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Heterologous Expression of Human SLC1A5v2 as a Functional Glutamine Transporter in *Escherichia coli*

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Neutral and non-essential amino acid, glutamine (Gln), plays an essential role in supplying nitrogen to all the amino acids and nucleotides in the mammalian body. Gln is also the most important carbon source that provides intermediates for gluconeogenesis and fatty acid synthesis and supplements the tricarboxylic acid cycle in fast-growing cancer cells. Among the known 14 Gln transporter genes, soluted carrier family 1 member 5 (SLC1A5) has been reported to be closely associated with cancer cell growth. Three variants (v1, v2, and v3) have been derived from SLC1A5. Here, we established a heterologous gene expression system for the active form of human SLC1A5 variant-2 (hSLC1A5v2) in *Escherichia coli*. v2 is the smallest variant that has not yet been studied. Four expression systems were investigated: pBAD, pCold, pET, and pQE. We also addressed the problem of codon usage bias. Although pCold and pET overexpressed hSLC1A5v2 in *E. coli*, they were functionally inactive. hSLC1A5v2 using the pBAD system was able to catalyze the successful transport of Gln, even if it was not highly expressed. Initial activity of hSLC1A5v2 for [^{14}C] Gln uptake in *E. coli* reached up to 6.73 μmole·min^{-1}·gDW^{-1} when the cell was induced with 80 mM L-arabinose. In this study, we demonstrated a heterologous expression system for the human membrane protein, SLC1A5, in *E. coli*. Our results can be used for the functional comparison of SLC1A5 variants (v1, v2, and v3) in future studies, to facilitae the developement of SLC1A5 inhibitors as effective anticancer drugs.

Keywords: **Heterologous expression, human SCL1A5v2, *Escherichia coli*, glutamine transporter**

Introduction

Glutamine (Gln) is the most abundant free amino acid in the mammalian body [1], reaching about 0.7 mmol/l in the blood [2]. This quantitative abundance is closely related to its role in the temporary storage of nitrogen. Most of the nitrogen in living organisms is contained in proteins and nucleic acids, which are mainly incorporated through Gln. In mammals, Gln is converted to glutamate (Glu) in cells, which provides amino groups to all non-essential amino acids, except asparagine [3]. Asparagine is produced by the direct donation of an amino group from Gln. In *Escherichia coli*, where all amino acids can be synthesized *de novo*, ammonia is first assimilated into Gln or Glu, which then donates an amino group to other amino acids (Fig. 1) [3]. To synthesize asparagine, tryptophan, and histidine, Gln provides an amino group directly (Fig. 1A) and indirectly via Glu to synthesize all other amino acids (Fig. 1B).

Gln also directly participates in *de novo* nucleotide synthesis. In purine synthesis, two Glns are used to form one inosine monophosphate (IMP) to complete the purine ring (Fig. 1C) [4]. Another Gln is used to produce guanosine monophosphate from IMP (Fig. 1C) [4]. In pyrimidine synthesis, one Gln is used during uridine triphosphate (UTP) production, and the other is used to form cytidine triphosphate from UTP (Fig. 1D) [4].

Gln provides carbon and energy sources. it is deami-

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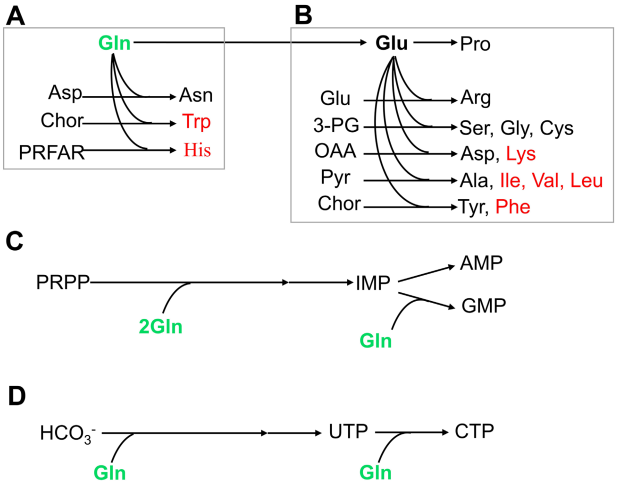

Fig. 1. De novo synthesis of amino and nucleic acids in *Escherichia coli*. During amino acid biosynthesis, (A) glutamine supplies amino group to produce asparagine, tryptophan, and histidine, and (B) glutamate derived from glutamine supplies amino group to produce arginine, serine, glycine, cysteine, aspartate, lysine, alanine, isoleucine, valine, leucine, tyrosine, phenylalanine, and proline. 3-PG, 3-phosphoglyceric acid; OAA, oxaloacetate; Pyr, pyruvate; Chor, chorismate; PRFAR, phosphoribulosyl-formimino-5-aminoimidazole-4-carboxamide ribotide phosphate. Red letters indicate the essential amino acids in humans. During biosynthesis glutamine supplies nitrogen to produce (C) purine and (D) pyrimidine. PRPP, phosphoribosyl pyrophosphate; IMP, inosine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate

nated to form α-ketoglutarate, which supplements the tricarboxylic acid (TCA) cycle. It also supports fatty acid synthesis, and 25% of the acetyl-CoA used for fatty acid synthesis under normoxia is derived from Gln [5]. These roles have become increasingly important in cancer cells. In cancer cells, most glucose is metabolized into lactate instead of acetyl-CoA as it becomes hypoxic due to rapid cell growth, leading to the Warburg effect [6]. Lactate is a dead-end material, and cancer cells use Gln to supplement the TCA cycle [7]. In cancer cells, Gln, but not glucose, is necessary for gluconeogenesis, energy production, and fatty acid synthesis [5, 8]. It functions as a signaling molecule that activates the mechanistic target of rapamycin complex-1 (mTORC1) pathway [9]. mTORC1 activates the eukaryotic translation initiation factor-3 and ribosomal protein S6 kinase β-1, which promote cell growth. Therefore, in fast-growing cancer cells, Gln is required not only to produce amino acids and nucleic acids but also as a source of carbon and energy.

To perform these roles, Gln must first enter the cell. There are 14 Gln transporter genes in the human genome, which belong to four SoLute-Carrier families: SLC1, SCL6, SLC7, and SLC38 (Table S1). They have a broad spectrum of substrates with similar properties for transporting amino acids. SLC1A5 is overexpressed in various types of cancers [10]. SLC1A5 catalyzes the transport of Gln in addition to alanine, serine, and cysteine. SLC1A5 is an $[Na^+ \cdot AA]_{ex}/[Na^+ \cdot AA]_{in}$ antiporter that couples the uptake of extracellular amino acids (plus Na^+) with the export of intracellular amino acids (plus Na^+) (Table S1) [11, 12].

Human SLC1A5 (hSLC1A5) is located on chromosome 19q13.3 and contains eight exons (Fig. S1A). Three variants of hSCL1A5 have been reported in the NCBI database (Fig. S1B). hSLC1A5 was re-named as variant-1 (hSLC1A5v1: NM_005628), which has the longest polypeptide of 541 aa by coding sequence (CDS) containing all eight exons. Variant-2 (hSLC1A5v2: NM_001145144) has the smallest polypeptides of 313 aa by CDS from exon 3. Variant-3 (hSLC1A5v3: NM00145145) is translated from exon 2 and is a 339 aa long polypeptide. Expression of hSLC1A5 is controlled by retinoblastoma protein (pRb) and multifunctional transcription factor c-Myc. pRb represses excessive cell growth by inhibiting the cell cycle activation factor, E2F-3, and preventing cell cycle progression. pRb expression is low in cancer cells, which results in excessive cell growth and overexpression of hSLC1A5 [13]. In cancer cells, c-Myc directs mitochondrial metabolism using external Gln for cell survival [14] and increases the expression of Gln transporters, including hSLC1A5 [15]. Expression of hSLC1A5 is increased by hypoxia independent factor 2a under hypoxic conditions [16] in cancer cells.

So far, studies on hSLC1A5 have focused on the transporter hSLC1A5v1. Yoo *et al.* [16] reported that hSLC1A5v3 is located in the mitochondrial inner-membrane and promotes cancer cell growth [16]. However, to date, no studies have focused on hSLC1A5v2.

In this study, we implemented a successful heterologous gene expression system for hSLC1A5 in *E. coli*. hSLC1A5v2 was cloned into various vector systems, and the expression conditions were optimized. Finally, a direct uptake experiment confirmed that the hSLC1A5v2 produced in this study could act as a functional trans-

porter in *E. coli*.

Materials and Methods

Strains and cultivation

E. coli strains used in this study are listed in Table S2. All strains were cultured in Luria-Bertani (LB) (Becton-Dickinson, USA), gM9, or eM9 medium. gM9 medium was M9 medium [17], with L-glutamine (20 mM; Junsei, Japan) instead of NH_4Cl as the nitrogen source. eM9 medium was M9 medium supplemented with acid-hydrolyzed casein (0.1%, w/v; Neogen, USA) and L-tryptophan (0.005%, w/v; Deajung, Korea). Glycerol or glucose (50 mM; Duksan, Korea) was added as a carbon source. The antibiotics chloramphenicol (15 μg/ml), ampicillin (50 μg/ml), and kanamycin (25 μg/ml) were added as required.

Cloning

All plasmids and primers used in this study are listed in Table S2. To clone hSLC1A5v2, pCMV6-XL6 containing SLC1A5 (NM_005628; Human Untagged Clone, Canada) was used as the PCR template. pMB61 vector was used as a PCR template to replace the *ori*-region of pRARE2 p15A with that of pBR322. Plasmids pMB155 (pColdI::hSLC1A5v2), pMB156 (pQE80L::hSLC1A5v2), pMB157 (pET30b::hSLC1A5v2), pMB171 (pBAD30-RBS::hSLC1A5v2), and pMB153 (pRARE2-pBR322ori) were confirmed via sequencing.

Induction conditions

pMB155, pMB156, and pMB157 were expressed in Rosetta2 (DE3) (Novagen, USA; Cat# 71402) cells. pMB171 was expressed in LMB073 cells containing pMB153. Further, 2% (v/v) of subcultures was inoculated into LB or M9 medium and grown aerobically for 2 h at 30 or 37℃ and, 180 rpm. hSLC1A5v2 expression was induced at an OD_{600} of 0.5 or 0.8. pMB155 was induced after chilling in an ice bath for 40 min, and the culture was incubated at 15℃.

Western blotting

hSLC1A5v2 was detected using 1:2,000 diluted 6x His tag primary (ThermoFisher, USA; Cat# MA1-21315) and 1:100,000 diluted goat anti-mouse IgG (H+L) HRP (ThermoFisher; Cat# G21040) antibodies with pMB155, pMB156, and pMB157. For pMB171, 1:400 diluted SLC1A5-specific antibody (450–541 aa; Abcam, USA; ab187692) and 1:10,000 diluted goat anti-rabbit IgG (H&L) HRP antibody (Abcam, USA; ab6721) were used as the primary and secondary antibodies, respectively.

[^{14}C] Glutamine uptake assays

Induced and harvested bacterial cultures were resuspended in ice-cold potassium buffer (100 mM K_2HPO_4/KH_2PO_4, 1 mM $MgSO_4$, pH7) at an OD_{600} of 5. The assay was performed as described by Kim and Unden [17]. The suspension was pre-incubated at 37℃ for 5 min, and [^{14}C] glutamine (281 mCi/mmol; Moravek, Inc., USA) was added to 100 μM in 100 μl cell-suspension and incubated for 1 min. The reaction was quenched by adding 900 μl of 0.1 M LiCl. Cells were separated by vacuum filtration through membrane filters (ADVANTEC, Japan, Cat#A020A025A) and washed twice with stop solution. Radioactivity was determined using a liquid scintillation counter (Beckman Coulter, USA). Transport activities were assessed based on the intracellular concentration of [^{14}C] glutamine using 462.9 mg dry weight/l of *E. coli* cells with OD_{600} of 1.0.

Results and Discussion

hSLC1A5v2 can be expressed in pCold and pET systems but does not show any transporter activity

Four different expression vectors, pET, pQE, pCold, and pBAD, with various advantages and limitaions in terms of gene expression, were selected for the heterologous expression of hSLC1A5v2 (Table S3). hSLC1A5v2 was cloned into pColdI (pMB155), pQE80L (pMB156), and pET30b (pMB157) and its expression was investigated in the Rosetta 2 (DE3) strain.

First, the expression conditions were screened in gM9 medium at different cultivation temperatures (30 and 37℃), induction time points (OD_{600} 0.5 and 0.8), and isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations (0.2 and 0.5 mM) (Table S4). Western blotting analysis revealed that hSLC1A5v2 expression was the highest when IPTG (0.5 mM) was added at OD_{600} of 0.8 (Figs. 2A and C) in pColdI and pET systems. However, hSLC1A5v2 was not produced under any condition in pQE80L (Fig. 2B). It was difficult to express hSLC1A5v2 in pQE vector because of the strict expression control

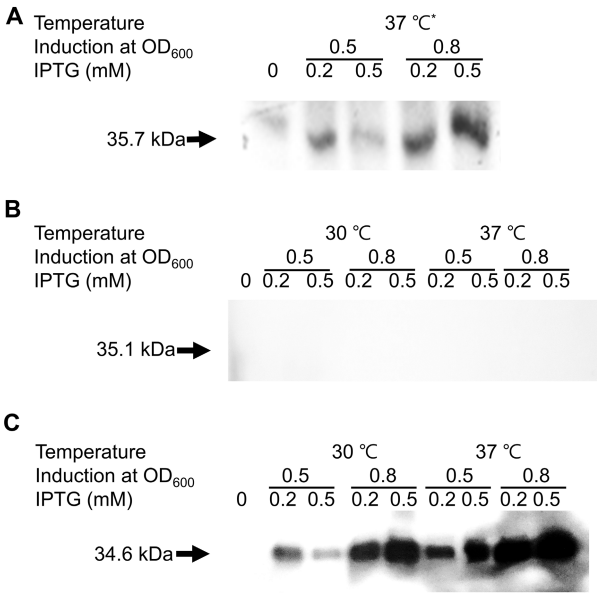

Fig. 2. Western blotting of solute carrier family 1 member 5 variant-2 (SLC1A5v2) expressed from (A) pMB155 (pColdI:: hSLC1A5v2), (B) pMB156 (pQE80L::hSLC1A5v2), and (C) pMB157 (pET30b::hSLC1A5v2). *E. coli* Rosetta 2 (DE3) harboring each plasmid was grown in the Luria–Bertani (LB) medium at 30 or 37℃. Expression was induced with 0.2 or 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD_{600} of 0.5 or 0.8 for 4 h. 37℃*, Cells containing pColdI were grown at 37℃ and further incubated at 15℃ after IPTG induction.

system (mounting double *lac* operators).

Next, the uptake of [^{14}C] Gln via hSLC1A5v2 into *E. coli* cells was measured to examine whether the expressed proteins functioned properly. hSLC1A5v2 produced in both pColdI and pET30b did not transport Gln (Fig. S2), although western blotting identified its expression (Figs. 2A and, C). Uptake of [^{14}C] Gln after induction was lower than that without induction (Fig. S2). As *E. coli*, the background cell, possesses its own Gln transporter, the reference value showed quite high basal activity for Gln uptake (~2 U·g DW^{-1}) (Fig. S2). Thus, a decrease below the reference value indicated that cell collapse and hSLC1A5v2 failed to properly build on the cell membrane of *E. coli*.

As expected, strong and fast expression of the pET system could cause this problem, but unexpectedly, similar results were obtained in the pCold system, which was considered suitable for reluctant genes. Induction at low temperatures has the advantage of increasing the expression of target proteins and lowering that of other host proteins. Galluccio *et al.* [18] reported the successful expression of hSLC1A5v1 with pColdI. However, they only confirmed its expression using western blotting. In contrast, we showed that the pCold system is not suitable for the heterologous expression of *hSLC1A5*. Membrane proteins require machines for protein folding and membrane translocation, which probably stop during induction at low temperatures, resulting in the misfolding, dysfunction, or aggregation of hSLC1A5v2, or cell membrane collapse.

hSLC1A5v2 expressed from pBAD system is capable of transporting gln in *E. coli*

We cloned hSLC1A5v2 into the pBAD system (pMB171) under the control of araBAD promoter. As this vector can be used in any strain of *E. coli* as a host, the Δ*glnH* mutant (LMB073) was used as a background strain, in which *glnH* encodes the substrate-binding component of GlnHPQ which is the main Gln transporter in *E. coli* [19]. pRARE2, solves the codon usage bias, was modified because both pBAD and pRARE2 have the same replication origin, p15A. To avoid plasmid incompatibility [20], p15A-ori of pRARE2 was replaced with pBR322-ori, resulting in pMB153 (pRARE2-pBR322ori). Gene induction regimes were screened with different culture media (gM9 with glycerol and LB), temperatures (30 and 37℃), induction points (OD_{600} 0.5 and 0.8), and arabinose concentrations (0.05–0.1 mM or 10–100 mM) (Table S4 and 5). Expression did not occur at low concentrations of arabinose (0.05–0.1 mM) (Fig. S3A), but was unexpectedly achieved at high concentrations (≥ −10 mM) (Fig. S3B). Spadiut *et al.* [21] also reported the use of a high concentration of arabinose (0.05–1.0%; 3.33–66.6 mM) to induce heterologous protein expression in pBAD. Several western blotting experiments showed that growth in LB at 30℃, OD_{600} of 0.8 and arabinose concentration of 10–80 mM was suitable for expressing hSLC1A5v2 in pBAD (Fig. 3A; Fig. S3B). Finally, the activity of hSLC1A5v2 was analyzed via the Gln uptake assay using a cell suspension. The results showed that hSLC1A5v2 from pBAD was capable of catalyzing [^{14}C]Gln uptake, which was the highest (6.73 μmol·min^{-1}·gDW^{-1}) when induced with 80 mM arabinose (Fig. 3B). hSLC1A5v2 was expressed as a functional protein from pBAD, unlike in the case of pET and pCold systems, in which Gln uptake was abolished

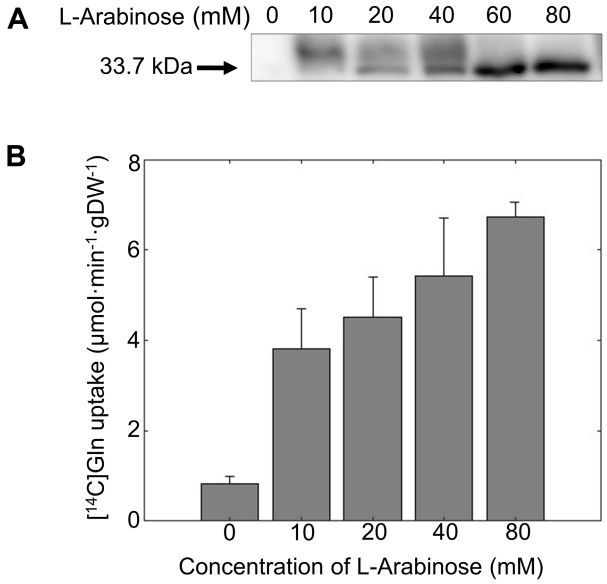

Fig. 3. Western Blotting (A) and [^{14}C]Gln uptake assay (B) of SLC1A5v2 expressed from pMB171 (pBAD30::RBS::SLC1A5v2). Gln uptake-deficient *E. coli* (*ΔglnH*) transformed with both pMB171 (pBAD30::RBS::SLC1A5v2) and pMB153 (pRARE-ori pBR322) were grown in LB medium at 30℃ at OD_{600} of 0.8. SLC1A5v2 was induced with L-arabinose (0–80 mM) for 2 h. Uptake of 100 μM [^{14}C]Gln into whole cell was measured for 1 min.

by induction. Spadiut *et al.* [21] compared the activities of heterologously expressed cytosolic enzymes in pET, pCold, and pBAD systems and reported the best activity in pCold. This is probably because membrane proteins, such as SLC1A5v2, require the expression of some host protein such as membrane translocation proteins in the Sec system. Therefore, pBAD is a suitable system for the heterologous expression of hSLC1A5v2, a human-derived membrane protein.

Escherichia coli-pBAD system can be used for the comparative analysis of three variants in different locations

This study showed that v2 of hSLC1A5 catalyzes Gln uptake in *E. coli*, while v1 and v3 have been identified as Gln transporters in human cells [16, 22]. The three variants were likely to migrate to different subcellular locations because of their different N-terminus (Fig. S1). To properly understand the function of any protein in eukaryotic cells, it is necessary to determine its subcellular localization. Many *in silico* databases have been developed to predict this, but their accuracy does not seem reliable. Recently, Jiang *et al.* [23] summarized these databases and evaluated their accuracy based on experimental results. Therefore, we used the databases that scored high accuracy (> 60%) in that by Jiang [23] to predict the location of SLC1A5v2 (Table S6). v1 and v3 were used for verification because their locations were experimentally proven as the plasma membrane and mitochondrial inner membrane, respectively [16, 22]. However, no database accurately predicted these locations (Table S6).

Yoo *et al.* [16] predicted the cleavage positions of presequence for mitochondrial targeting at the R44 and K45 amino acids at the N-terminus of SLC1A5v3 using PrediSi (Table S6) and experimentally verified R44 and K45 as MTS (mitochondrial targeting sequences). Mitochondrial proteins contain an arginine (or positively charged)-rich N-terminus targeting signal (presequence) between 20 and 60 aa at the N-terminus [24]. The 44^{th}/45^{th} amino acids of v3 corresponded to the 18^{th}/19^{th} amino acids at the N-terminus of v2 and the 64^{th}/65^{th} of v1 (Table S6). The 18^{th}/19^{th} position of v2 is outside the range of 20–60 aa at the N-terminus, so it will not be able to target mitochondria, as is 64^{th}/65^{th} of v1, so v2 is likely to migrate to the plasma membrane. However, experimental evidence is still required to determine an accurate location.

If v2 is placed into the plasma membrane like v1 or into the mitochondrial membrane like v3, it will have functional differences. Along with the identification of Gln transporters, it is necessary to analyze the differences in their capabilities. This study established an optimal route for measuring the activity of hSLC1A5 in *E. coli*, which can be used to analyze the transport characteristics of v1, v2, and v3 in *E. coli* in detail in future studies. Moreover, it can be used to identify methods to prevent the entry of Gln into cancer cells.

In this study, we used four vectors, pBAD, pCold, pET and pQE to express hSLC1A5v2 in *E. coli*. hSLC1A5v2 expression in its active form was possible only from the pBAD system, but protein expression was observed in the western blots of pBAD, pCold, and pET systems. This result indicates that pBAD is more effective than pCold for the heterologous expression of human membrane proteins. The hSLC1A5v2 from pBAD system could transport Gln in *E. coli in vivo*, and the initial activity of [^{14}C] Gln uptake reached up to 6.73 μmole·min^{-1}·gDW^{-1}. Our method for the establishment of a

functional expression system for hSLC1A5 that supports cancer cell growth can be used to for the identification of anticancer targets and development of anticancer drugs.

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Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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