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GLUT10 – lacking in arterial tortuosity syndrome – is localized to the endoplasmic reticulum

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Keywords:	GLUT10, arterial tortuosity syndrome, endoplasmic reticulum
Abstract:	GLUT10 belongs to a family of transporters that catalyze the uptake of sugars/polyols by facilitated diffusion. Loss-of-function mutations in the SLC2A10 gene encoding GLUT10 are responsible for arterial tortuosity syndrome (ATS). Since the subcellular distribution of the transporter is dubious, we aimed to clarify the localization of GLUT10. In silico GLUT10 localization prediction indicated its presence in the endoplasmic reticulum (ER). A perinuclear distribution of GLUT10 was demonstrated by immunofluorescence. Colocalization with mitochondrial marker was not observed. Immunoblotting revealed that GLUT10 protein was present in the microsomal fraction of the cells. Expression of exogenous, tagged GLUT10 in fibroblasts from an ATS patient revealed a colocalization with ER markers. The results demonstrate that GLUT10 is present in the ER.

GLUT10 – lacking in arterial tortuosity syndrome – is localized to the endoplasmic reticulum

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ABSTRACT

GLUT10 belongs to a family of transporters that catalyze the uptake of sugars/polyols by facilitated diffusion. Loss-of-function mutations in the *SLC2A10* gene encoding GLUT10 are responsible for arterial tortuosity syndrome (ATS). Since the subcellular distribution of the transporter is dubious, we aimed to clarify the localization of GLUT10. *In silico* GLUT10 localization prediction indicated its presence in the endoplasmic reticulum (ER). A perinuclear distribution of GLUT10 was demonstrated by immunofluorescence. Colocalization with mitochondrial marker was not observed. Immunoblotting revealed that GLUT10 protein was present in the microsomal fraction of the cells. Expression of exogenous, tagged GLUT10 in fibroblasts from an ATS patient revealed a colocalization with ER markers. The results demonstrate that GLUT10 is present in the ER.

Keywords: GLUT10, arterial tortuosity syndrome, dehydroascorbic acid, endoplasmic reticulum, nuclear envelope, Fe^{2+}/2-oxoglutarate dependent dehydrogenases.

INTRODUCTION

The Major Facilitator Superfamily (MFS) of membrane transporters consists several thousands of membrane transporters, including the *SLC2A* (solute carrier 2A) gene family encoding glucose transporter (GLUT) proteins. GLUT family members – presently 14 proteins – are grouped into three different classes based on their sequence similarities; GLUT10 encoded by *SLC2A10* gene belongs to class 3 [1]. Loss-of-function mutations in *SLC2A10* are responsible for arterial tortuosity syndrome (ATS, OMIM #208050), which is a monogenic autosomal recessive heritable connective tissue disorder characterized by elongation and generalized tortuosity of the major arteries. The clinical phenotype also includes aneurysms of large arteries and stenosis of the pulmonary artery. Patients usually present dysmorphic facial features and other connective tissue abnormalities such as hyperextensible skin and joint laxity. Histopathological analysis shows extreme disorganization and fragmentation of elastic fibers in the arterial wall. To date, all of *SLC2A10* gene mutations cause a loss of function of GLUT10 [2-7]. However, the precise subcellular localization, and the exact physiological role of GLUT10 in the ATS pathogenesis remain still debated. Three mechanisms have been proposed to explain the link between defective GLUT10 activity and the arterial defects described in ATS. For a detailed description of these hypotheses see [8]. Briefly, the first hypothesis, based on the perinuclear localization of GLUT10 in human fibroblasts, assumes that the GLUT10 is a glucose transporter in the nuclear envelope. Glucose-dependent upregulation of the TGFβ signaling inhibitor proteoglycan decorin is missing in ATS, also explaining the upregulation of the TGFβ pathway observed in ATS cells [2]. The second theory suggests that GLUT10 is involved in transport of dehydroascorbic acid (DAA; the oxidized form of the potent antioxidant ascorbic acid, AA) across the mitochondrial inner membrane [9]. Upon being transported into mitochondrial matrix, DAA is reduced to AA, which can eliminate reactive oxygen species (ROS), thereby protecting the cell from oxidative stress. A third hypothesis postulated that GLUT10 acts as a DAA transporter in the ER membrane [10,11]. DAA, in turn, is reduced to AA in the ER lumen, where it acts as a cofactor of Fe^{2+}/2-oxoglutarate dependent dioxygenases. These enzymes catalyze the hydroxylation of prolyl and lysyl residues by, reactions crucial for maturation and folding of several extracellular matrix proteins.

Recently we demonstrated that *in vitro* translated GLUT10 protein functions as a DAA transporter in proteoliposomes [8]. Furthermore, DAA uptake in plasma membrane

permeabilized fibroblasts was greatly diminished in cells from ATS patients [8], suggesting that GLUT10 is localized in the endomembranes. Since there is no consensus regarding the subcellular distribution of GLUT10, the aim of the present study was to unambiguously clarify the localization of this transporter.

METHODS

Animals

Male CD-1 mice (20–25 g body weight), male Hartley guinea pigs (250–300 g body weight) and male Wistar rats (180–230 g body weight) were purchased from Charles River Hungary (Isaszeg). Guinea pigs were scorbutized as described in [12]. Use of experimental animals has been approved by the Department of Epidemiology and Animal Welfare of Budapest and Pest County Agricultural Office.

Preparation of subcellular fractions from the liver

Subcellular fractionation of guinea pig, mouse, rat and human liver was performed as described earlier [13]. ER-derived vesicles (microsomes) were prepared from liver homogenates as described in [13]. Human microsomes were obtained from Thermo Fisher Scientific, Waltham, MA USA 02451.

Cell cultures

Skin fibroblasts from an ATS patient and unrelated healthy donors were established from skin biopsies as previously reported [2,14]. The ATS patient was homozygous for the c.1334delG microdeletion [2]. Written informed consent was obtained from the patient and healthy individuals for skin biopsy procedure. This study was approved by the medical ethical committee of the University Hospital Spedali Civili of Brescia, Italy, and was performed in accordance with the Declaration of Helsinki Principles.

Dermal fibroblasts cultures were grown *in vitro* at 37°C in a 5% CO_2 atmosphere in Earle's Modified Eagle Medium (MEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 µg/ml penicillin and streptomycin (Life Technologies, Carlsbad, CA, USA). Cells were analyzed at the same *in vitro* passage number (5^{th} to 7^{th}).

Subcellular fractionation of human fibroblasts

Subcellular fractions were prepared from human control fibroblast cells as reported earlier [15] with minor modifications. The trypsinized cells were resuspended in sucrose-HEPES buffer (0.34 M sucrose, 10 mM HEPES; pH 7.4) and sonicated 5 times for 15 seconds at 4° C

using Sonic 300 Dismembrator (35% pulse cycle). Cell homogenates were centrifuged for 10 minutes at 1000g. The postnuclear supernatant were centrifuged for 20 minutes at 18000 g. Microsomes were recovered by ultracentrifugation for 60 minutes at 195000 g. Pellets were resuspended in 20 mM MOPS buffer (pH 7.2) and maintained in liquid N_2.

Western blots were carried out as described previously [13]. 20 µg protein was loaded from each fraction. The membranes were incubated with antibodies against GLUT10 (1:1000, Abcam, Cambridge, UK), VDAC1 (1:1000; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), cyclophilin D (1:2000; MitoSciences, Eugene, Oregon, USA) Grp94, Grp78 and GAPDH (1:5000; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA).

Construction of a tagged-pG10 expression vector

10 ng of the pG10 expression vector [8,14] were used as PCR template to amplify the full-length coding sequence of *SLC2A10,* from the Kozak consensus sequence to the last amino acid codon. The PCR product was gel-purified and directly inserted into the pEF6/V5-His-TOPO™ expression vector according to the manufacturer's instructions (Life Technologies). Prior to transfection, the pG10-tag plasmid was sequenced to verify the correct in-frame insertion of GLUT10 with the C-terminal V5-His amino acids present on the expression vector. Transient transfection of pG10-tag into skin fibroblasts of an ATS patient (P1, [8,14]) was achieved using the TurboFect transfection reagent in accordance with the manufacturer's instruction (Thermo Scientific).

Indirect immunofluorescence analysis

To analyze the GLUT10 distribution, control fibroblasts were grown 48 h and reacted for 2 min with 3% paraformaldehyde/0.5% Triton, 20 min with 3% paraformaldehyde, washed with 100 mM glycine/PBS, blocked for 30 min with 5% BSA and immunoreacted overnight at + 4° C with 20 µg/ml polyclonal rabbit anti-human GLUT10 antibody (Alpha Diagnostic Int. Inc., San Antonio, TX). After washing in PBS, cells were incubated for 1 h at room temperature with 1:1000 anti-rabbit secondary antibody conjugated to Alexa Fluor 488. The double staining was performed immunoreacting with 2µg/ml anti-cytochrome C monoclonal antibody (clone 6H2-B4) for 2 h and with an anti-mouse secondary antibody conjugated to Alexa Fluor 594. The signals were acquired by a cooled digital camera, DS Qi1, (Nikon, Japan) mounted on a Nikon Eclipse Ti inverted fluorescence microscope. The experiments were repeated three times.

The co-localization of GLUT10 with the protein disulfide isomerase (PDI) was analyzed in transfected ATS fibroblasts grown for 48 h. Cells were fixed in cold methanol and immunoreacted for 2 h with 1:100 rabbit polyclonal anti-PDI antibody (Novus Biologicals), which labels ER, and 1 μg/ml anti-V5 monoclonal antibody (Sigma Chemicals). Cells were incubated for 1 h with 1:1000 anti-rabbit and anti-mouse secondary Abs conjugated to Alexa Fluor® 488 and 594, respectively. IF signals were acquired by a CCD camera (SensiCam-PCO Computer Optics GmbH, Germany) mounted on Zeiss fluorescence Axiovert microscope. The experiments were repeated three times.

In silico analysis

The sequence of GLUT10 was retrieved from the Uniprot database (http://www.uniprot.org/). Prediction softwares used: Target P: http://www.cbs.dtu.dk/services/TargetP/ [16]; Mitoprot II: http://ihg.gsf.de/ihg/mitoprot.html [17]; Predotar: https://urgi.versailles.inra.fr/predotar/predotar.html [18]; Psort II: http://psort.hgc.jp/form2.html [19]; MultiLoc/TargetLoc: http://abi.inf.uni-tuebingen.de/Services/MultiLoc/ [20]; ngLOC: http://genome.unmc.edu/ngLOC/index.html [21]; YLoc: http://abi.inf.uni-tuebingen.de/Services/YLoc/webloc.cgi [22] and CELLO v2.5: http://cello.life.nctu.edu.tw/ [23]. The reliability of the prediction tools has been double checked by testing GLUT1-4 proteins and the ER marker protein CYP2E1 (data not shown).

RESULTS

In silico prediction of subcellular localization of GLUT10

Since previous studies led to controversial (or) debated results [9-11,24] concerning the cellular localization of GLUT10, our main objective was to assess this aspect. Our anticipatory *in silico* search for the prediction of the subcellular distribution of GLUT10 indicated a relatively high probability of ER localization and an absolutely low probability of mitochondrial occurrence for GLUT10 (Table I).

GLUT10 is predominantly present in the microsomal fraction

Henceforth, the subcellular localization of GLUT10 was investigated on subcellular fractions gained by differential centrifugation. Immunoblotting of the subcellular fractions from human control fibroblasts revealed that GLUT10 protein was also exclusively present in the microsomal fraction (ER-derived). The ER marker Grp94 appeared in the same fraction, while the mitochondrial and cytoplasmic markers – VDAC1, cyclophilin D and GAPDH, respectively – were absent or only slightly present in the microsomal fraction (Fig. 1A).

GLUT10 was also detected by immunoblotting in mouse, rat, guinea pig and human liver (Fig. 1B,C). Immunoblotting of the subcellular liver fractions from gulonolactone oxidase deficient human and guinea pig revealed the enrichment of GLUT10 in the microsomal fraction. Interestingly, GLUT10 was hardly present in the liver of ascorbate synthesizing rat and mouse. On the other hand, guinea pigs suffering from scurvy did not present higher GLUT10 levels than the corresponding controls. The ER marker Grp78 was present, while the mitochondrial marker VDAC was absent in the same fraction (data not shown).

GLUT10 shows a reticular pattern with perinuclear abundance in fibroblasts

Immunofluorescence of human control skin fibroblasts confirmed the perinuclear abundance of GLUT10, reported in previous studies [2,14]. A reticular distribution of the protein could be also observed, which was present in the area of the nucleus as well (Fig. 2). Investigation of the colocalization of GLUT10 and the mitochondrial protein

cytochrome c showed the lack of colocalization (Fig 2). It should be noted that in fibroblasts from ATS patients GLUT10 could not be detected by immunocytochemistry [2,5,14], while cytochrome c was present in a pattern similar to control cells (data not shown).

GLUT10 colocalizes with the ER marker protein disulfide isomerase

Our previous efforts for the demonstration of GLUT10 colocalization with an ER marker have been failed. Although several ER markers have been tested, GLUT10 immunoreaction somehow quenched the labeling of the ER marker proteins, most probably due to the different cell-fixing protocols required for the transporter and ER proteins tested. To overcome this problem tagged GLUT10 was transiently transfected into skin fibroblasts of ATS patient. This approach, revealed the colocalization of GLUT10 and the ER marker protein protein disulfide isomerase investigated by immunofluorescence (Fig. 3).

DISCUSSION

Although mutations in *SLC2A10* that encodes the transporter GLUT10 have been unequivocally identified as the genetic cause of ATS, the underlying pathogenetic mechanisms have not been clarified in details. The determination of the intracellular localization of GLUT10 is crucial for further clarifying the pathomechanism of ATS. Our results reported here demonstrate that GLUT10 localizes to the ER and nuclear envelope (NE) of fibroblasts.

The breakthrough paper of the field already showed the perinuclear abundance of GLUT10 in human fibroblasts, deciphered as localization in the NE [2]. However, a subsequent study found that GLUT10 was present in the Golgi apparatus and mitochondria in smooth muscle cells and insulin-stimulated adipocytes [9]. Here we confirmed the perinuclear localization, but a reticular distribution was also present in both the cytoplasm and nucleoplasm. The former can simply be explained by the presence of GLUT10 in the ER, while the latter phenomenon might be due to the nucleoplasmic reticulum, which has been reported in many cell types including fibroblasts [25]. Immunoblotting of subcellular fractions also revealed that GLUT10 was present in the microsomal fraction, containing ER-derived vesicles. The colocalization of GLUT10 with PDI was shown by immunofluorescence in GLUT10-reexpressing ATS fibroblasts confirming the presence of GLUT10 in ER.

Our results go against the mitochondrial localization of GLUT10 [9]. GLUT10 was not colocalized with mitochondrial marker in immunocytochemistry (Fig. 2), the protein was not present in the mitochondrial fraction of fractionated fibroblasts (Fig. 1), and the uptake of DAA was not decreased in mitochondria from ATS fibroblasts (unpublished data). The distinct localization might also be due to the species difference. However, according to *in silico* predictions, the mitochondrial localization has a low probability (Table I). Moreover, mitochondria are reportedly equipped by other AA and DAA transporters [26-29], which queries the importance of GLUT10 as a mitochondrial transporter [30].

The question arises: which function of DAA/AA is missing in the ER/NR lumen and/or nucleoplasm in ATS patients? Besides the general antioxidant role, AA acts also as a cofactor for 2-oxoglutarate/Fe^{2+} dependent dioxygenases [31]. Prolyl- and lysyl-

hydroxylases involved in the posttranslational modification of collagen and other extracellular matrix proteins, such as elastin, belong to this group of the enzymes [32], as many nucleoplasmic DNA and histone demethylases [33]. DAA has been nominated as an electron acceptor in the process of oxidative protein folding, *i.e*. in the formation of disulfide bonds [34]. Thus, shortage of AA in the nucleoplasmic and ER lumenal compartments can depress the production of extracellular matrix proteins at both epigenetic and posttranslational levels [24]. Indeed, changes of gene expression have been demonstrated by a recently published transcriptome analyses in ATS fibroblasts. The alterations affected the expression of several genes involved not only in TGFβ signaling and extracellular matrix homeostasis but also in specific pathways that control cell energy balance and the oxidative stress response [14]. In summary, the present results and our previous findings [8,14] reinforce the hypothesis that ATS is a vitamin C compartmentalization disease. Further studies are needed to elucidate the details of molecular mechanisms impaired by DAA depletion in these intracellular compartments.

LIST OF ABBREVIATIONS

AA, ascorbic acid; ATS, arterial tortuosity syndrome; DAA, dehydroascorbic acid; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; NE, nuclear envelope; NIDDM, non-insulin-dependent-diabetes-mellitus; NR, nucleoplasmic reticulum; ROS, reactive oxygen species; VDAC, voltage dependent anion channel.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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LEGEND TO FIGURES

Figure 1. *GLUT10 is present in the microsomal fraction of human fibroblasts and liver from various species*

(A) Immunoblotting of subcellular fractions prepared from control fibroblasts showed that GLUT10 protein was present in the microsomal (ER) fraction. The purity of the fractions was checked by immunoblotting of marker proteins; Grp94 for microsomes (ms), voltage dependent anion channel 1 (VDAC1) and cyclophilin D for mitochondria (mit), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the cytosol (cyt). (B) Immunoblotting of subcellular fractions of human liver. Abbreviations: Hom; homogenisate; PMSN, post-mitochondrial supernatant. (C) Immunoblotting of GLUT10 protein in the microsomal fractions from livers of different species. Abbreviations: M, mouse; R, rat; GP, guinea pig; sGP, scorbutic guinea pig. Representative blots out of four are shown.

Figure 2. *GLUT10 shows a perinuclear reticular abundance in human fibroblasts and does not colocalize with mitochondria*

Immunofluorescence in human control fibroblasts confirmed the perinuclear abundance of GLUT10 accompanied with a reticular pattern. Control fibroblasts were decorated with GLUT10 (green), cytochrome C (red) or both antibodies and images were acquired by fluorescent microscopy. GLUT10 did not show colocalization with mitochondria.

Figure 3. *GLUT10 colocalizes with the ER marker PDI*

Tagged GLUT10 was transiently transfected in fibroblasts of an ATS patient. The co-localization of GLUT10 (red) with PDI (green) was analyzed by immunofluorescence.

	Target P	Mitoprot	Predotar	PSORT II	MultiLoc / TargetLoc	ngLOC	yLoc	Cello
Location	**Probability of Location**							
Plasma membrane				43.5 %	0.12	14.46	99.8 %	4.855
ER	0.982		0,89	39.1 %	0.63		0.1 %	0.008
Extracellular space				4.3 %	0.03		0.1 %	0.061
Lysosome					0.06		0.0 %	0.029
Golgi apparatus				4.3 %	0.14		0.0 %	
Peroxisome					0.01		0.0 %	0.007
Mitochondrion	0.014	0.0097	0,00	4.3 %	0.0		0.0 %	0.009
Cytoplasm					0.0	40.73	0.0 %	0.010
Nucleus					0.0	8.909	0.0 %	0.004

Table I. Subcellular localization of GLUT10 predicted by an in silico analysis

The output of each prediction software is an estimated probability. The higher the output scores the higher the probability that the protein (GLUT10) is localized in the certain compartment. Empty cells: the prediction tool does not give probability score for this localization.

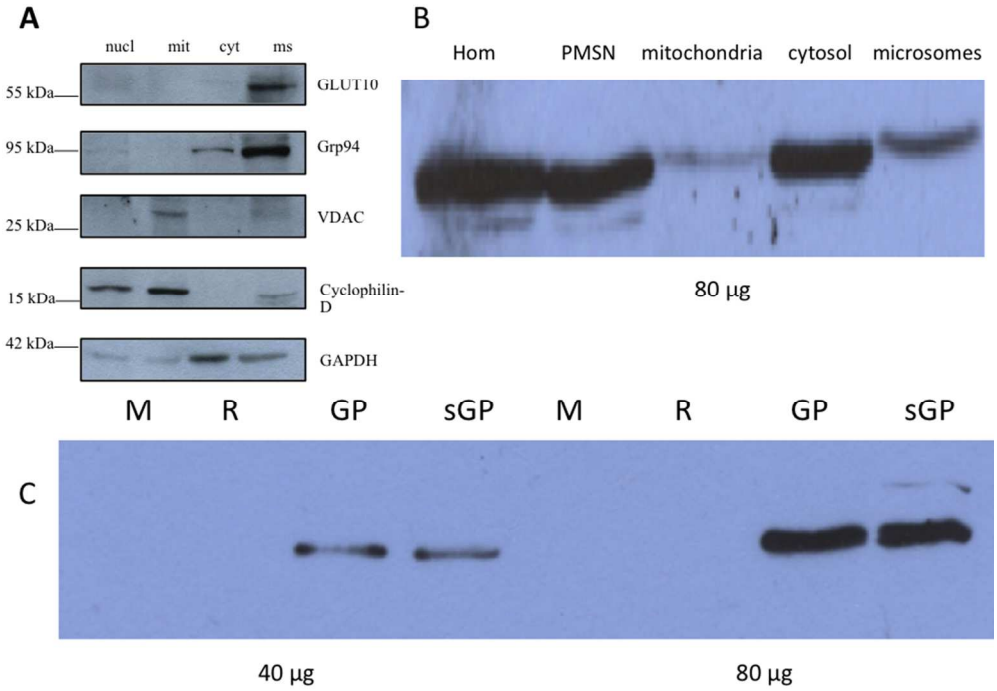

Figure1

352x264mm (72 x 72 DPI)

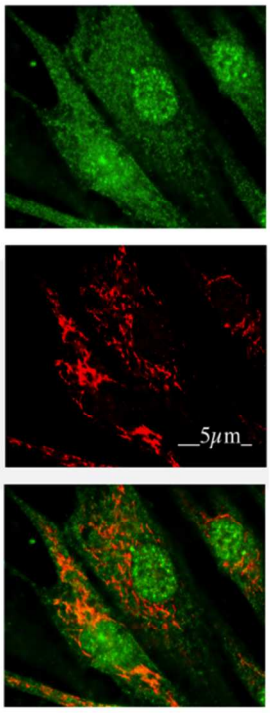

Figure2

352x264mm (72 x 72 DPI)

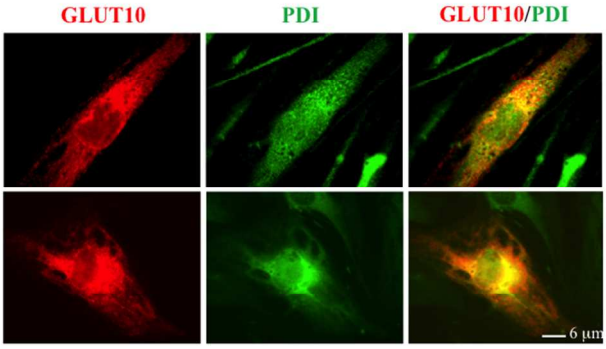

Figure3

352x264mm (72 x 72 DPI)