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LAT1 is the transport competent unit of the LAT1/CD98 heterodimeric amino acid transporter

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ABSTRACT

LAT1 (SLC7A5) and CD98 (SLC3A2) constitute a heterodimeric transmembrane protein complex that catalyzes amino acid transport. Whether one or both subunits are competent for transport is still unclear. The present work aims to solve this question using different experimental strategies. Firstly, LAT1 and CD98 were immuno-detected in protein extracts from SiHa cells. Under oxidizing conditions, i.e., without addition of SH (thiol) reducing agent DTE, both proteins were revealed as a 120 kDa major band. Upon DTE treatment separated bands, corresponding to LAT1(35 kDa) or CD98(80 kDa), were detected. LAT1 function was evaluated in intact cells as BCH sensitive [^{3}H]His transport inhibited by hydrophobic amino acids. Antiport of [^{3}H]His was measured in proteoliposomes reconstituted with SiHa cell extract in presence of internal His. Transport was increased by DTE. Hydrophobic amino acids were best inhibitors in addition to hydrophilic Tyr, Gln, Asn and Lys. Cys, Tyr and Gln, included in the intraliposomal space, were transported in antiport with external [^{3}H]His. Similar experiments were performed in proteoliposomes reconstituted with the recombinant purified hLAT1. Results overlapping those obtained with native protein were achieved. Lower transport of [^{3}H]Leu and [^{3}H]Gln with respect to [^{3}H]His was detected. Kinetic asymmetry was found with external Km for His lower than internal one. No transport was detected in proteoliposomes reconstituted with recombinant hCD98. The experimental data demonstrate that LAT1 is the sole transport competent subunit of the heterodimer. This conclusion has important outcome for following studies on functional characterization and identification of specific inhibitors with potential application in human therapy.

1. Introduction

SLC7A5 is a neutral amino acid transporter also known as LAT1 (Christensen, 1990; Pochini et al., 2014). It belongs to the SLC7 family, accounting for 13 members divided in cationic amino acid transporters (CATs) and light subunits of amino acid transporters (LATs). The peculiar property of the latter group of proteins consists in forming covalent heterodimer with larger polypeptides belonging to the SLC3 family, a small group of type II membrane glycoproteins (Palacin and Kanai, 2004; Verrey et al., 2004). In particular, SLC7A5 interacts with SLC3A2, known as CD98 or 4F2hc, via a conserved disulfide between residues C164 of hLAT1 and C109 of hCD98, forming a heterodimer (Fort et al., 2007; Wagner et al., 2001).

LAT1 is a polypeptide of 507 amino acids, corresponding to a molecular mass of 55 kDa, with 12α-helical transmembrane segments (Prasad et al., 1999); it is broadly expressed and mainly localized in basolateral membranes of polarized epithelia (Bode, 2001; Broer, 2002; Yanagida et al., 2001). Important exceptions are the luminal and abluminal membranes of BBB (blood–brain barrier) and the brush border membranes of placenta, i.e. maternal side (del Amo et al., 2008). CD98 is a 630 amino acid polypeptide with an apparent molecular mass of 68 kDa on SDS-PAGE (Galluccio et al., 2013). The role of LAT1/CD98 heterodimer in mediating amino acid transport across the plasma membrane has been assessed in cell systems, so far. However, the precise role of CD98 in the intrinsic transport function of LAT1 is still controversial

Abbreviations: CATs, cationic amino acid transporters; LATs, light subunits of amino acid transporters; CD98, cluster of differentiation 98; BBB, blood brain barrier; BCH, 2-amino-2-norbornanecarboxylic acid; $C_{12}E_8$, octaethylene glycol monododecyl ether; DTE, dithioerythritol; TX-100, Triton X-100.

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(Kanai et al., 1998; Palacin and Kanai, 2004). On the one hand, CD98 has been considered crucial for LAT1 transport activity (Kanai et al., 1998; Prasad et al., 1999). On the other hand, some authors claimed that involvement of CD98 in transport is linked to maturation and trafficking of LAT1 protein in plasma membrane (Franca et al., 2005; Mastroberardino et al., 1998; Nakamura et al., 1999; Wagner et al., 2001). Moreover, in mouse hepatocarcinoma cells, over-expressed LAT1 has been showed to mediate leucine transport without involvement of CD98 (Campbell and Thompson, 2001). Thus, the contribute of CD98 to transport function represents still an open issue. LAT1/CD98 heterodimer mediates a sodium independent amino acid antiport with preference for large amino acids such as Trp, Phe, Tyr and His even though smaller amino acids such as Met, Val, Leu, Ile are also transported (Kanai et al., 1998; Yanagida et al., 2001). Moreover, LAT1/CD98 has been suggested also as a transporter of non amino acid substrates, such as L-dopamine, gabapentin and thyroid hormones (del Amo et al., 2008; Kageyama et al., 2000). It is specifically inhibited by BCH that has been used to discriminate its activity from that of other amino acid transporters in cells (Kim et al., 2002). The three dimensional structure of CD98 has been recently solved (Fort et al., 2007), while crystals of LAT1 are not available, as for all the mammalian amino acid transporters. Some homology structural models have been built on the basis of the arginine/agmatine transporter, AdiC, from *Escherichia coli* and the ApcT from *Methanococcus jannaschii*, whose structures have been solved by X-ray crystallography (Gao et al., 2010). The interest in shedding light on the functional and regulatory properties of LAT1 is strengthened by its well documented over-expression in many tumors, together with the glutamine transporter ASCT2 (SLC1A5). Indeed, tumor survival is characterized by increased absorption of amino acids, used both as oxidative fuel and signaling molecules (Fuchs and Bode, 2005; Ganapathy et al., 2009; Wang et al., 2013). Therefore, LAT1 represents an important novel target for chemotherapy since inhibitors of this transporter are potential anticancer drugs (Baniasadi et al., 2007; Fan et al., 2010; Shennan and Thomson, 2008). Recently, an elegant approach of Virtual High Throughput Screening of drug libraries has been described (Geier et al., 2013). To unravel dark sides of this transporter on both functional and structural point of views, heterologous over-expression of the human proteins LAT1 and CD98 have been performed. Abundant expression was obtained by cloning hLAT1 and hCD98 in the plasmids pH6EX3 (Brizio et al., 2000) and pGEX4T1 (Galluccio et al., 2013). In the present work, hLAT1 mediated transport has been investigated in intact cells as well as in proteoliposomes reconstituted with protein extracted from SiHa cells or recombinant hLAT1 protein. Using these combined strategies, it was shown that hLAT1 is the only competent transport unit of the heterodimer.

2. Materials and methods

2.1. Materials

His Trap HP columns, thrombin, PD-10 columns, ECL plus and Hybond ECL membranes were purchased from GE Healthcare; SiHa cell line was kindly provided by Dr. Massimo Tommasino (IARC/CIRC WHO, Lyon France); culture media and Fetal Bovine Serum were purchased from Life Technologies; radiolabeled amino acids were purchased from ARC (American Radiolabeled Chemicals); anti-hLAT1, anti-hCD98 and anti-rabbit IgG HRP conjugate from Cell Signaling; all the other reagents are from Sigma–Aldrich.

2.2. Cell culture

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

2.3. Extraction of LAT1/CD98 complex from SiHa cells

LAT1/CD98 complex was solubilized, from SiHa pellets, with RIPA buffer and incubated for 30 min on ice. After centrifugation (12,000 × *g*, 15 min, 4 °C) proteins were quantified using Lowry method, analyzed on SDS-PAGE 10% and transferred onto nitrocellulose membranes for western blot.

2.4. Western blot analysis

hCD98 and hLAT1 were immuno-detected incubating membranes with anti-LAT1 antibody 1:2000 or anti-CD98 1:1000 over night at 4 °C. The reaction was detected by Electro Chemi Luminescence (ECL) assay after 1 h incubation with secondary antibody anti-rabbit 1:5000.

2.5. Transport measurement in cells

SiHa cells were seeded onto 12 well plates up to 80% confluence. For transport assay, cells were rinsed twice with transport buffer: 20 mM Tris–HCl pH 7.4, 200 mM glucose and 2 mM DTE where indicated. Radiolabeled 5 μM [^{3}H]His was added and the transport reaction terminated at the indicated times by discarding the uptake buffer and rinsing the cells with the same ice-cold buffer (0.5 ml) plus 1 mM BCH. Cells from each well were solubilized in 500 μl of 1% TX-100. Cell extracts were counted for radioactivity.

2.6. Purification of hCD98 and hLAT1

hCD98 was over-expressed with a GST tag in *E. coli* and purified as previously described (Galluccio et al., 2013). hLAT1 was over-expressed in *E. coli* and purified as previously described (Galluccio et al., 2013) with the following modifications: after cell lysate solubilization and centrifugation (12,000 × g, 10 min, 4 °C) the supernatant was applied on a His Trap HP column (5 ml Ni Sepharose) equilibrated with 10 mL buffer (20 mM Tris–HCl pH 8.0, 10% glycerol, 200 mM NaCl, 0.1% sarkosyl, and DTE 2 mM) using ÄKTA start. Column was washed with 10 mL of buffer (20 mM Tris–HCl pH 8, 10% glycerol, 200 mM NaCl, 0.1% n-dodecyl β-D-maltoside and 2 mM DTE). Protein was eluted with the same buffer plus 400 mM imidazole; 2.5 mL were pooled and desalted on a PD-10 column.

2.7. Reconstitution of the hCD98–hLAT1 complex extracted from SiHa cells into liposomes

The hCD98/hLAT1 complex was extracted from SiHa cells membrane with RIPA buffer and reconstituted in liposomes by removing the detergent using the batch-wise method. Mixed micelles containing detergent, protein and phospholipids were incubated with 0.5 g Amberlite XAD-4 at room temperature for 40 min (Scalise et al., 2012). The initial mixture contained: 150 μg of cell extract, 100 μL of 10% $C_{12}E_8$, 100 μL of 10% egg yolk phospholipids (w/v) in the form of liposomes prepared as previously described (Indiveri et al., 1994), 20 mM Tris–HCl pH 7.5 and 10 mM L-His, except were differently indicated, in a final volume of 700 μL. Cell extracts were treated or not with 10 mM DTE.