Construction and characterization of the rat neutral amino acid transporter

HA – SNAT3 fusion protein

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Abstract: Sodium-coupled neutral amino acid transporter 3 (SNAT3) is a member of SLC38 gene family which primarily expresses in astrocytes in the brain and retina. It transports L-glutamine predominantly and plays an important role in glutamate-glutamine cycle in the brain and liver. In order to easily detect the expression and localization of SNAT3 on the membrane, we constructed a eukaryotic expression plasmid with an HA tag at the N-terminus of rat SNAT3 in the mammalian expression vector pBK-CMVΔ (1098 – 1300) by PCR and double restriction endonuclease digestion techniques. After transiently transfected the plasmid into human embryonic kidney cells (HEK293T), expression of HA-SNAT3 fusion protein was detected by western blotting. The result suggested that the HA-SNAT3 fusion protein could normally express on the membrane. The plasmid pBK-CMVΔ (1098 – 1300)-HA-SNAT3 will provide a useful tool for the investigation of structure and function of SNAT3 in the future.

Key words: neutral amino acid transporter; HA-SNAT3 fusion protein; construction and characterization

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1 Introduction

Sodium-coupled neutral amino acid transporter 3 (SNAT3) belongs to the SLC38 gene family, a main branch of solute carrier families in mammals. The SLC38 gene family contains 11 members in human of which have been characterized and functionally subdivided into System A (SNAT1, SNAT2 and SNAT4) and System N (SNAT3, SNAT5 and SNAT7). System A transporters are responsible for transporting small aliphatic amino acids while System N subtypes have much narrower substrate profiles with preference for L-glutamine, L-histidine and L-asparagine. In addition, System N has the ability to counter transport both Na⁺ and H⁺. However, System A transports substrates coupled to the uptake of Na⁺ with a stoichiometry of 1:1. Previous studies have shown that the SLC38 gene family transporters are essential for the uptake of nu-
trents and play key roles in inhibitory and excitatory neurotransmission in the CNS ammonium detoxification and gluconeogenesis in the liver and response to acidosis in the renal.

SNAT3, formerly named SN1 (Slc38a3) [1] exhibits functional and regulatory properties of classically defined system N transporters. SNAT3 contains 504 amino acid residues with a predicted molecular mass of 55 kDa. SNAT3 predominantly transports L-glutamine followed by L-histidine and L-asparagine [2]. Abundant expression of SNAT3 protein is detected in astrocytes throughout the retina and brain except neurons and oligodendrocytes [11-22]. SNAT3 mRNA is rich in kidney [11-22] skeletal muscle [13,25] and placenta [13,25] while its protein is not detected in heart or skeletal muscle [11-12]. SNAT3 has been suggested to be responsible for glutamine transport in glutamate-glutamine cycle [12] which is crucial for the survival of excitatory neurons by removing glutamate from the synaptic cleft rapidly to avoid excitotoxic effects of glutamate [13]. It’s an essential process in the complex balance of neuronal amino acid metabolism. The properties and distribution of SNAT3 in the liver are also suitable for the known functional organization of the liver acinus in which the glutamate-glutamine cycle participates in the detoxification of ammonia from the portal blood. If not cleared in due course, ammonia will be toxic to the CNS and become an inducing factor in hepatic encephalopathy [17-40]. SNAT3 has been reported to play a role in the renal response to acidosis as the kidney is a major site of glutamine utilization especially during systemic acidosis [20-24]. Furthermore, SNAT3 can contribute to the provision of glutamine to the fetus in the early stage of gestation [26].

Despite the importance of SNAT3 in the mammalian physiology, relatively little is known about its structure and function. Hydrophathy plotting indicates that SNAT3 contains 11 transmembrane helices (TM) with an intracellular N terminus and an extracellular C terminus. SNAT3 mediates glutamine transport that is energetically coupled to the cotransport of 1 Na⁺ and the antiport of 1 H⁺ which makes the overall transport mechanism electroneutral and be governed by pH and Na⁺ gradients [13,22]. Previous study has shown that asparagine 76 of SNAT3 is critical for substrate-induced ion conductance and affects the binding of the cosubstrate Na⁺. Besides, this residue is likely to be localized in the translocation pore in the center of the membrane [28]. In addition to coupled transport, SNAT3 was suggested to mediate four modes of uncoupled ion movement across the membrane [25]. More recent findings revealed that glucocorticoids can upregulate SNAT3 expression in the kidney in response to metabolic acidosis [26]. The transport activity of SNAT3 is reported to be rapidly reduced on PKC activation by phorbol esters via a specific trafficking mechanism in oocytes [26].

It’s very difficult to get the specific antibody of membrane proteins like SNAT3 because of their structural complexity, so some tags such as HA, myc and GFP are linked to the target proteins. According to the same idea, the HA-SNAT3 fusion protein was constructed to examine SNAT3 expression in our study. We added an HA tag to the N-terminal of rat SNAT3 in the eukaryotic expression vector pBK-CMVΔ (1098 – 1300) [26] and the expression of SNAT3 was identified by western blotting. The successful construction of pBK-CMVΔ (1098 – 1300) -HA-SNAT3 plasmid will be of great benefit to the investigation of SNAT3’s structure and function.

2 Materials and methods

2.1 Materials

pBK-CMVΔ (1098 – 1300) -SNAT3 (pBK-CMVΔ-SNAT3) is a plasmid which contains the cDNA coding of rat SNAT3 and is stored by Molecular and Cell Biology Laboratory of Shanghai Normal University. Oligonucleotide primers were synthetized by SanGon. Human Embryo Kidney cells (HEK293T, ATCC number CRL 11268) were purchased from the Typical Culture Collection cell bank, Chinese Academy of Sciences. E. coli

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2. 3. 2 Transformation

2. 3. 1 Double enzyme digestion and T4 Ligase ligation

After the gel purification, PCR product and pBK-CMV Δ-SNAT3 plasmid were respectively double digested by EcoR I and Sac I at 37 ℃ for 3 h. The 50 μL of reaction mixture contained 5 μL of 10 × NEB Buffer 1, 2 μL of each primer (12. 5 ng/μL) and 72 μL of sterile ddH₂O in a final volume of 200 μL. The forward primer (5' - GTTCAGGAATTCATGAGGCGCTTTG - CAGAC-3') contained 27 bases of the HA tag initiation codon ATG and an EcoI site. The reverse primer (5' - CCTTTCGAGCTCAGTATAGATGGGCAG - 3') contained a Sac I site. Both primers were checked for hairpin formation minimal self-priming and sense/antisense formation with the help of Primer Premier 5. 0 software. Negative control reactions were carried out with the same system but the template was replaced by sterile ddH₂O.

Thermal cycler was programmed for an initial denaturation step (94 ℃ 5 min) and 35 circles of denaturation (94 ℃ 40 s) annealing (66. 2 ℃ 40 s) and extension (72 ℃ 10 min) followed by a final extension step (72 ℃ 10 min) and cooling to 16 ℃.

After electrophoresis on 1% agarose in TAE (Tris-acetate-EDTA) buffer followed by ethidium bromide staining, the PCR products were excised. Nucleic acids were extracted and purified with Gel midi purification kit according to the supplier’s instructions.

2. 3 Construction of pBK-CMVΔ-HA-SNAT3

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2. 3. 2 Transformation

E. coli DH5α competent cells were transformed with the ligation product by heat shock (42 ℃ 85 s) followed by an incubation on ice (3 min). Cells were allowed to be recovered in LB medium (37 ℃ 45 min at 150 r/min) without selection and plated on LB agar-solidified medium with kanamycin (30 ng/μL). After 15 h culture at 37 ℃ the grown colonies were inoculated to liquid culture and plasmid DNA was extracted from DH5α cells with a plasmid mini kit according to the provided procedures.

2. 3. 3 Identification

To verify the positive colony containing target plasmids bacterial PCR was carried out. After purification, the plasmid DNA was double digested by EcoR I and Sac I for 3 h at 37 ℃. A primary screening was per-
formed with gel electrophoresis. Then the plasmid of pBK-CMVΔ-HA-SNAT3 was sequenced.

2.4 Cell Culture and Transient expression

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 4 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator under an atmosphere of 5% CO₂ in air. When the confluence was about 80% ~ 90% plasmids of pBK-CMVΔ-SNAT3 or pBK-CMVΔ-HA-SNAT3 were transiently transfected into cells using Lipofectamine 2000 according to the manufacturer’s protocols.

2.5 Western blotting

After transfection for 36 h, HEK293T cells were washed 2 ~ 3 times with ice-cold PBS containing 0.1 mmol/L CaCl₂, 1 mmol/L MgCl₂. Then the cells were harvested and suspended in RIPA lysis buffer containing 100 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS and protein inhibitor. Freeze-thawing technique was used to break the cells. The intact cells and nucleus were removed by centrifugation at 1000 g for 15 min at 4 °C. 100 μL of the supernatant was taken as total proteins. After the supernatant was centrifuged at 30000 g for 30 min at 4 °C the pellet was resuspended in 100 μL of buffer containing 40 mmol/L Tris-Cl (pH 7.6), 5 mmol/L MgCl₂ and 0.4 mmol/L EGTA as membrane proteins. Protein concentrations were determined with BCA protein assay kit according to the manufacturer’s instructions. 3 μg of both total and membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. For detection of SNAT3-HA fusion protein, the membrane was incubated with anti-HA tag mouse monoclonal antibody (1:3000) in TBST containing 0.1% Tween 20 and 2.5% skim milk. Besides, Anti-GAPDH mouse monoclonal antibody (1:3000) was used to test Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference because of its constitutive expression in almost all tissues. After rinsed with TBST containing 0.1% Tween 20 the membrane was reacted with HRP-conjugated goat anti-mouse IgG secondary antibody (1:60000). Bands were visualized using an enhanced chemiluminescence detection method with ECL Western blotting detection kit.

3 Results and conclusions

3.1 construction and identification of pBK-CMVΔ-HA-SNAT3

The plasmid pBK-CMVΔ-SNAT3 has a length of 6743 bp. Using it as a template we got the PCR fragment containing an HA tag at the end of the N-terminus of SNAT3. The size of this fragment was supposed to be 975 bp. Agarose gel electrophoresis revealed an amplified fragment of about 1.0 kb (Fig. 1 A Lane 2) which was consistent to the excepted size. No positive band was observed in the negative control (Fig. 1 A Lane 3).

The original plasmid pBK-CMVΔ-SNAT3 and the PCR fragment were double digested with EcoR I and Sac I restriction enzymes respectively. Agarose gel electrophoresis showed that the plasmid pBK-CMVΔ-SNAT3 was dissected to two fragments: one was about 1.0 kb containing the initiation codon ATG; the other one was about 5.7 kb containing the vector of pBK-CMVΔ and a part of SNAT3 N-terminus (Fig. 1B Lane 2). And the size of PCR fragments after digestion was about 1.0 kb (Fig. 1B Lane 3).

After excised and extracted from the agarose gel respectively the 5.7 kb of the vector fragment and the 1.0 kb PCR product were ligated by T4 DNA Ligase. The ligation system was transformed directly into E. coli DH5α competent cells. After bacterium fluid PCR some positive colonies containing pBK-CMVΔ-HA-SNAT3 were picked up (Fig. 1C Lane 2 to Lane 7). The purified plasmid was double digested by EcoR I and Sac I to further verify pBK-CMVΔ-HA-SNAT3 was constructed successfully. Lane 2 to Lane 5 in Fig. 1 D showed two
fragments after agarose gel electrophoresis. The one about 6.0 kb was the pBK-CMVΔ-SNAT3 fragment which was equal to the positive control (Fig. 1D, lane 6). The other one about 1.0 kb contained the HA gene and was shorter than that of pBK-CMVΔ-SNAT3 because of the removal of the 112 bp junk sequence during PCR process. The map of recombinant plasmid of pBK-CMVΔ-HA-SNAT3 was shown in Fig. 2. Finally the constructed plasmid of pBK-CMVΔ-HA-SNAT3 was verified by DNA sequencing.

(A) Amplification of HA. Lane 1: DL 10000 DNA marker; Lane 2: PCR fragment; Lane 3: negative control without templates;

(B) Double digestion of PCR fragment and pBK-CMVΔ-SNAT3. Lane 1: DL 10000 DNA marker; Lane 2: pBK-CMVΔ-SNAT3; Lane 3: PCR fragment; (C) Bacterium fluid PCR. Lane 1: DL 10000 DNA marker; Lane 2–5: PCR fragment from different colonies; Lane 6: pBK-CMVΔ-SNAT3 as positive control; Lane 7: negative control without templates; (D) Double enzyme digestion of constructed pBK-CMVΔ-SNAT3 plasmids results by EcoR I and Sac I. Lane 1: DL 10000 DNA marker; Lane 2–5: pBK-CMVΔ-SNAT3; Lane 3: pBK-CMVΔ-SNAT3 as positive control

Figure 1 Amplification of HA and identification of pBK-CMVΔ-HA-SNAT3 by bacterium fluid PCR and double enzyme digestion

Figure 2 Map of recombinant plasmid pBK-CMVΔ-SNAT3
3.2 Western blotting

After the expression constructs of the HA-tagged SNAT3 (pBK-CMVΔ-SNAT3) and the untagged SNAT3 (pBK-CMVΔ-SNAT3) were respectively transfected into HEK293T cells for 36 h, expression of SNAT3 protein was detected by western blotting using primary anti-HA tag monoclonal antibody. The band at about 55 kDa was observed in pBK-CMVΔ-SNAT3-transfected cells (Fig. 3, Lane 1 and 2), which was equal to the expected size. At the same time, no band could be detected in pBK-CMVΔ-SNAT3-transfected cells (Fig. 3, Lane 3 and 4) while the internal reference GAPDH at 36 kDa was viewed in every lane. The result suggested that the HA-SNAT3 fusion protein can be normally expressed on the membrane.

Lane 1: total protein of HEK293T cells transfected with pBK-CMVΔ-HA-SNAT3; Lane 2: membrane protein of HEK293T cells transfected with pBK-CMVΔ-HA-SNAT3; Lane 3: total protein of HEK293T cells transfected with pBK-CMVΔ-SNAT3; Lane 4: membrane protein of HEK293T cells transfected with pBK-CMVΔ-SNAT3

Figure 3 HA-SNAT3 expression detected by western blot

4 Discussion

SNAT3 is a transmembrane protein with 11 TM which plays a pivotal role in the provision of glutamine with sodium and proton coupled manner in the glutamate-glutamine cycle, an essential process in the brain, liver and kidney. There’s no effective commercial antibody prepared for SNAT3 so far, which limits the study of SNAT3’s structure and function.

HA tag is derived from a human influenza hemagglutinin HA molecule corresponding to amino acids 96–106 and is used as general epitope tag in expression vectors. As a short peptide with only 9 amino acid residues, HA tag has little effect on the structure and function of target proteins. Besides, anti-HA tag monoclonal antibody is efficient and cheap, which makes HA tag a beneficial tool for study of the structure and function of membrane proteins. In this work, we constructed a recombinant vector pBK-CMVΔ-SNAT3 by connecting an HA tag to the N-terminus of SNAT3 and the successfully-constructed fusion protein was detected by western blotting using anti-HA monoclonal antibody.

In conclusion, a mammalian expression vector with HA-SNAT3 fusion protein has been constructed in our present study so we can use it to detect the expression and localization of SNAT3 or its mutant transporters by testing HA tag. In a word, the successful construction of pBK-CMVΔ-SNAT3 will be helpful to the research on the structure and function of SNAT3 in the future.

References:

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HA-SNAT3


为了方便检测SNAT3在细胞膜上的表达和定位，本研究采用PCR扩增和酶切连接的方法将HA标签连接到质粒pBK-CMVΔ(1098-1300)-SNAT3中大鼠SNAT3的N端，构建了真核生物表达载体pBK-CMVΔ-SNAT3。用脂质体转染法将该表达载体瞬时转染进人胚肾细胞(HEK293T cells)，通过Western blotting检测HA-SNAT3融合蛋白的表达。结果表明，HA-SNAT3能够正确在细胞膜上表达。pBK-CMVΔ-SNAT3表达载体的成功构建对于进一步研究SNAT3的结构和功能提供了有效方法。