The Secretory Pathway Ca\(^{2+}\)/Mn\(^{2+}\)-ATPase 2 Is a Golgi-localized Pump with High Affinity for Ca\(^{2+}\) Ions*

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Received for publication, January 27, 2005, and in revised form, March 25, 2005
Published, JBC Papers in Press, April 14, 2005, DOI 10.1074/jbc.M501026200

Accumulation of Ca\(^{2+}\) into the Golgi apparatus is mediated by sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs) and by secretory pathway Ca\(^{2+}\)-ATPases (SPCAs). Mammals and birds express in addition to the housekeeping SPCA1 (human gene name ATP2C1, cytogenetic position 3q22.1) a homologous SPCA2 isoform (human gene name ATP2C2, cytogenetic position 16q24.1). We show here that both genes present an identical exon/intron layout. We confirmed that hSPCA2 has the ability to transport Ca\(^{2+}\), demonstrated its Mn\(^{2+}\)-transporting activity, showed its Ca\(^{2+}\)- and Mn\(^{2+}\)-dependent phosphoprotein intermediate formation, and documented the insensitivity of these functional activities to thapsigargin inhibition. The mRNA encoding hSPCA2 showed a limited tissue expression pattern mainly confined to the gastrointestinal and respiratory tract, prostate, thyroid, salivary, and mammary glands. Immunocytochemical localization in human colon sections presented a typical apical juxtanuclear Golgi-like staining. The expression in COS-1 cells allowed the direct demonstration of \(56\text{Ca}^{2+}(K_{D, 5} = 0.27 \text{ mM})\) or \(56\text{Mn}^{2+}\) transport into an A23187-releasable compartment.

In vertebrates, three families of P-type Ca\(^{2+}\)-ATPases control intracellular Ca\(^{2+}\) homeostasis: PMCAs, SERCAs, and SPCAs. All these Ca\(^{2+}\) pumps contribute to the removal of activator Ca\(^{2+}\) from the cytoplasm after stimulation, thus decreasing the cytoplasmic Ca\(^{2+}\) concentration back to baseline levels. PMCAs extrude Ca\(^{2+}\) from the cytoplasm into the extracellular medium, whereas SERCAs accumulate Ca\(^{2+}\) into the endoplasmic reticulum. Ca\(^{2+}\) uptake into the Golgi apparatus is mediated by both SERCA and SPCA Ca\(^{2+}\) pumps (for a review, see Ref. 1). The high concentration of Ca\(^{2+}\) in the lumen of the intracellular organelles is not only a source of activator Ca\(^{2+}\) for cytosolic processes but is also indispensable for proper transcription, translation, translocation, folding, and processing of secreted proteins (2). In the Golgi apparatus, luminal Ca\(^{2+}\) is also required for intra-Golgi membrane transport, transport between the Golgi and ER, and endosome fusion. Although the pivotal role of the ER as a Ca\(^{2+}\) store is well established, the view that also the Golgi apparatus can act as an agonist-releasable Ca\(^{2+}\) store is much more recent (3, 4). Also the knowledge of the Golgi-specific SPCA-type Ca\(^{2+}\)-ATPases is much more limited than that of the well characterized SERCA and PMCA pumps. The PMR1 gene of Saccharomyces cerevisiae (5) is the first member of the SPCA family that has been described. The Pmr1 protein was localized to the Golgi apparatus or one of its subcompartments (6). Pmr1 is an ion-motive ATPase that supplies the secretory pathway with Ca\(^{2+}\) and Mn\(^{2+}\) ions required for glycosylation, sorting, and ER-associated protein degradation (7, 8). Genes homologous to the S. cerevisiae PMR1 have been reported for a number of other fungi.

The characterization of the animal PMR1 homologues is more recent. Although the cDNA of the putative rat PMR1 homologue was cloned in 1992 using a SERCA-derived probe (9), the authors failed to functionally characterize this protein. Direct evidence that the Pmr1 homologue in animals is able to transport Ca\(^{2+}\) and Mn\(^{2+}\) was presented for the Caenorhabditis elegans homologue by Van Baelen et al. (10). Ton et al. (11) demonstrated the Ca\(^{2+}\) transport activity of the human homologue hSPCA1a (GenBank\textsuperscript{TM} accession number AF181120). Later studies indicated that hSPCA1d isoform (GenBank\textsuperscript{TM} accession number AY268375) is also capable of transporting Mn\(^{2+}\) ions (12). The ability to transport Mn\(^{2+}\) at an appreciable rate is a characteristic not shared by the SERCA or PMCA Ca\(^{2+}\) transport ATPases. Like Pmr1 in yeast, the SPCA1 protein is localized in the Golgi apparatus.

The human ATP2C1 gene that encodes hSPCA1 was recently identified as the defective gene in Hailey-Hailey disease (Online Mendelian Inheritance in Man (OMIM) accession number 169600) (13, 14). Hailey-Hailey disease is an autosomal dominant skin disorder that is characterized by suprabasal acantholysis of keratinocytes, resulting in epidermal blister formation. Up till now a total of 70 different mutations have been described in Hailey-Hailey disease patients (15). These mutations are scattered throughout the entire gene with no apparent clustering. The symptoms of Hailey-Hailey disease strongly resemble those of Darier-White disease (OMIM accession number 124200), which is caused by mutations in one of the SERCA2 gene (ATP2A2) alleles (16). The link between defects in intracellular Ca\(^{2+}\) pumps and epidermal pathogenesis indicates that Ca\(^{2+}\) inside the lumen of intracellular stores plays an essential role in preserving skin integrity.

* This work was supported by the Interuniversity Attraction Poles Programme-Belgian Science Policy PS/05 and by Fonds voor Wetenschappelijk Onderzoek Vlaanderen Grant G.0166.04. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: PMCA, plasma membrane Ca\(^{2+}\)-ATPase; SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; SPCA, secretory pathway Ca\(^{2+}\)-ATPase; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; RT, reverse transcription; DTT, dithiothreitol; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; TGN, trans-Golgi network.
The human genome also contains a second gene (ATP2C2) encoding a secretory pathway Ca\(^{2+}\)/Mn\(^{2+}\)-ATPase (hSPCA2) that has not been characterized except for a recent study published while the present work was submitted (17). This group revealed hSPCA2 as a Ca\(^{2+}\) pump upon heterologous expression in yeast. Complementation studies in yeast suggested the importance of hSPCA2 in cellular Mn\(^{2+}\) detoxification. hSPCA2 was shown to be expressed in neuronal cells. In contrast to the report of Xiang et al. (17) we showed that hSPCA2 displays a high apparent affinity for Ca\(^{2+}\) similar to those values documented for hSPCA1a expressed in yeast (11) and for hSPCA1d expressed in COS-1 cells (12). Furthermore the Ca\(^{2+}\)- or Mn\(^{2+}\)-dependent activation of phosphoenzyme formation with \(\gamma\)\(^{32}\)P\textit{ATP} and the ability to pump both Ca\(^{2+}\) and Mn\(^{2+}\) were also demonstrated. Finally the expression of hSPCA2 at both mRNA and protein levels was analyzed showing that hSPCA2 has a much more restricted tissue distribution than hSPCA1 and colocalizes with Golgi-specific markers in colon epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Generation of the Expression Construct**—The hSPCA2-encoding expression construct was generated by PCR using Clontech Advantage 2HF polymerase. Human colon cDNA was used as a template. Two overlapping fragments were amplified separately using primers that introduce a HindIII restriction site at the 5′-end (indicated in lowercase in the sequence shown below). The forward primer was NewhSPCA2F (5′-CTGTTGGAAGGCTGGCTTCATGGCGCAG-3′), and the reverse primer was Overlap2hSPCA2R1 (5′-TATATATCCTCAGGTTATGTCAATCTCCATCCTCACTGGCCAG-3′). The 3′-fragment was amplified using the forward primer Overlap2hSPCA2F1 (5′-CTTGGCGGATGGAAGTGGGCTTAATATTGTCAATCTCCATCCTCACTGGCCAG-3′) and the reverse primer was Overlap2hSPCA2F1R2 (5′-ATCTGAATCTCCTTCTGGGTGCTACTT-3′). The hybridization of Cell Culturing and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 3.8 mM d-glucose, 85 mM sodium penicillin, 85 mM sodium streptomycin, and 1% non-essential amino acids. HeLa cervical carcinoma cells were obtained from the ATCC (Manassas, VA) and grown in Ham's F-12 medium supplemented with 10% fetal bovine serum, 3.8 mM d-glucose, 85 mM sodium penicillin, and 85 mM sodium streptomycin. Human colon carcinoma cells (Caco2) were purchased from the ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 3.8 mM d-glucose, 2.5 mM sodium penicillin, and 2.5 mg/mL streptomycin. Human primary keratinocytes were isolated from human foreskin obtained from circumcision operations. Briefly the tissue was cut into small pieces and dispersed in dispase. The epidermis was peeled from the dermis and incubated in Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS before collecting the cells by centrifugation (18). Keratinocytes were cultured in Keratinocyte-SFM medium (Invitrogen) supplemented with bovine pituitary extract (25 μg/mL) and epidermal growth factor (0.1–0.2 ng/mL). To induce differentiation, the extracellular Ca\(^{2+}\) concentration was raised from 0.09 mM in the normal medium to 1.2 mM, and the cells were cultured for 14 days.

**For 45Ca\(^{2+}\) fluxes, COS-1 cells were seeded in 12-well gelatin-coated dishes at a density of ~30,000 cells/cm\(^2\) and investigated 8 days later. For 32P-flux studies, cells were seeded in 100-mm culture dishes at 2.5 × 10\(^4\) cells/plate.

**Transfections**—COS-1 cells were transiently transfected using Gene Juice transfection reagent (Novagen). Transfection was performed with 1 μg of plasmid DNA (in 10 μL of OptiMEM medium) in a 12-well plate. After 1 day, the cells were fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. The cells were permeabilized by incubation in 0.2% Triton X-100 for 2 min at room temperature. After three washes with PBS, non-specific binding was blocked by incubation for 1 h in PBS containing 3% bovine serum albumin and 1:100 normal goat serum. Primary antibodies were diluted in 1% bovine serum albumin in PBS and incubated for 1 h followed by three washes with PBS. As negative controls, coverslips were incubated with preimmune serum at the same dilution as the immune serum. Secondary antibodies were added in 1% bovine serum albumin in PBS and incubated for 1 h. Secondary antibodies were goat anti-rabbit Alexa Fluor 488 or 594 or goat anti-mouse Alexa Fluor 488 or 594 (Molecular Probes). Finally cells were washed, and the coverslips were mounted in Vectashield (Vector Laboratories) to inhibit water loss and sealing. Confocal scans were performed using a STORM \(^{\text{TM}}\) 840 scanner (Amersham Biosciences). Quantification was performed using ImageQuant \(^{\text{TM}}\) software (Amersham Biosciences).

**Northern Dot-blot**—The DNA hybridization probe was generated using \(\text{Pfu}\) Ultra polymerase (Stratagene) in 20-μL PCRs using 10 ng of plasmid DNA of the hSPCA2 expression construct as template. PCR products were \(^{32}\)P-labeled by the inclusion of 5 μL of \(\text{o}^{32}\)PdCTP (10 μCi/μL). The primers used for ATP2C2 were ATP2C2NBlotPrim (5′-GGCGGCTACATGCTGAG-3′) and ATP2C2NBlotR (5′-CCATGAGGAGGCCTGGCAATG-3′). The hybridization probe was separated from unincorporated \(^{32}\)P-labeled nucleotides using mini-Quick Spin columns (Roche Diagnostics). A human MTE \(^{\text{TM}}\) (multiple tissue expression) array was purchased from Clontech. Probes were hybridized according to the manufacturer's instructions, and the labeling was detected by exposure to Biomax film (Eastman Kodak Co.).

**Immunocytochemistry**—Cryosections (5 μm) of human colon tissue or cells grown on gelatin-covered coverslips were washed several times with PBS and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. The cells were permeabilized by incubation in 0.2% Triton X-100 for 2 min at room temperature. After three washes with PBS, non-specific binding was blocked by incubation for 1 h in PBS containing 3% bovine serum albumin and 1:100 normal goat serum. Primary antibodies were diluted in 1% bovine serum albumin in PBS and incubated for 1 h followed by three washes with PBS. As negative controls, coverslips were incubated with preimmune serum at the same dilution as the immune serum. Secondary antibodies were added in 1% bovine serum albumin in PBS and incubated for 1 h. Secondary antibodies were goat anti-rabbit Alexa Fluor 488 or 594 or goat anti-mouse Alexa Fluor 488 or 594 (Molecular Probes). Finally cells were washed, and the coverslips were mounted in Vectashield (Vector Laboratories) to inhibit water loss and sealing. Confocal scans were performed using an Olympus Cell \(^{\text{TM}}\) fluorescent microscope. Subcellular structures could be identified using monoclonal antibodies directed against components of the ER or the Golgi. A SERCA2 antibody (ID8, Affinity BioReagents) was used to visualize the ER, whereas the Golgi apparatus was identified using anti-TGN46 antibodies (Serotec).

**Preparation of Crude Cell Extracts and Isolated Membranes**—For total protein extractions, cells were washed twice in PBS before lysis in...
freshly prepared SDS extraction buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA, and 2% SDS supplemented with the protease inhibitors 0.28 mM phenylmethylsulfonyl fluoride, 0.83 mM benzamidine, and a 1/100 g/ml concentration of each of leupeptin, aprotinin, and pepstatin A). After a 30 min incubation on ice, the insoluble material was pelleted at 6000 g and discarded. The supernatant was used for further experiments. Microsomes from transfected COS-1 cells were prepared as described by Verboomen et al. (20). Microsomes from tissues were prepared as follows. Fresh or frozen tissue was rinsed in cold PBS supplemented with 1 mM EDTA and allowed to equilibrate for 10 min on ice in hypotonic Buffer A (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 2 mM DTT, 1 mM EDTA, and the protease inhibitor mixture given above). Homogenization was performed using a Teflon/glass Potter homogenizer. An equal volume of Buffer B (10 mM Tris-HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, and the protease inhibitor mixture given above) was added, and homogenization was continued. Cellular debris and nuclei were centrifuged at 4000 g for 15 min at 4 °C. The supernatant was recentrifuged at 100,000 g for 40 min at 4 °C followed by resuspension of the microsomal pellet in Buffer C (10 mM Tris-HCl, pH 7.5, 2 mM DTT, 150 mM KCl, and 250 mM sucrose) (21). Quantification of total protein was done using the bicinchoninic acid method (Pierce) using the manufacturer's instructions. When compounds in the samples interfered with this method, the protein was precipitated with trichloroacetic acid prior to quantification by the Lowry method.

Western Blotting—Microsomes or crude cell extracts were loaded on NuPage™ bis-Tris 4–12% gradient gels or NuPage 7% Tris acetate gels (Invitrogen). After electrophoresis, the separated proteins were transferred to Immobilon-P membranes. The blots were quenched in TBST (Tris-buffered saline containing 0.05% Tween) supplemented with 5% (w/v) nonfat dry milk. When necessary, the primary antibody solution was preincubated with 10 μg of the immunogenic peptides for 30 min. After three washes in TBST, incubation with the primary antibody in TBST was performed at room temperature. After labeling with the secondary antibody coupled to alkaline phosphatase (dilution, 1:8000), the labeled bands were detected using Vistra ECF (Amersham Biosciences) as substrate. Detection of the fluorescence was performed using a STORM 840 scanner (Amersham Biosciences) in combination with ImageQuant software (Amersham Biosciences).

Isotope Fluxes—45Ca2+ fluxes were performed on cells permeabilized with saponin. Cells grown in 12-well, gelatin-coated plates were treated for 10 min with 20 μg/ml saponin in permeabilization buffer (120 mM KCl, 30 mM imidazole-HCl, pH 6.8, 2 mM MgCl2, 1 mM ATP, and 1 mM EGTA) at 25 °C. After a wash with the same buffer without saponin, the non-mitochondrial stores were loaded with 45Ca2+ (10 μCi/ml) or 54Mn2+ (10 μCi/ml) for 90 min in a buffer containing 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, 5 mM Tris, 10 mM NaN3, 0.44 mM EGTA, and 100 nM thapsigargin (SERCA-specific inhibitor). To obtain a free Mg2+ concentration of 0.5 mM and the indicated free Ca2+ and Mn2+ concentrations, the total MgCl2, CaCl2, and MnCl2 concentrations were calculated using the CaBuf program (ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip) developed by G. Droogmans in our laboratory. These calculations are based on the dissociation constants given by Fabiato and Fabiato (22) for Ca2+ and by Martell and Smith for Mn2+ (23). After the loading phase, cells were washed twice with 1 ml of efflux medium (120 mM KCl, 30 mM imidazole-HCl, pH 6.8, 1 mM EGTA). Subsequently the efflux medium was replaced every 2 min to monitor the leak of Ca2+. At the end of each experiment, the remaining 45Ca2+ in the

FIG. 1. Intron-exon layout of ATP2C2. Nucleotide sequence of the exon-intron boundaries and the size of the introns are shown. Exon sequences are indicated in uppercase, whereas introns are in lowercase. The deduced amino acid sequence at each junction is displayed below the exon sequences. Exon-intron boundaries are indicated by slashes, and the conserved GT and AG nucleotides flanking the introns are shown in boldface.
cells was released by the addition of 1 ml of 2% (w/v) SDS for 30 min.

Formation of the Phosphoenzyme Intermediate—Microsomes (10 μg) were phosphorylated on ice in a 100-μl reaction mixture containing 1 μl of 2 Ci/μl [γ-32P]ATP (Amersham Biosciences), 160 mM KCl, 17 mM Hepes, pH 7.0, 1 mM DTT, 5 mM NaN₃, and the appropriate concentrations of Ca²⁺, Mn²⁺, and EGTA. The reaction was stopped after 20 s by the addition of ice-cold stop solution (6% trichloroacetic acid, 10 mM phosphoric acid, and 1 mM ATP). The protein was allowed to precipitate on ice for 30 min and pelleted by centrifugation at 4 °C for 3 min at 13,200 rpm. Pellets were washed two times with stop solution and finally with 0.2 M sodium acetate before solubilization in modified SDS-PAGE sample buffer (150 mM Tris-HCl, pH 7.4, 8 mM EDTA, 3% SDS, 20% sucrose, 0.14 mg/ml bromphenol blue, and 10 mM DTT). Phosphorylated proteins were separated by acid 7.5% SDS-PAGE according to Sarkadi et al. (24). After fixation of the gels in 7.5% acetic acid they were dried between sheets of gel drying film (Promega) and exposed to a PhosphorImager™ screen (Amersham Biosciences) for quantification. Background phosphorylation due to endogenous Ca²⁺ pumps in this expression system is negligible (25, 26).

RESULTS ATP2C2 Encodes a P-type ATPase—The GenBank™ entry of the cDNA sequence KIAA0703 (GenBank™ accession number AB14603, Ref. 27) was the first indication of a putative second isoform of the human secretory pathway Ca²⁺-ATPase. The existence of a second SPCA isoform has been mentioned previously in the literature (11, 28), but no further studies were published until the recent study by Xiang et al. (17). Our initial studies showed that overexpression of the protein encoded by the KIAA0703 cDNA in COS-1 cells did not result in the formation of a functional Ca²⁺-ATPase (data not shown). It is now clear that the reason behind this failure lies in the faulty N terminus. The KIAA0703 sequence contains three putative in-frame ATG start codons at positions 441, 501, and 606. We assembled expression constructs for each of the three putative protein products. All three proteins could be overexpressed, but this did not result in an enhanced Ca²⁺ transport capacity, indicating that non-functional proteins were formed (data not shown). Moreover these proteins were not localized in the Golgi but instead were found in the ER, and they were susceptible to enhanced degradation because Western blotting showed an intense additional band corresponding to a smaller proteolytic fragment (data not shown).

Further analysis of chromosome 16 suggested the existence of two alternative exons located more upstream in the genomic sequence. The first two exons of the KIAA0703 cDNA were replaced by these two new exons, thus generating a new cDNA clone whose nucleotide sequence was deposited in GenBank™ under accession number AY791884. In contrast to the corresponding sequence in KIAA0703, the revised 5’-end of the new cDNA clone was highly similar to that of the SPCA2 mRNA sequence from rat found in the data base (GenBank™ accession number AF484685). In addition, the first two exons of ATP2C2 are separated by a large intron of about 30 kb, which
is similar in size to the corresponding intron of ATP2C1.

The complete genomic exon/intron layout is shown in Fig. 1. ATP2C2 spans a region of 95.5 kb on human chromosome 16 (16q24.1, GenBank™ accession number AC022165) and consists of 27 exons ranging in size from 36 to 393 bp. The introns are between 86 and 29,785 bp in length. The putative translation initiation ATG codon (ACCATGG) is embedded in a Kozak consensus sequence (29). Gene transcription and processing results in a 3386-bp cDNA of which 2841 bp comprise the open reading frame. The 946-amino acid sequence of the encoded protein is identical to that studied by Xiang et al. (17).

Expression Pattern of the ATP2C2 mRNA—Northern dot-blot hybridization analysis was performed to compare the

FIG. 3. Ratio RT-PCR of ATP2C1/ATP2C2 transcripts in different human tissues and cell lines. A, typical example of a ratio RT-PCR on LNCaP cells expressing both genes. Amplicons derived from ATP2C1 and ATP2C2 were discriminated by restriction digestion and electrophoresis. Lanes RNA – and RT – show the negative controls. The sample in lane RNA – contained no template. Lane RT – is an RNA sample that was incubated in parallel with the reverse transcription but without added reverse transcriptase. Aliquots of the PCR product were not treated (uncut) or cut with MseI (specific for ATP2C1), ScrFI (specific for ATP2C2), or their combination. The bands were quantified by fluorescent staining of the gel. B, relative quantification of ATP2C1 and ATP2C2 messengers by ratio RT-PCR in different human cell types and tissues. Results are the mean ± S.E. of three or four independent experiments. Col car, colon carcinoma; Kerat, keratinocytes; HBE, human bronchial epithelium.

FIG. 4. Overexpression of hSPCA2 in COS-1 cells. A, Western blot of extracts of COS-1 cells transfected with empty vector or with a hSPCA2 construct. Lanes 1 and 2 were loaded with 5 and 18 µg, respectively, of SDS extract of control COS-1. Lanes 3 and 4 contain 5 and 18 µg, respectively, of extract of COS-1 cells transiently overexpressing hSPCA2. The blots were immunostained for hSPCA2 using the XIB antibody (1:1000) or hSPCA1 using the hSPCA1cytc antibody (1:3000). SERCA2b was detected using a polyclonal antibody (1:2000) described previously (35). B, immunocytochemical images of hSPCA2-overexpressing COS-1 cells. a, SPCA2-overexpressing COS-1 cells stained with the XIB preimmune serum (1:1000), b, hSPCA2-overexpressing COS-1 cells stained with the XIB antibody (1:1000) preincubated with 10 µg of the antigenic peptides to block specific signals. The middle panels show a double staining for hSPCA2 (c) and the Golgi marker TGN46 (d). The bottom panels show a double staining for hSPCA2 (e) and the ER-resident protein SERCA2 (f). The antibodies used were anti-hSPCA2 XIB preimmune (1:1000), anti-hSPCA2 XIB (1:1000), anti-TGN46 (1:300), and anti-SERCA2 (1:300). All pictures were overlaid with the 4,6-diamidino-2-phenylindole staining pattern of the same field to visualize the nuclei. The scale bar represents 20 µm.

FIG. 5. Western blot of endogenously expressed hSPCA2 in human tissues. Western blot using anti-hSPCA2 (XIB, 1:1000) is shown. First lane, 30 µg of colon microsomes; second lane, 30 µg of lung microsomes; third lane, 15 µg of hSPCA2-overexpressing COS-1 cells as a positive control (+). The signal was abolished when the primary antiserum was preincubated with 10 µg of the immunogenic peptides (peptide inhibition).
expression pattern of the novel gene with that of its previously identified family member ATP2C1 (Fig. 2). Whereas there was little variation in the level of the ubiquitous signal of ATP2C1 (Fig. 2B), the new paralogue presented large differences in the relative expression levels among various tissues (Fig. 2A). The strongest signals were detected throughout the gastrointestinal tract from the stomach (B5) to the rectum (C6) with the most intense signal in the rectum (C6). Other positive tissues include trachea (H7), fetal lung (G11), prostate tissue (E8), thyroid gland (D9), salivary gland (E9), and mammary gland (F9).

To better quantify the relative expression levels of ATP2C1 and ATP2C2 mRNA in different tissues and cell lines, we used a ratio RT-PCR protocol. In this method, a common set of primers is used to co-amplify homologous fragments of related sequences (30). Fig. 3A shows a typical experiment in which the relative amount of both messengers is determined by restriction analysis of the PCR products. Ratio RT-PCR was performed on mRNA samples from a number of human tissues (lung, colon, and colon carcinoma) that tested positive for ATP2C2 mRNA when cultured under proliferating conditions (data not shown). However, when keratinocytes were allowed to differentiate (by growing them to confluency in the presence of high extracellular Ca²⁺ concentration), the expression of both genes could be demonstrated (Fig. 3B).

Characterization of the hSPCA2 Protein—The hSPCA2-specific antibodies (XIB) were generated against a mixture of two SPCA2-specific peptides that are not conserved in hSPCA1. The antiserum labeled a band on Western blots of hSPCA2-overexpressing COS-1 cells but not of control COS-1 cells (Fig. 4A). The specificity of the antiserum was proven by the absence of the immunoreaction with preimmune serum and its suppression by preincubation of the serum with the immunogenic peptides. The immunoreactive protein migrated slightly below the predicted Mr value of 103,293. A similar anomalous faster migration has also been observed for the C. elegans SPCA homologue (10). Immunostaining of Western blots for other organellar Ca²⁺ pumps demonstrated that overexpression of hSPCA2 did not alter the expression levels of the endogenous Ca²⁺-ATPases SERCA2b and hSPCA1 (Fig. 4A). The overexpressed protein was localized in a juxtanuclear region in COS-1 cells. This region corresponded to the Golgi apparatus as shown by the colocalization experiments depicted in Fig. 4B, middle panels. SERCA2, which predominantly stains the ER, showed a much more diffuse, reticular staining pattern (Fig. 4B, bottom panels).

In the next series of experiments, the endogenous hSPCA2 protein was studied in human colon. Fig. 5 shows a Western blot performed with microsomes isolated from human colon and lung. The protein could only be demonstrated in colon, a tissue with one of the highest hSPCA2 mRNA expression levels. The
immunoreactive band migrated slightly higher than the protein overexpressed in COS-1 cells. This could be due to post-translational modifications. An additional band of lower mobility was present in colon microsomes, but this band was nonspecific because it was not inhibited by preincubation of the antibody with the immunogenic peptides (Fig. 5). Immunohistochemical staining of human colon cryosections resulted in hSPCA2-specific labeling in close proximity of the apical pole of the nuclei of colon epithelial cells. This juxtanuclear staining colocalized with the Golgi marker TGN46 (Fig. 6). hSPCA1 showed a similar juxtanuclear staining, indicating that hSPCA1 and hSPCA2 reside in the same or closely juxtaposed subcellular compartments. The Golgi marker TGN46 appeared to label all epithelial cells because all nuclei were associated with a Golgi-like staining. Additionally, cells stained for the Golgi marker were also positive for both hSPCA1 and hSPCA2.

hSPCA2 Is a Functional Ca\(^{2+}\) - and Mn\(^{2+}\) -transporting Enzyme—The hSPCA2 protein was functionally characterized by heterologous overexpression in COS-1 cells. The transient formation of a phosphorylated intermediate is a key feature of all P-type ion transport ATPases. To visualize the phosphorylated hSPCA2 intermediate, control cells (transfected with empty vector) and hSPCA2-overexpressing COS-1 cells were phosphorylated in vitro using \([\gamma-32P]\)ATP in the presence of 5 \(\mu M\) Ca\(^{2+}\) or 5 \(\mu M\) Mn\(^{2+}\). The effects of 100 nM thapsigargin (TG) (A, top and bottom panels) and 200 mM hydroxylamine (B) on the formation of the phosphointermediate is also shown.

To study the ion transport activity in overexpressing cells we performed \(^{45}\text{Ca}^{2+}\) fluxes on permeabilized cells (10). In a first series of experiments we measured the Ca\(^{2+}\) accumulation in the stores of overexpressing COS-1 cells as a function of time. Permeabilized COS-1 cells were incubated for different time periods in a medium resembling the cytosolic ionic composition and containing \(^{45}\text{Ca}^{2+}\). To exclude the interference of the endogenous SERCA2b pump, the experiments were performed in the presence of 100 nM thapsigargin. Fig. 8A shows that the time-dependent loading of \(^{45}\text{Ca}^{2+}\) in hSPCA2-overexpressing COS-1 cells was higher than in control cells. This difference increased with time both in absolute and relative terms. Because the cells are more prone to detach during prolonged incubation time, the loading time was restricted to 90 min as described previously (12).

To determine the Ca\(^{2+}\) dependence of the hSPCA2-mediated Ca\(^{2+}\) uptake, permeabilized cells were loaded at different free Ca\(^{2+}\) concentrations for a fixed time of 90 min, and the difference in the level of \(^{45}\text{Ca}^{2+}\) uptake of hSPCA2-overexpressing cells and control cells was measured (Fig. 8B). Half-maximal activation (\(K_{0.5}\)) of the enzyme was observed at 0.27 \(\mu M\) free Ca\(^{2+}\) concentration.

Because previous reports on the SPCA1 pump of humans (12) and C. elegans (10) have shown that SPCA1 can act as a Mn\(^{2+}\) pump, we tested whether hSPCA2 also functions as a Mn\(^{2+}\)-transporting enzyme. Control cells and hSPCA2-overexpressing cells were loaded with \(^{54}\text{Mn}^{2+}\) for 90 min in the presence of 100 nM thapsigargin, and the efflux of \(^{54}\text{Mn}^{2+}\) was followed for 18 min. The higher uptake of \(^{54}\text{Mn}^{2+}\) in hSPCA2-overexpressing cells provided direct evidence that hSPCA2 can act as a Mn\(^{2+}\)-transporting ATPase (Fig. 8C). The efflux of accumulated Mn\(^{2+}\) was accelerated by the ionophore A23187 (10 \(\mu M\), demonstrating that the Mn\(^{2+}\) ions had been transported into a membrane-delineated compartment. In the presence of A23187, the efflux curves of control cells and hSPCA2-overexpressing cells converged, showing that the amount of background \(^{54}\text{Mn}^{2+}\) binding was identical and that the difference between the two curves was specifically due to an enhanced Mn\(^{2+}\) transporting capacity of hSPCA2-expressing cells.

DISCUSSION

In this study we confirmed the finding of Xiang et al. (17) that human tissues can express a second isoform of secretory pathway Ca\(^{2+}\)-ATPase, named hSPCA2, encoded by the \(\text{ATP2C2}\) gene. We further explored its tissue-specific and cellular expression pattern and present its functional characteristics upon heterologous expression in COS-1 cells. A TBLASTN search with an SPCA signature peptide sequence (IQEYRSEKSLEELTK) revealed that mammalian genomes (\(\text{Homo sapiens, Pan troglodytes, Canis familiaris, Bos taurus, Rattus norvegicus, and Mus musculus}\) all contain two different SPCA-encoding genes. Also the chicken genome (\(\text{Gallus gallus}\) ) appears to contain two related genes. We did not find any indication for the presence of a second SPCA isoform in the fish \(\text{Danio rerio, Fugu rubripes, and Tetraodon nigroviridis}\). This is remarkable because in general, as a result of an additional genome duplication, euteleost fishes seem to have larger gene families than tetrapods (31). Whereas euteleost genomes contain more SERCA genes than mammals, this is apparently not the case for the SPCA genes. Invertebrates (\(\text{Drosophila melanogaster, Anopheles gambiae, Aphis mellifera, and C. elegans}\) also seem to possess only one SPCA1 gene, but these lower organisms also contain only a single SERCA gene. Given the complete description of the human genome, the \(\text{ATP2C2}\) gene is probably the last functional gene to be identified in the multigene family of type 2 Ca\(^{2+}\)-transport ATPases. This superfamly consists of three SERCA-encoding genes (\(\text{ATP2A1-3}\)), four PMCA genes (\(\text{ATP2B1-4}\)), and two SPCA-encoding genes (\(\text{ATP2C1}\) and -2).
Characterization of Human SPCA2

hSPCA1 and hSPCA2 mRNAs present different tissue distributions. hSPCA1 was recognized as a housekeeping enzyme (13), and this observation was confirmed and extended by the result shown in Fig. 2B. The expression pattern of hSPCA2 mRNA is much more heterogeneous. The highest levels occur in the gastrointestinal tract and in a number of secretory tissues as determined by Northern dot-blot hybridization and by ratio RT-PCR. Also the tissue distribution of expressed sequence tag clones in the data base is compatible with this pattern. These results are different from the data published by Xiang et al. (17) who showed abundant hSPCA2 protein expression in brain and testes. In our studies, the mRNA expression of hSPCA2 in brain and testes was in the lower range (Fig. 2).

Because skin tissue was not represented in the RNA blots used and because of the central role of the hSPCA1 pump in Hailey-Hailey disease, we investigated the co-expression of hSPCA1 and hSPCA2 in a separate set of experiments on isolated keratinocytes using PCR. We demonstrated that hSPCA2 is expressed at the mRNA level in differentiated keratinocytes. The co-expression of hSPCA2 and hSPCA1 could help to explain the relatively high contribution of thapsigargin-insensitive Ca2+ accumulation in the Golgi of keratinocytes (33) and lung-derived 16HBE14o– cells (34). The proper function of the secretory pathway in keratinocytes may be crucial for correct delivery of cell adhesion components (15). The fact that hSPCA2 is not able to compensate for the decreased level of hSPCA1 in keratinocytes of Hailey-Hailey disease patients may suggest a specific function for this pump distinct from that of hSPCA1. This hypothesis fits with the observation that a complete knock-out of the ATP2C1 gene in mice results in an embryonic lethal phenotype.2

The yeast Pmr1 pump has been located to the medial Golgi compartment (6, 7). hSPCA1 is also a Golgi-resident protein (11) and comigrates with markers for the trans-Golgi compartment (32). hSPCA2 shares the property of Golgi localization with hSPCA1, but the exact localization inside the Golgi has not been studied yet. In epithelial cells of human colon tissue, both hSPCA isoforms show the same Golgi-like localization. However, the resolution of immunofluorescence microscopy may not be adequate to determine whether both pumps are in the same subcompartments of the Golgi. Reports on the mouse SPCA1 indicate that it can also be present in post-Golgi compartments (35).

The co-expression of hSPCA2 with hSPCA1 suggests that hSPCA2 supplements the role of hSPCA1 in the thapsigargin-insensitive Ca2+/Mn2+ accumulation pathway in the Golgi of a number of cell types. The expression in secretory cells suggests that hSPCA2 assists in the function of hSPCA1 or that it performs an as yet unknown specialized function in those cell types in which the Golgi apparatus is important not only for the basic cell biological maintenance of cell function but also for the specific secretory task these cells perform in the organism. The large number of epithelial cells expressing hSPCA1 and hSPCA2 in colon epithelium cannot be accounted for by the number of mucus-secreting cells only, indicating that these Ca2+ pumps also are present in enterocytes and play an important role in their function.

Xiang et al. (17) have reported a slightly different localiza-

2 T. Doetschman and G. E. Shull, unpublished data.

bol used. C, 54Mn2+ loading of permeabilized COS-1 cells transfected with hSPCA2 or empty vector as a control. Cells were loaded for 90 min at 1 μM free Mn2+. After two washes with efflux medium, passive Mn2+ efflux was measured for 10 min. The ionophore A23187 (10 μM) was added after 10 min. The graph is representative of four independent experiments.
tion of hSPCA2. They found hSPCA2 in TGN-derived vesicles in rat hippocampal neurons. We showed a predominantly juxtanuclear Golgi-like distribution in human colon epithelial cells. This difference most probably reflects variations in the architecture of the secretory pathway in different cell types.

Our functional studies of the overexpressed protein in mammalian cells confirmed the findings of Xiang et al. (17) that hSPCA2 is a P-type ATPase capable of transporting both Ca\(^{2+}\) and Mn\(^{2+}\) with high affinity. Half-maximal (\(K_{0.5}\)) stimulation of the enzyme occurred at a free Ca\(^{2+}\) concentration of 0.27 \(\mu\)M, which is in the same range as that described for the human SPCA1a protein (0.26 \(\mu\)M) (11), hSPCA1d (0.20 \(\mu\)M) (11), and its homologue in C. elegans (0.25 \(\mu\)M) (10). The yeast homologue Pmr1 shows a slightly higher apparent affinity for Ca\(^{2+}\) (\(0.1 \mu\)M) (8). When hSPCA2 is overexpressed in yeast, a lower half-maximal stimulation of hSPCA2 was further investigated by testing its ability to be phosphorylated in the presence of Ca\(^{2+}\) and Mn\(^{2+}\). In each case, a phosphoprotein intermediate could be demonstrated. Furthermore the formation of the phosphointermediate was sensitive to hydroxyamine, showing that the phosphorylation occurs on the catalytic carboxyl group of an aspartate residue, thus excluding the possibility that it was the result of protein kinase activity.

In conclusion, we functionally characterized a second isoform of SPCA-type ATPase. Both hSPCA2 overexpressed in COS-1 cells and hSPCA2 endogenously present in colon epithelial cells displayed a Golgi-like juxtanuclear distribution. hSPCA2 was shown to be capable of transporting Ca\(^{2+}\) with high affinity in a thapsigargin-independent way. Further studies are required to characterize the specific properties of hSPCA2 and its function in cellular divalent ion homeostasis.

Acknowledgments—We thank I. Willems, M. Schuermans, and T. Layten for excellent technical assistance. A. Floirizone, M. Crabbe, and S. De Swaef are acknowledged for maintaining the cell cultures. The Department of Morphology and Molecular Pathology (Katholieke Universiteit Leuven) is acknowledged for providing the human tissue sections.

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