similar to the PMCA of erythrocytes IOVs (ePMCA, empty triangles). The activity of the recombinant ATP13A2 (empty circles) not differ from that obtained by the endogenous PMCA from CHO cells transfected with the empty vector (filled circles). Data points are the average of three to five experiments. The lines are the best fit to the data given by the Hill equation.

measured using either inside out vesicles (IOVs) from erythrocytes, or microsomal vesicles from CHO cells stable expressing the human PMCA isoform 4xb (h4PMCAxb). As shown in Fig. 2, the activity of the PMCA enzyme gradually increased with the concentration of Ca^{2+}, reaching half maximal activity at about 1 μM of free Ca^{2+}. By contrast, the Ca^{2+} uptake activity of microsomes expressing the recombinant ATP13A2 was not significantly different from that of microsomal vesicles from CHO control cells transfected with the empty vector indicating that in these conditions no uptake of Ca^{2+} was associated with the expressed ATP13A2.

3.3 Phosphorylation Reaction

The first step of the catalytic cycle of all well characterized P-type Ca^{2+} pumps involves the Ca^{2+} dependent transfer of the γ–phosphate from ATP to the enzyme to form a phosphoenzyme intermediate. As shown in Fig. 3 a strong band was observed when microsomes carrying the recombinant PMCA were phosphorylated with ATP in the presence of Ca^{2+}. Likewise, a band corresponding to the PMCA phosphoenzyme was observed in erythrocyte IOVs. In contrast the phosphorylation pattern of membrane vesicles containing the recombinant ATP13A2 was similar to that of control membranes, indicating that no phosphorylation attributable to the ATP13A2 protein had occurred. Thus, the expressed ATP13A2 was not activated by Ca^{2+} to form the phosphorylated intermediate.

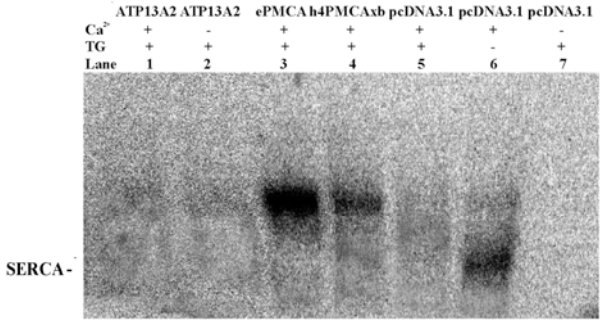

Fig. 3 Formation of the phosphorylated intermediate.
Phosphoenzyme formation was carried out as described under "Material and Methods". Lane 1, recombinant ATP13A2 with Ca^{2+}; lane 2, recombinant ATP13A2 without Ca^{2+}; lane 3, PMCA from erythrocyte IOVs with Ca^{2+} (ePMCA); lane 4, recombinant PMCA with Ca^{2+} (h4PMCAxb); lane 5, control empty pcDNA3.1 vector with Ca^{2+}; lane 6, pcDNA3.1 membranes as in lane 5, but the phosphorylation was carried in the absence of thapsigargin allowing the visualization of the SERCA phosphoenzyme; lane 7, control empty pcDNA3.1 vector without Ca^{2+}.

4. Discussion

The P_5-ATPases have been associated with the homeostasis of intracellular Ca^{2+} and this fact has led to the idea that they are Ca^{2+} transporters. Here, it was investigated calcium as a possible substrate of the human ATP13A2 enzyme by comparing the Ca^{2+} transport activity and Ca^{2+}-dependent phosphoenzyme formation of ATP13A2 with that of the related Ca^{2+} specific P_2-ATPase PMCA. Neither of the two Ca^{2+}-dependent activities were detected in the recombinant ATP13A2. These results suggest that although P_5-ATPases influence many Ca^{2+} related functions, Ca^{2+} is not the substrate transported by the human P_5-ATP13A2. Mutations in the ATP13A2 gene underlay an autosomal recessive form of early onset parkinsonism [3]. Expression of ATP13A2 in animal models of PD is sufficient to rescue neurodegeneration associated with α-synuclein (α-syn) aggregation [19], which is relevant to this study since it was used the same construct for the expression of ATP13A2 and thus it implies that no additional factors are needed in order to observe the biological response.

Because it has been hypothesized that P_{5A} and P_{5B} pumps may have different ion specificities [20], the lack of Ca^{2+} dependent activities of P_{5B} -ATP13A2 should not be generalized to the whole P5 subfamily. Moreover, recently it was reported that yeasts lacking Ypk9p, the yeast ATP13A2 homolog, were unaffected by Ca^{2+} but were more sensitive to high concentrations of cadmium, manganese, nickel and selenium [7]. Yet another study has shown that the manganese toxicity can be alleviated by the expression of the Ypk9p protein. On the basis of these results it has been proposed that the yeast Ypk9p functions as a manganese transporter to protect cells from

excess Mn^{2+} exposure [19]. On the other hand, it was recently published that the deletion of the P_{5B}-ATPase CATP-5 of Caenorhabditis elegans is responsible for the tolerant phenotype seen in the presence of the toxic spermidine analog norspermidine, raising the possibility that the polyamines are the substrates transported by P_{5B}-ATPases [21].

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