PAT1 (SLC36A1) shows nuclear localization and affects growth of smooth muscle cells from rats

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The proton-coupled amino acid transporter (PAT1) is a 53-kDa protein encoded by the SCL36A1 gene and is a member of the solute carrier family 36 (3, 28). PAT1 mRNA is encoded by 11 exons with the translation start site in exon 2 and the termination site in exon 11. The last exon encodes the COOH terminus of the protein as well as a large 3′-untranslated region (7). Transport of proline, alanine, glycine, and GABA via PAT1 substrate, in A7r5 cells suggested an alternative role for PAT1 in SMCs than in transport. To shed light on the function of PAT1 in A7r5 cells, experiments with downregulation of the PAT1 level by use of a siRNA approach were conducted. The growth rates were evaluated, and knockdown of PAT1 led to induced cellular growth, suggesting a role for PAT1 in regulating cellular proliferation of SMCs.

PAT1; Slc36a1; smooth muscle cells; cellular localization; growth regulation

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not to be the case. Instead, we speculated whether PAT1 might play a role in SMC growth. The aim of the present study was therefore to investigate the function and expression of PAT1 in various models of SMCs.

MATERIALS AND METHODS

Unless otherwise stated, chemicals and reagents mentioned in this section were from Sigma-Aldrich (St. Louis, MO).

Cell culture and animals. SMC A7r5 cells, derived from embryonic rat aorta, were used in cell passages 12–35 [European Collection of Cell Cultures (ECACC), Salisbury, UK]. Caco-2 cells were from ATCC (Manassas, VA), and primary SMCs were derived from the aorta and the colon of male Wistar rats (250–280 g) obtained from the Department of Pharmacology and Pharmacotherapy at the University of Copenhagen. Animals were kept under standard laboratory conditions with free access to standard laboratory food and tap water. The study on animal tissue was conducted according to the guidelines of the Danish Animal Experiments Inspectorate.

Cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO2 atmosphere. Once a week, the rat cells were divided by trypsinization in ratio of 1:2 or 1:3, and the medium was replaced two to three times a week. Caco-2 cells were divided in a ratio of 1:5 two to three times a week.

Isolation of aortic SMCs was carried out as described earlier (25). Briefly, the aorta was rinsed with HBSS buffer (pH 7.4; Invitrogen, Carlsbad, CA). Embedded in DMEM, the adventitia and heart were removed, and aorta was cut into smaller pieces. The pieces of tissue were collected in DMEM containing collagenase type II (66 U/ml; Worthington Biochemical, Lakewood, NJ) and incubated for 6 h at 37°C and 5% CO2. When confluent, the cells were cultured using the same protocol as described for the A7r5 cells. Isolation of SMCs from rat colon were carried out as earlier described (16). In short, after removal of the rat colon from rat, the tissue was washed several times in HBSS buffer, pH 7.4. Mucosa and serosa were removed, and the tissues were cut into small pieces and incubated overnight in normal growth medium containing collagenase type II (66 U/ml; Worthington Biochemical, Lakewood, NJ) and incubated for 6 h at 37°C and 5% CO2. When confluent, the cells were cultured using the same protocol as described for the A7r5 cells.

RNA interference. Cells were transfected with small interfering RNA (siRNA) duplexes directed against PAT1, GAPDH, or GFP mRNA (Invitrogen), using HiperFect according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Transfection complexes were formed by 5- to 10-min incubation at room temperature. Briefly, 5 × 104 cells were seeded into 24-well plates and siRNA complexes [37.5 ng siRNA and 3 μl Hiperfect in OptiMEM (100 μl); GIBCO, Carlsbad, CA] were added. The transfection was repeated after 24 h. To avoid cell confluence, the cells were transferred to six-well plates 48 h after the first transfection. The number of cells in each well was counted 48, 72, and 96 h after the first transfection. The results are reported as means ± SE from nine different transfections for each siRNA, meaning three different transfections and three different counts for each time point.

PAT1 transfection. The A7r5 cells were transfected with the mammalian expression vector pCDNA3.1+ (Invitrogen) or with pCDNA3.1+ containing either the human PAT1 cDNA sequence (hPAT1; a kind gift from Prof. V. Ganapathy, Dept. of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA) or a truncated version of hPAT1 lacking a 3′-terminal untranslated region (hPAT1-truncated (7); a kind gift from S. Frolund, Dept. of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark). The transfections were performed using GenePorter2 according to the manufacturer’s protocol (Genlantis, San Diego, CA). Briefly, 1 × 107 cells were seeded in a 24-well plate and allowed to grow for 20 h. Four hours prior to transfection, growth medium was removed and replaced with serum-free medium; 24 h after seeding, DNA complexes (1 μg DNA, 25 μl DNA Diluent B, 3.5 μl GenePorter2, and 21.5 μl OptiMEM) were added (total transfection volume 250 μl). The cells were allowed to grow for an additional 24 h in the presence of DNA complexes, after which they were transferred to permeable 12-well plate polycarbonate filters (Corning, Corning, NY). Immunostaining and confocal laser scanning microscopy were performed 2 days after transfection. The transfections were repeated twice.

Whole cell extract. Cells were collected by trypsinization and centrifugation and washed twice with ice-cold PBS. Unless otherwise stated, all procedures were carried out at 4°C. The cell pellet was resuspended in five times the volume of the pellet in RIPA buffer (10 mM Tris-HCl, pH 8.0, 0.14 M NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 10 μg/ml proteinase inhibitors, 1 mM PMSF, and 10 mM Na-pyrophosphate) and incubated on ice for at least 15 min. Kidney, liver, and skeletal muscles from the quadriceps, heart muscles from the right atrium, smooth muscles from the jejunum, colon, and rectum were harvested from male Wistar rats produced by Charles River (Sülzfeld, Germany). RIPA buffer (300 μl) was added to ~5 mg of rat tissue and homogenized by sonication using a Branson Sonifier Cell Disruptor 15B (Branson, Danbury, CT) for 20 s three to five times with a power input of 5. The proteins were separated from cell debris by centrifugation for 10 min at 13,000 g, and the protein containing supernatant was collected and analyzed by Western blot analysis. The PAT1 levels in the different cell types are reported as means ± SE from a minimum of three preparations from three different animals.

Cellular fractionation of A7r5 cells. Cellular fractionation was performed as previously described (26). Unless otherwise stated, all procedures were carried out at 4°C. Approximately 1 × 107 confluent A7r5 cells were collected by trypsinization and centrifugation and washed twice with PBS. The pellet was suspended in five times the volume of the pellet with buffer N (15 mM Tris-HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 250 mM sucrose, 1 mM PMSF, 2.5 mM protease inhibitors, and 1 mM Na-pyrophosphate) and incubated for 5 min. The supernatant (cytoplasmic fraction) was collected by centrifugation at 37°C, 3,000 g for 20 s three to five times with a power input of 5. The pellet was washed twice with buffer N, resuspended in five times the volume of the pellet with buffer N + 0.6% (vol/vol) NP-40, and incubated for 10 min. The supernatant (nucleoplasm fraction) was collected by centrifugation for 5 min at 3,000 g, and the washes of the pellet with buffer N were repeated. The pellet was suspended in five times the volume of the pellet in buffer N, incubated for 10 min at 37°C, and separated in three tubes. MNase1 (5 U; Invitrogen) was added to each tube and incubated at 37°C for 5, 15, or 30 min followed by incubation on ice for 10 min and centrifugation (10 min at 10,000 rpm). The resulting supernatants were collected (S2 fraction, heterochromatin), and the pellets (fraction P) were suspended in buffer P [15 mM Tris-HCl, pH 7.5, 60 mM KCl, 450 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 1 mM PMSF, 2.5 mM protease inhibitors (Roche, Basel, Switzerland) and 1 mM Na-pyrophosphate]. The collected protein samples were analyzed by Western blot analysis. DNA was purified from fraction P by incubating the samples with proteinase K (100 μg/ml; overnight at 37°C) followed by Na-acetate and ethanol precipitation. The degree of DNA digestion was analyzed by gel electrophoresis on a 1% agarose (Invitrogen) gel contain ethidium bromide (Invitrogen).

Western blot analysis and antibodies. The protein concentration was determined by the Bradford assay prior to SDS-PAGE, and equal amounts of total protein from the different cell lysates were mixed with Laemmli-SDS-sample buffer and heated at 95°C for 10 min. Western blot analysis was performed by electrophoresis on Mini-
PROTEIN TGX gels (Bio-Rad, Hercules, CA) and transfer to PVDF membranes according to the manufacturer’s protocol (Thermo Fischer Scientific, Waltham, MA). Proteins were visualized via chemiluminescence and a FluorChem Q imagine system (Alpha Innotech, Santa Clara, CA). The following primary antibodies were used: rabbit polyclonal anti-GAPDH FL-335 0.2 mg/ml in a 1:200 dilution (Santa Cruz, Santa Cruz, CA), rabbit polyclonal anti-PAT1 raised against either the human NH2-terminal (N-PAT1) peptide sequence MSTQR-LRNEEYHYSSTDSS 0.78 mg/ml in a 1:200 dilution or an intermediate sequence (I-PAT1) 1.06 mg/ml in a 1:200 dilution (both from 21st Century Biochemicals, Marlboro, MA). I-PAT1 contains a mix of antibodies raised against an intermediate peptide sequence FVS-RAPEHELVLVDFL and IVSRPVERFELVL found in rat and mouse. The secondary antibody was polyclonal goat anti-rabbit immunoglobulin-HRP 1.5 mg/ml in a 1:1,000 dilution (Invitrogen). All antibodies were used according to the manufacturers’ instructions.

**Immunofluorescence.** Cells were seeded and grown on either collagen-coated (3.39 μg/cm²) glass coverslips in six-well plates or permeable 12-well polycarbonate filters. Seeding concentration was 2.5 × 10⁵ cells per six-well plate and 3.0 × 10⁶ cells per filter for analyses of confluent cells 7 days after seeding. For analyses of nonconfluent cells, 1.0 × 10⁶ cells were seeded per six-well plate and analyzed 24 h after seeding. Cells deprived of amino acids were incubated in PBS supplemented with Ca²⁺ and Mg²⁺ (100 mM CaCl₂ + 100 mM MgCl₂) 16 h prior to analyses.

Before immunostaining, cells were washed with PBS, fixed for 15 min at room temperature with 4% formaldehyde (Alfa Aesar, Ward Hill, MA) in PBS containing 0.2% Triton X-100 and washed three times with PBS. Fluorescent labeling was performed as described previously (21, 34). Primary antibodies used were rabbit polyclonal anti-PAT1 raised against either the NH2-terminal (N-PAT1) or an intermediate sequence (I-PAT1), rabbit polyclonal anti-GAPDH FL-335. Secondary antibody was goat anti-rabbit IgG (H+L; Invitrogen). Actin filaments were visualized using phalloidin 488 Alexa (2 mg/ml) in a 1:200 dilution (Invitrogen). The cell nucleus was stained with propidium iodide (8 μg/ml; 2 min at room temperature), and the cells were mounted in Aqua/Polymount coverslipping medium. Three-dimensional data sets were acquired using a confocal laser scanning microscope (CLSM) equipped with an Axiovert 100M microscope under ×20 or ×63 magnification, using 1.4 NA Plan apochromatic objectives.

**Radiolabeled flux experiments in A7r5 cells.** A7r5 cells were seeded at a density of 3.1 × 10⁴ cells/cm² on the bottom of tissue culture 12-well plates and cultured for 7 days. Cells were used in passages 12–18. L-[³H]Proline (PerkinElmer, Waltham, MA) uptake studies in A7r5 cells were performed in HBSS buffer supplemented with 0.05% (wt/vol) BSA at pH 6 or 7.5. The concentrations of labeled L-[³H]proline and D-[¹⁴C]mannitol (PerkinElmer) were 6.67 nM and 8.5 μM, respectively, in all uptake or flux experiments, and the specific activity was kept at 0.5 μCi/ml. The sodium dependency of L-[³H]proline uptake was investigated using the procedure as described above but applying sodium-free HBSS (1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 5.4 mM KCl, 0.4 mM KH₂PO₄, 137.5 mM NaCl, 5.56 mM D-glucose). The uptake rates of nonlabeled 0 to 5,000 μM L-proline and D-[¹⁴C]mannitol into A7r5 cells were carried out in an HBSS buffer adjusted to pH 7.4. The pH-dependent uptake was measured with L-[³H]proline and D-[¹⁴C]mannitol added to HBSS solutions, which were buffered with either 10 mM MES (used for pH 5.5, 6.0, or 6.5) or 10 mM HEPES (used for pH 7.0, 7.4, or 8.0). The pH values of all HBSS solutions were adjusted with HCl (1 M) or NaOH (1 M) solutions. Before initiation of the uptake experiments, the cells were equilibrated with prewarmed HBSS buffer at 37°C for 15 min at a desired pH value according to the experiment. The uptake experiments were initiated by adding pH-adjusted HBSS buffer with L-[³H]proline and D-[¹⁴C]mannitol and a relevant compound such as L-proline, GABA, or 5-HTP in a specified concentration. The uptake studies were performed for 10 min at 37°C under agitation, after which the medium was removed and the cells were rapidly washed three times with fresh, ice-cold HBSS. Cells were lysed in 300 μl of lysis buffer A (1% Triton X-100, 150 mM NaCl, and 50 mM Tris, pH 8.0) for 2 min. Thereafter, the cell lysates were filled into scintillation vials and 2 ml of Ultima GoldTM scintillation cocktail (PerkinElmer) was added. The samples were shaken prior to liquid scintillation counting to quantify the amount of radioactivity accumulated in the cell monolayers. The data are presented as means ± SE based on three repetitions (n = 3) for each independent cell passages (n = 3).

**Statistical analysis.** The statistical analysis was performed on GraphPad Prism software (version 5.01) and GraphPad inStat (version 3.0) or Excel (version 14.1.2). Statistical comparisons of mean values were made using paired two-tailed Student’s t-test or one-way ANOVA followed by Dunnett’s or Tukey’s multiple comparison test in the case of comparing mean vs. control. The level of significance was 95%; hence, P < 0.05 was considered significant; n was the number of repetitions within a given cell passage.

**RESULTS**

**PAT1 expression in smooth muscles.** The presence of PAT1 protein in A7r5 cells, rat heart, and smooth and skeletal muscles was investigated by Western blot analysis using a PAT1 antibody directed toward the NH2-terminal part of PAT1 (N-PAT1) (Fig. 1). Total protein lysates were prepared from the different muscle types as well as from rat liver, kidney, and human Caco-2 cells as a control. In Fig. 1, the protein levels are normalized to the liver samples. The highest expression level of PAT1 protein was, as expected, observed in the intestinal Caco-2 cells (P < 0.05). PAT1 was detected in smooth muscle tissue derived from colon and jejunum and in skeletal muscle at a level comparable to that of the liver. Expression levels in the kidney and A7r5 cells were about

![Fig. 1. Western blot analysis of proton-coupled amino acid transporter 1 (PAT1) protein levels in Caco-2 and A7r5 cells as well as in in rat tissues (liver, kidney, heart, skeletal muscle, and smooth muscle from jejunum and colon). Results are reported as means ± SE of 3–5 independent experiments in arbitrary units (AU) relative to liver PAT1 expression. Expression levels were tested using ANOVA with Dunnett’s posttest using the liver level as control. **P < 0.01.](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.00322.2013)
one-half that in the liver (not significantly different), and the lowest level of PAT1 was observed in the heart.

Proline uptake in A7r5 cells is not proton coupled or inhibited by PAT1 inhibitors. To investigate whether PAT1 is involved in amino acid transport in A7r5 cells, the L-[3H]proline uptake rate was measured at pH 6.0 and 7.4 in the absence or presence of GABA or 5-HTP, a PAT1 substrate and inhibitor, respectively (Fig. 2, A and B). The uptake rate of L-[3H]proline was not affected by the presence of GABA or 5-HTP at either pH 6.0 or pH 7.4 (P > 0.05). Only a surplus of nonlabeled L-proline was able to inhibit the L-[3H]proline uptake significantly compared with the control at both pH 6.0 and 7.4 (P < 0.05; Fig. 2A).

Uptake of L-[3H]proline was measured at pH 7.4 in the presence or absence of sodium. The uptake of L-[3H]proline in the presence of sodium was 0.45 ± 0.04 fmol·cm⁻²·min⁻¹ and 0.25 ± 0.04 fmol·cm⁻²·min⁻¹ in the presence of unlabeled L-proline and sodium. When sodium chloride was replaced with choline chloride, the uptake of L-[3H]proline was 0.24 ± 0.03 and 0.24 ± 0.03 fmol·cm⁻²·min⁻¹ in the presence of unlabeled proline or choline chloride, respectively. The uptake of L-[3H]proline in A7r5 cells is thus sodium dependent.

The uptake of L-[3H]proline was moreover measured as a function of extracellular pH ranging from pH 5.5 to 8.0 (Fig. 2C). L-[3H]proline uptake increases with increasing extracellular pH values of the uptake buffer, and the uptake was significantly higher at pH 6.5, 7.0, 7.5, and 8 than at pH 6.0 (P < 0.05). The kinetic parameters for cellular L-[3H]proline uptake were estimated based on the concentration-dependent proline uptake (Fig. 2D). The uptake of L-[3H]proline was measured over a concentration range of 0 to 5 mM. Kinetic analysis performed by nonlinear regression of carrier-mediated uptake data revealed a Michaelis constant (Km) of 1.3 ± 0.3 mM and a maximal uptake rate of transport (Vmax) of 16.1 ± 1.2 pmol·cm⁻²·min⁻¹ (r² = 0.945). The uptake of L-[3H]proline was thus concentration dependent. Collectively, this suggests that uptake of L-[3H]proline in A7r5 cells is carrier mediated and sodium and proton dependent but not inhibited by GABA and 5-HTP. Although the proline uptake is pH dependent, it is the opposite of the pH dependency known for PAT1, indicating that cellular proline uptake was not mediated by PAT1.

Nuclear localization of PAT1 in SMCs. Fluorescent immunostaining and confocal microscopy showed PAT1 localization in the same region as the cell nucleus in A7r5 cells (Fig. 3A). The cytoskeleton was visualized by staining actin filaments. Two different PAT1 antibodies directed toward either N-PAT1 or an intermediate part of PAT1 (I-PAT1) were used. The control (no primary antibody) showed the specificity of the secondary antibody. The intensity of the laser at 530 nM was twice as high as the other representations in order to highlight the specificity. The magnification is similar to that of actin.

Fig. 2. Uptake rate of L-[3H]proline (6.67 nM, 0.5 Ci/ml) in A7r5 was determined over a 10-min period. A: L-[3H]proline uptake rate in the presence of 5-hydroxytryptophan (5-HTP) or 5 mM proline at pH 6.0 or 7.4. B: L-[3H]proline uptake in the presence of GABA at pH 6.0 or 7.4. C: uptake rate of L-[3H]proline at different pH values of the extracellular buffer. D: concentration-dependent proline uptake rate. Experiments were performed in triplicate in 3–4 cell passages; values are given as means ± SE. Data were tested using one-way ANOVA followed by Tukey’s posttest. ***p < 0.001.
The specificity of the antibodies was supported by immunostaining of PAT1 in Caco-2 cells, where PAT1 expression was detected mainly in the apical cell membrane, as expected (Fig. 3B). Intracellular localized PAT1 influences growth and amino acid sensing (12, 17). PAT1 localization might therefore be influenced by cell proliferation or starvation. However, fluorescent immunostaining of PAT1 in dividing A7r5 or confluent A7r5 deprived of nutrients by incubating the cells in isotonic PBS supplemented with calcium and magnesium for 16 h before fixation did not reveal any alteration of the PAT1 localization (Fig. 4A). The cells were also stained with C-PAT1 with similar results (not shown). The images are overexposed to highlight a potential localization of the PAT1 in the cytoplasmic compartment or intracellular organelles such as lysosomes. The morphology was similar to the results obtained for cells grown to 40% (not shown), but not as elongated as cells grown at 100% confluence (Fig. 1). During cell division PAT1 seemed to be associated with chromatin (Fig. 4A, row 2, indicated by arrow and inset). To ensure that these observations were not cell line specific or due to the cancerous state of A7r5 cells, PAT1 localization was investigated in primary SMCs derived from either rat colon or aorta. In vitro cultured SMCs derived from colonic muscularis propria, grown for 7 days on collagen-coated coverslips (passage 4), showed a similar distribution of N-PAT1 with predominant localization within the nucleus, as the signal colocalized with the propidium iodide signal. The cells were also stained with C-PAT1 with similar results (not shown). The immunocytochemical staining was repeated with two different passages (4 and 5) both with similar results. In SMCs from aortic muscularis propria grown for a week on collagen prior to fixations (passage 5), the distribution of N-PAT1, seemed to be predominantly within the nucleus. The cells were also stained with C-PAT1 with similar results (not shown). The sections illustrate a potential localization of the PAT1 in the cytoplasmic compartment together with the GAPDH signal. The morphology was similar to the results obtained for A7r5 cells grown on collagen, as the actin filaments were elongated (not shown). The immunocytochemical staining was repeated for three different passages (passages 5–8), all with similar results.

To confirm the nuclear localization of PAT1, cellular fractionation studies were performed. Confluent A7r5 cells were separated into three fractions containing cytoplasmic, nuclear, and chromatin-bound proteins. The chromatin fraction was further divided into three fractions by MNase1 digestion to separate transcribed DNA from nontranscribed DNA. The amount of PAT1 protein in the different fractions was examined by Western blot analysis (Fig. 4B). The chromatin was digested for 5, 15, or 30 min by MNase1, leading to almost complete DNA digestion at the last time point (Fig. 4C). Low levels of PAT1 were observed in both the cytoplasm and the nucleoplasm, whereas the majority of PAT1 protein was purified together with the chromatin fraction independently of the degree of digestion, suggesting a uniform distribution of PAT1-bound DNA. No further analyses were made to decide at which level the MNase1 digestion had led to separation of transcribed and nontranscribed DNA, since the PAT1 chromatin association was independent of level of digestion. GAPDH was found mainly in the cytoplasmic and soluble nuclear fraction, and only trace amounts could be detected together with the chromatin-bound fraction (Fig. 4B).
PAT1 COOH-terminal direct nuclear localization in SMCs.

The PAT1 transcript contains a large terminal untranslated region the function of which is unknown (7). To investigate whether a localization signal could be located in this region of the PAT1 transcript, two different PAT1 constructs containing either the sequence for the entire human PAT1 transcript (hPAT1) or the PAT1 transcript without the untranslated 3′-sequence (hPAT1 truncated) were transiently transfected into A7r5 cells. As a control, A7r5 cells were transfected with the vector only (pcDNA3.1/H11001; Fig. 5A). The transfections were analyzed by confocal analysis of PAT1, and the images represent our observations from several cells seen in two different preparations. Figure 5B shows the differences between the PAT1 signal in nontransfected cells vs. the signal observed in positively transfected cells with either of the constructs. The images of positively transfected cells were overexposed to visualize the PAT1 level in the surrounding nontransfected cells. It is evident that the hPAT1-truncated protein lost the localization specificity and is distributed all over the cell, whereas hPAT1 is localized within the nucleus of A7r5. Figure 5C shows transfected cells with either plasmid both vertical and parallel to the optical axis. Here, it was possible to observe that hPAT1-truncated is not evenly distributed all over the cell, since less protein is seen in the nucleus and the majority seems to be localized within the cytoplasm. Transfection with hPAT1 leads to nuclear localization of almost all PAT1 protein. Figure 5D illustrates PAT1 in different sections of the cells from the nucleus toward the cell membrane. Again, it is evident that full-length hPAT1 was transported mainly to the nucleus, whereas the majority of the truncated hPAT1 protein was located within the cytosol, and only a low level of the expressed PAT1 was found within the cell nucleus. Attempts to create cells stably transfected with PAT1 were not successful, as positively transfected cells stopped proliferating (data not shown).

PAT1 regulates cellular growth in SMCs. To study a possible role of PAT1 in growth of SMCs, we downregulated PAT1 by using a siRNA approach and followed the cell proliferation for 4 days. A7r5 cells were transiently transfected with siRNA directed toward PAT1, GFP, or GAPDH mRNA. The uniformity and specificity of PAT1 siRNA- and GAPDH siRNA-transfected SMCs were demonstrated by fluorescent immunostaining (Fig. 6A). The negative controls, which were treated under similar conditions but without the additions of siRNA
nucleotides, confirmed that the delivery method did not induce any changes in the distribution of the specified proteins; the signals still colocalized with the nuclear propidium iodide stain. Both antibody solutions N-PAT1 and C-PAT1 targeting different epitopes of the PAT1 protein showed reduced levels of intensity in the A7r5 cells transfected with siRNA targeting PAT1 translation (C-PAT1 not shown). The signal intensity was considerably reduced compared with both the negative controls and cells transfected with siRNA targeting the translation of the endogenous proteins.

Transfection normally causes changes in cell growth due to the harsh treatment with a transfection agent. To ensure that transfection with siRNA did not greatly obscure the normal growth of A7r5 cells, the effect of the treatment was analyzed by comparing the growth of cells transfected with siRNA directed toward GFP (noncoding in A7r5 cells) with the growth rate of untreated cells (Fig. 6B). The transfection with GFP-siRNA showed a similar minor reduction of cells growth at all time points analyzed. When the growth rate of A7r5 cells treated with PAT1 siRNA to that of A7r5 siRNA-GFP was compared, we found cells with a lower level of PAT1 to proliferate faster than the cells with a normal growth rate. A significant difference in growth rate was observed 4 days after PAT1 siRNA transfections compared with the GFP siRNA transfections ($P < 0.05$; Fig. 6B). As expected, GAPDH siRNA-transfected cells stopped growing.

The level of PAT1 mRNA is influenced by the availability of amino acids or stress in human skeletal muscles (11, 12). However, no difference of the PAT1 protein level could be detected when we examined whether cellular proliferation had...
any effect on PAT1 levels in A7r5 cells (Fig. 6C). Whole cell extract was prepared from confluent or dividing A7r5 cells, and the PAT1 levels in the extracts were examined by Western blot analysis. GAPDH levels were tested as a loading control, and Caco-2 cells were used as a control cell line (Fig. 6C).

DISCUSSION

After the initial cloning of PAT1 from rat brain (28) it became apparent that PAT1 is a multifunctional protein acting as a membrane transporter of amino acids and some drug substances (14, 18) and an amino acid sensor (7, 11, 15, 17, 23). Here, we show that PAT1 is expressed in SMCs and is located in the nucleus associated with chromatin and independent of cell proliferation or starvation. The 3′-untranslated region of the PAT1 transcript appears to be important for nuclear localization. Knockdown studies of PAT1 in A7r5 cells indicated a role in growth regulation, and on the basis of our results we propose PAT1 to have an inhibitory effect on growth of SMCs.

PAT1 is localized within the nucleus in SMCs and the localization is directed by the 3′-untranslated region of the PAT1 transcript. The presence of the mammalian PAT1 mRNA in liver, kidney, heart, and skeletal muscles has previously been reported (1, 3, 7, 22, 29). Here, we confirmed that the PAT1 protein is expressed in SMCs albeit not as abundantly as in epithelial cells. Surprisingly, in SMCs PAT1 was localized in the cell nuclei associated with chromatin. It is possible that a small fraction of the nuclear PAT1 was placed within the nuclear envelope, although we found that the majority was chromatin bound in A7r5 cells, independently of cellular starvation or confluence of the cells. Nuclear localization of PAT1 was confirmed in primary SMCs derived from rat aorta and colon. The uptake studies confirmed that transport of the prototypical PAT1 substrate proline into A7r5 cells did not show any characteristics of PAT1-mediated transport. The uptake of proline showed a reverse pH dependency of that of PAT1 and lack of inhibition by known PAT1 inhibitors; hence, PAT1 does not seem to be associated with the cell membrane or involved in cellular uptake of PAT1 substrates in SMCs. PEPT1, a peptide transport protein, is likewise located in the nuclei of SMCs (2) and in the membrane of epithelial cells (5). The role of these proton-coupled nutrient transporters in the nucleus of SMCs remains unknown.

Here, we show that the nuclear localization of PAT1 is dependent on a 3′-untranslated region of the PAT1 transcript, showing the importance of a sequence that until now has been of unknown function. How the interplay between localization, signal sequences, and protein modifications is directed and dependent on cell type leading to nuclear localization in SMCs, intracellular localization in kidney cells (33), and membrane localization in intestinal epithelial cells (7) is a challenging and still unanswered question.
PAT1 is involved in cellular proliferation in SMCs. Besides cellular uptake of amino acids and drug substances, PAT1 plays a role in cell growth. Overexpression of PATH, a PAT1 analog, in D. melanogaster causes a slight overgrowth of the fly’s eye, and depletion of PAT1 causes reduced proliferation of MCF-7 breast cancer cells (17, 23). Similarly, our observations indicate a role of PAT1 in cell growth, albeit with an opposite regulatory effect than earlier observed; PAT1 depletion caused faster cell growth rather than reduced growth, suggesting a yet undiscovered regulatory function of PAT1 within SMCs, maybe within transcription regulation or DNA organization during cell division.

Since a decrease of PAT1 protein increases cellular proliferation, we wondered whether the PAT1 protein level would differ between dividing and confluent cells. When the protein levels in ~30% or 100% confluent cells were compared, no difference was registered. A lack of differences in protein levels was also observed previously in studies investigating the expression levels of transport proteins of skeletal muscle biopsies (12). Here, it was observed that an increased level of available nutrition followed by starvation triggered an increase of the PAT1 mRNA level 1 h after addition of nutrients, and the PAT1 mRNA level was restored to normal 2 h later. For other transporters, such as LAT1, CD98, and SNAAT2, mRNA levels were increased in a similar manner as PAT1. However, only a minor, increase, if at all, in the protein levels of the transporters was observed, suggesting that the change in mRNA levels was not reflected by a similar change in protein levels, or a high turnover number of the proteins might mask a possible change in PAT1 protein level.

PAT1 has until now been associated with functions involving amino acids independent of localization. Thus, it has been speculated that PAT1 has a possible function of as a sensor of the availability of amino acids or as a signaling protein informing the TORC1 machinery about the presence of amino acids (11, 15, 17, 23). In agreement with earlier studies (15, 17, 23), the present study demonstrates a role of PAT1 in growth regulation. However, no indications suggesting an involvement of amino acids in this process was observed, although there is the possibility that PAT1 allows cell division when the availability of amino acids is sufficient.

It is not possible to transfer the knowledge of PAT1 in one cell type to another, making it extremely challenging to map the function and localization of PAT1 in cells. Understanding how the protein is transcribed, translated, and modified in the different cells types might hold the key to understanding as well as a lot of information about the possibilities of protein processing in general.

In conclusion, this study has demonstrated that PAT1 is expressed in SMCs with a nuclear localization. The protein appears to be involved in regulating cell growth rather than cellular transport of amino acids.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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