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N-linked Glycosylation of human SLC1A5 (ASCT2) transporter is critical for trafficking to membrane

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ABSTRACT

The human amino acid transporter SLC1A5 (ASCT2) contains two N-glycosylation sites (N163 and N212) located in the large extracellular loop. In the homology structural model of ASCT2 these Asn residues are extracellularly exposed. Mutants of the two Asn exhibited altered electrophoretic mobility. N163Q and N212Q displayed multiple bands with apparent molecular masses from 80 kDa to 50 kDa. N163/212Q displayed a single band of 50 kDa corresponding to the unglycosylated protein. The presence in membrane of WT and mutants was evaluated by protein biotinylation assay followed by immunoblotting. The double mutation significantly impaired the presence of the protein in membrane, without impairment in protein synthesis. [^{3}H]glutamine transport was measured in cells transiently transfected with the WT or mutants. N163/212Q exhibited a strongly reduced transport activity correlating with reduced surface expression. The same proteins extracted from cells and reconstituted in liposomes showed comparable transport activities demonstrating that the intrinsic transport function of the mutants was not affected. The rate of endocytosis of ASCT2 was assayed by a reversible biotinylation strategy. N212Q and N163/212Q showed strongly increased rates of endocytosis respect to WT. ASCT2 stability was determined using cycloheximide. N163Q or N163/212Q showed a slightly or significantly lower stability with respect to WT. To assess trafficking to the membrane, a brefeldin-based assay, which caused retention of proteins in ER, was performed. One hour after brefeldin removal WT protein was localized to the plasma membrane while the double mutant was localized in the cytosol. The results demonstrate that N-glycosylation is critical for trafficking.

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1. Introduction

Amino acid flux through biological membranes and distribution to tissues are both finely controlled by numerous transporters belonging to different solute carrier (SLC) families. Many of these amino acid transporters are characterized functionally [1–5] but much is still unknown concerning their structure/function relationships, protein–protein interactions and trafficking. Among these transporters, SLC1A5, known as ASCT2, is one of the most attractive to study due to its involvement in cell signaling and relevance to human pathology. ASCT2 was proposed to be a sodium dependent antiporter accepting alanine, serine and cysteine as the major substrates and also glutamine, alanine, serine, asparagine, threonine, valine and methionine [6,7]. More recent studies performed in proteoliposomes on the rat or human proteins revealed that ASCT2 is functionally asymmetrical since the substrate alanine, valine and methionine can be only inwardly transported [8,9]. The ability of ASCT2 to transport glutamine, along with its ubiquitous tissue expression, suggests that this transporter is a key player in glutamine homeostasis [10,11]. The potentially important role of ASCT2 in human cancer emerged from the finding that it is over-expressed in several malignancies together with SLC7A5, also known as LAT1 [1,12,13]. Over-expression of ASCT2 and LAT1 increases glutamine uptake allowing cancer cells to produce metabolic energy from glutamine by a modified biochemical pathway, typical of cancer cells, involving glutaminase and some enzymes of the citric acid cycle [14,15]. ASCT2 has been proposed, in association with LAT1, to be a plasma membrane component of the mTOR signaling pathway [16,17]. Therefore, ASCT2 represents a potential pharmacological target in cancer as well as in pathologies linked to derangements of the mTOR pathway, such as obesity, type 2 diabetes and neurodegeneration [18]. Over the years, several studies have attempted to identify potent and

Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; BFA, brefeldin-A; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; TX-100, Triton X-100; NP-40, nonidet; MeAIB, α-(methylamino)isobutyric acid; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; ER, Endoplasmic reticulum; MESNA, 2-mercaptoethanesulfonic acid

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specific inhibitors of ASCT2 by combining bioinformatics and experimental strategies [19–21]. However, the main challenge in performing large scale screening of pharmacological compounds that interact with ASCT2 is the lack of three-dimensional structure of the transporter. A crystallographically derived structure of plasma membrane transporters of high eukaryotes is not available, with the exception GLUT1, which was solved using an inactive form of the protein [22]. Therefore, data on structure/function relationships for ASCT2 are derived from homology structural models built using, as template, the glutamate transporter Glt_{ph} from *Pyrococcus horikoshii*, which possesses enough similarity to human and rat ASCT2 [23]. From these studies it was deduced that the rat orthologue, consisting of 16 cysteine (Cys) residues in its primary structure, harbors the typical metal binding motif, CXXC, which shows high reactivity towards mercury compounds [24]. The human orthologue contains only 8 Cys residues and lacks of the CXXC motif. Even though the overall sequence identity between the rat and human orthologues is more than 79%, local alignment shows a stretch of 31 amino acids (aa 200–229), where identity is lower than 14%, suggesting that the human and rat orthologues have different properties and, hence, the rat protein cannot be used as a model for the human transporter. The presence of several Cys residues, however, does allow the human orthologue to interact with SH reagents and this completely abolishes transport activity. These data indicate that the Cys residues are important for transport and are accessible to SH reagents [25]. There is considerably less information on the regulation of transport activity and cell expression of ASCT2 compared to structural features [1]. Very recently it was shown that transport reaction is modulated by membrane potential and that the sodium coupled glutamine/amino acid antiport is electrogenic. Moreover, internal sodium also regulates transport by allosteric effects. Furthermore, it has been demonstrated that ASCT2 interacts with the scaffold protein PDZK1 [25] suggesting that protein–protein interactions may be involved in regulation of the transporter. Some information is available on ASCT2 promoter and regulation via a Raf-MEK-ERK kinase cascade [26]. A completely unexplored aspect of regulation of ASCT2 and most amino acid transporters is the trafficking to plasma membrane. N-glycosylation has been linked to trafficking of transporters [27–29] and represents the most frequent post translational modification of human proteins since more than 50% of proteins are glycosylated [30,31]. We hypothesized that glycosylation could play a role in the trafficking of ASCT2 in mammalian cells and role of glycosyl moieties of the protein in the process of delivery to plasma membrane was assessed. Two putative glycosylation sites were predicted by bioinformatics and were subject to site-directed mutagenesis. The effects on function and targeting of both WT and mutant isoforms of ASCT2 are reported here and represent, to our knowledge, the first evidence on the role of glycosyl moiety in trafficking for a human amino acid transporter.

2. Materials and methods

2.1. Materials

Human embryonic kidney HEK293 cells were obtained from the American Type Culture Collection (ATCC). Tissue culture media, fetal bovine serum, Alexa Fluor 594 anti rabbit and transfection reagent Lipofectamine were obtained from Life Technologies. PNGase F, restriction endonucleases and other cloning reagents from NEB (New England Biolabs); sulfo-NHS-SS-biotin from Thermo Scientific; ECL plus, Hybond ECL membranes from GE Healthcare; L-[^{3}H] glutamine from Perkin Elmer; the anti rabbit IgG HRP conjugate from Cell Signaling; the QIAEX II Gel Extraction Kit from Qiagen; the Polyjet transfection reagent was from SignaGen Laboratories; the protease inhibitor mix from Roche; the rabbit anti-ASCT2 from Millipore, the plasmid p3XFLAG-CMV-7.1, $C_{12}E_8$, Amberlite XAD-4, egg yolk phospholipids (3-sn-phosphatidylcoline from egg yolk), Sephadex G-75, L-glutamine, cycloheximide, rabbit anti-FLAG, MESNA, streptavidin beads and all the other reagents were from Sigma-Aldrich.

2.2. Site-directed mutagenesis

The cDNA of ASCT2 was subcloned into p3XFLAG-CMV-7. p3XFLAG-CMV-7.1 is a 4717bp expression vector used to establish transient expression of N-terminal FLAG fusion protein in mammalian cell. The vector encodes three-FLAG epitope (DYKDHDGDYKDHDIDYKDDDDK) at the N-terminus inserted after Met. The plasmid is a shuttle vector for *Escherichia coli* with ampicillin resistance and contains a CMV promoter. This construct was used to introduce the mutations in the ASCT2 protein. The amino acid replacements were performed with complementary mutagenic primers (N163Q forward CTCCGCCGCCAT CCAAGCCTCCGTG, N163Q reverse CACGGAGGCTTGGATGGCGGCGGAG, N212Q forward TATGAAGAGAGGCAAATCACCGGAACC, N212Q reverse GGTTCCGGTGATTTGCCTCTCTTCATA) using the overlap extension method and the High-Fidelity PCR System [32,33]. The PCR products were purified by the QIAEX II Gel Extraction Kit (QIAGEN), digested with HindIII and KpnI (restriction sites added at the 5′ end of forward and reverse primers, respectively) and ligated into the same mammalian expression vector. All mutations were verified by DNA sequencing.

2.3. Cell culture

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 mM glutamine and 1 mM sodium pyruvate. Cells were grown on 10 cm^2 plates at 37 °C in a humidified incubator and a 5% CO_2 atmosphere.

2.4. SDS-polyacrylamide gel electrophoresis and western blotting

The human cells or rat kidney cortex (where ASCT2 is expressed [9]) homogenate was solubilized in RIPA buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate) supplemented with protease inhibitors. The proteins were separated on a 12% polyacrylamide gel and transferred to nitrocellulose membrane. The nitrocellulose membrane was treated with blocking buffer for 1 h at room temperature. The membrane was then incubated in a solution of the primary antibody, a rabbit anti-FLAG or a rabbit anti-ASCT2 and diluted 1:5000 or 1:2000 with the blocking buffer overnight at 4 °C, respectively. The blot was washed three times at room temperature. It was then incubated with the secondary antibody, anti-rabbit IgG, a peroxidase-linked species-specific whole antibody diluted 1:10,000 with the blocking buffer for 1 h at room temperature and revealed by chemiluminescence.

2.5. Transport measurements in cells

HEK 293 cells were seeded onto 12 well plates and cultured using standard culturing conditions until they reached 80% confluence. Cells were transfected with Lipofectamine transfection reagent according to the manufacturer's procedures: 0.5 μg of p3XFLAG-CMV-7.1-ASCT2 WT, p3XFLAG-CMV-7.1-N161Q, p3XFLAG-CMV-7.1-N212Q and p3XFLAG-CMV-7.1-N163/212Q diluted in 50 μL Opti-MEM were combined with 1.5 μl of Lipofectamine in 50 μL Opti-MEM. After 20 min incubation at room temperature the mixture was added to cell culture for 5 h at 37 °C in a CO_2 incubator. After incubation the medium was replaced with DMEM supplemented with 10% (v/v) FBS, 1 mM Gln and 1 mM sodium pyruvate; 24 h after transfection, cells were used for transport assay of L-[^{3}H]glutamine. Cells were rinsed twice with warm transport buffer: 20 mM THCl pH 7.4, 3 mM K_2HPO_4, 1 mM $CaCl_2$, 5 mM glucose, 130 mM NaCl, 10 mM BCH, and 10 mM MeAIB. Radiolabeled 10 μM [^{3}H]glutamine was added and the transport reaction was terminated at the indicated times by discarding the uptake

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