Construction and identification of EGFP-SNAT2 fusion protein in mammalian expression vector

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Abstract: SNAT2 is the second member of the sodium-coupled neutral amino acid transporters (SNATs) of the SLC38 gene family. It plays an important role in Glutamate-glutamine cycle in the brain and gluconeogenesis in the liver. In order to detect expression and localization of SNAT2 on the membrane conveniently, the C terminus of enhanced green fluorescence protein (EGFP) was linked to the N terminus of SNAT2 in the mammalian expression vector p*BK-CMV*(Δ [1 098 – 1300]) by PCR and single restriction endonuclease digestion techniques. After the plasmid p*BK-CMV-EGFP-SNAT2* was transiently transfected into HEK293T cells for 36 hours, the expression and localization of EGFP-SNAT2 fusion protein were detected by western blot and laser scanning confocal microscope (LSCM). The result showed that EGFP-SNAT2 expressed and localized on the membrane normally. The construction of the expression vector of p*BK-CMV-EGFP-SNAT2* will be of great benefit to investigation of structure and function of SNAT2 in the future.

Key words: SNAT2; EGFP; construction; expression

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1 Introduction

SNAT2 (Sodium-coupled neutral amino acid transporter 2) belongs to the SLC38 gene family, which was subdivided into System A and System N according to their properties of transporting substrates. System A perfers to transport small, aliphatic amino acids, including SNAT1, SNAT2 and SNAT4, while system N, including SNAT3 and SNAT5, have narrower substrate profiles with prefering to transport of glutamine, histidine and asparagine. The two subtypes are also different in their patterns of regulation [1]. The molecular mechanism of System N is distinct from that of System A. System N transports substrates coupled to counter-transport of H^+ as well as co-transport of Na^+. However, System A subtype transports amino acids coupled to the uptake of Na^+ with a stoichiometry of 1∶1.

SNAT2 has been founded in every tissue tested using northern analysis [3]. SNAT2 may play an important

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role in the glutamate-glutamine cycle in the brain and gluconeogenesis in the liver [2]. On the other hand, SNAT2 appears to be the major regulated isoform in response to endocrine and nutrient stimuli [3]. It has been reported that alterations in glutamate transporters is responsible for several neurodegenerative disorders including amyotrophic lateral sclerosis, Huntington´s disease, Parkinson´s disease, and Alzheimer´s disease [14].

Except the physiological importance, little is known about its functional and structural properties. Hydropathy analysis predicts that SNAT2 contains 11 transmembrane helices (TM) with an intracelluar N terminus and an extracelluar C terminus [4]. The transport process mediated by SNAT2 is sensitive to the external pH, coupled the anion leak conductance, which can be inhibited by the transported amino acids [5]. Since the crystal structure of SNAT2 is unavailable so far, sequence homology was used to investigate the function and structure of SNAT2 [6]. The crystal structure of a bacterial homologue from *Aquifex aeolicus* (LeuTAa) was used as a model to unveil about the family of Na^+/Cl^- dependent neurotransmitter transporters [13], which indicates that the structure of SNAT2 is possible to be similar to LeuTAa. Mutation analysis in the past several years also identified various amino acid residues in SNAT2 that seem to be important for transport activity based on LeuTAa model. The conserved asparagine 82 in TM1 and threnonine 384 in TM 8 have shown to control the interaction of Na^+ with SNAT2 [2]. However, since a high-resolution structure of SNAT2 has not been obtained so far, it is still elusive to explain the currently available functional data.

It´s difficult to get the specific antibodies of some membrane proteins like SNAT2 nowadays for their structural complexity. A common method is indirectly detecting some tags or makers linked to the target protein. The green fluorescent protein (GFP) has become an important tag to detect the expression of some proteins, since it has no cytotoxicity and can be easily observed [9]. For example, the GFP reporter was introduced into the wild-type RS105 and hrpX mutant to identify HrpX regulon genes [11]. GFP gene were also used in the non-structural segment 1 (1) of Human Parvovirus B19 (PVB19) to visualizes PVB19-NS1 [12]. Enhanced green fluorescent protein EGFP is mutated from GFP, which is much more sensitive than GFP. Furthermore, it doesn´t affect the growth and function of the cells, so it´s widely linked to the target protein. The expression and localization of target proteins can be directly detected by the fluorescence microcopy or by western blot using commercial GFP polyclonal or monoclonal antibody.

In this study the EGFP-SNAT2 fusion protein was constructed in order to detect the expression and localization of SNAT2. The C terminus of EGFP was linked to the N terminus of SNAT2 in the mammalian expression vector p*BK-CMV* (Δ [1 098 – 1300]) by using PCR and single restriction endonuclease digestion techniques. Then expression and localization of SNAT2 were identified by western blot and laser scanning confocal microscope (LSCM). The successful construction of p*BK-CMV*Δ*-EGFP-SNAT*2 plasmid is meaningful for the investigation of SNAT2´s structure and function.

2　Materials and methods

2.1　Bacteria, plasmids and cell strains

p*BK-CMV*(Δ [1 098 – 1300])*-SNAT*2 (*pBK-CMV-*Δ*-SNAT*2) and p*MD-*19*T-EGFP* are stored by Molecular and Cell Biology Laboratory of Shanghai Normal University, Human Embryo Kidney cells (HEK293T, ATCC number CRL 11268) were purchased from the Typical Culture Collection cell bank, Chinese Academy of Sciences. *E. coli* (DH5α) competent cells used in the study was purchased from TIANGEN BIOTECH.

2.2 PCR

The PCR amplification of 750 bp cDNA sequence of *EGFP* from p*MD-19T-EGFP* was done with the following primers:

Egfp-F: 5′-GATTAAATTTGCGCGCATGGTGAGCAAGGGCGAG-3′.

Egfp-R: 5′-GAATATCTATGCGCGCCTTGTACAGCTCGTCCATG-3′.

The digestion sites of enzyme BssH Ⅱ — GCGCGC was introduced into the sequence for ligation. The PCR reaction mixture contained 2 μL of the template, 20 μL of *Egfp*-F (12.5 μmol · L^{-1}), 20 μL of *Egfp*-R (12.5 μmol · L^{-1}), 108 μL of sterile ddH_2O, and 150 μL of 2 × Taq Master Mix (from (CWBIO)). PCR was performed as followed: an initial denaturing at 94℃ for 5 min, then the EGFP was amplified by 30 cycles (94℃, 30 s; 66.1℃, 30 s; 72℃, 1 min) with the final extension at 72℃ for 10 min. The PCR products were analysed by 1% agarose gel and purified by gel midi purification kit (TIANGEN BIOTECH) according to the manufacture′s instructions.

2.3 Construction of p*BK-CMV-EGFP-SNAT2*

2.3.1 Digestion and ligation

After the gel purification, the *EGFP* fragment and p*BK-CMV-Δ-SNAT2* plasmid fragment were digested by BssH Ⅱ (NEB) at 50℃ overnight, respectively. The 50 μL of reaction mixture contained 7 μg of DNA fragment and 1 μL of BssH Ⅱ. Then the DNA products were purified in the method described previously. The ligation was performed in a final volume of 10 μL, in which contained 1 μL of ligation buffer, 0.5 μL of T4 Ligase (from NEB), 1.8 μL of *EGFP*, 1.1 μL of p*BK-CMV-Δ-SNAT2* plasmid fragment, and 5.6 μL of sterile ddH_2O.

2.3.2 Transformation

The mixtures of ligation were transformed into DH5α competent cells and plated on Luria-Bertani (LB) plates containing kanamycin (30 ng/μL). After 15 h culture at 37℃, the colonies were picked up. The plasmids were purified from DH5α cells using a plasmid mini kit (CWBIO) according to the provided procedures.

2.3.3 Identification

To verify the positive colony containing target plasmids, an upstream primer of *EGFP* (5′-GATTAAATTTGCGCGCATGGTGAGCAAGGGCGAG-3′) and a downstream primer of *SNAT2* (5′-GAAAGCCCAAGGATTGCACTGCCCACAATCG-3′) were used to amplify the bacterium fluid using PCR. After purification, the plasmid DNA was digested by BssH Ⅱ for 5 h at 50℃. The fragements were visualized on 1% agarose gel. Then the plasmid of p*BK-CMV-Δ-EGFP-SNAT2* was sequenced.

2.4 Confocal microscopy and Western blot

HEK293T cells were cultured in Dulbecco′s modified Eagle′s medium (DMEM, from Gibco) with 10% bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (4 mmol · L^{-1}) in a humidified incubator with 5% CO_2 at 37℃ for 24 h. When the confluency was about 80% – 90%, plasmids of p*BK-CMV-Δ-EGFP-SNAT2* were transiently transfected into cells using Lipofectamine2000 (from Invitrogen) according to the provided procedures. Confocal microscopy detection was carried out after incubation at 37℃ for 36 h. Single optical sections were taken and recorded digitally using a Zeiss LSCM confocal imaging system. HEK293 cells without transfection were also recorded as a negative control.

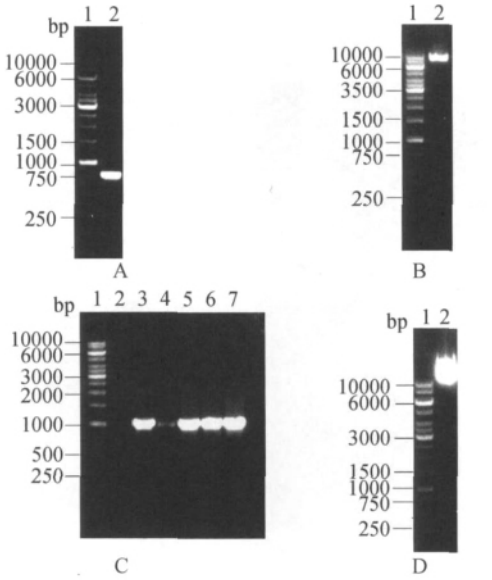
After transiently transfected by plasmid of p*BK-CMV-Δ-EGFP-SNAT2* for 36 h incubation, HEK293T cells were collected in 1 mL of PBS with protein inhibitor. Then cells were broken using frozen and thawed method. After centrifuged at 1 000 g at 4℃ for 15 min, the supernatant was collected as total proteins. After the

supernatant was centrifuged at 7 000 g at 4℃ for 30min, the pellet was resuspended in 100 μL of buffer containing 40 mmol · L^{-1} Tris-Cl (pH7.6), 5 mmol · L^{-1} $MgCl_2$ and 0.4 mmol · L^{-1} EGTA as membrane proteins. Protein concentrations were determined by BCA protein assay kit (from Pierce). 10 μg of total proteins and 4 μg of membrane proteins were separated by 10% SDS-PAGE, Anti-GFP tag rabbit polyclonal antibody (1∶6 500, from ImB) and HRP-linked goat anti-rabbit lgG secondary antibody (1∶5 500, from ImB) were used by western blot to detect SNAT2.

3 Results and conclusions

3.1 Construction and identification of p*BK-CMV Δ-EGFP-SNAT2*

The *EGFP* gene was amplified from p*MD-19T-EGFP* using PCR, the length of which was 749 bp (Fig. 1A, lane2). Lane 2 in Fig. 1B showed the purified p*BK-CMVΔ-SNAT2* fragment after BssH Ⅱ digestion, which is about 9 000 bp. Transformation into *E. coli* was performed after ligation. After bacterium fluid PCR, some positive clones contain p*BK-CMVΔ-EGFP-SNAT2* were picked up (Fig. 1C, lane 3, 5, 6 and 7). To verify p*BK-CMVΔ-EGFP-SNAT2* was constructed successfully further, the purified plasmid was digested using BssH Ⅱ. Two fragments were obtained after agarose gel electrophoresis (Fig. 1D, Lane 2). The one about 750 bp was the *EGFP* gene, the other one about 9 000 bp was the p*BK-CMVΔ-SNAT2* fragment. The map of recombinant plasmid of p*BK-CMVΔ-EGFP-SNAT2* was shown in Fig. 2. Finally, the plasmid of p*BK-CMV-Δ-EGFP-SNAT2* was sequenced.



A: Amplification of *EGFP*, Lane 1: DL 10 000 DNA marker, Lane 2: *EGFP* fragment; B: p*BK-CMV-Δ-SNAT2* fragment, Lane 1: DL 10 000 DNA Marker, Lane 2: fragments of purified p*BK-CMV-Δ-*SNAT2; C: Bacterium fluid PCR, Lane 1: DL 10 000 DNA Marker, Lane 2 ~ 7: PCR fragment from different colonies; D: Digestion of contructed p*BK-CMV-Δ-EGFP-SNAT2* plasmids results by BssH Ⅱ, Lane 1: DL 10 000 DNA Marker, Lane 2: p*BK-CMV Δ-EGFP-SNAT2* digested by BssH Ⅱ (750 bp for the *EGFP* fragment, 9 000bp for the p*BK-CMVΔ-SNAT2* fragment)

Figure 1　Amplification of *EGFP* and identification of p*BK-CMV-EGFP-SNAT2* by bacterium fluid PCR and enzyme digestion

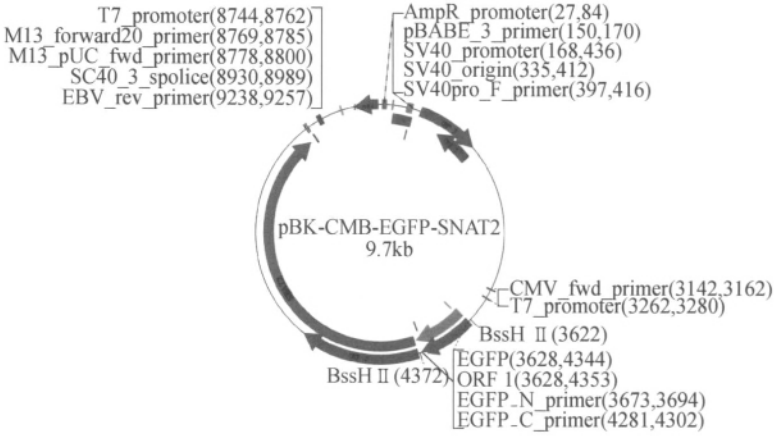
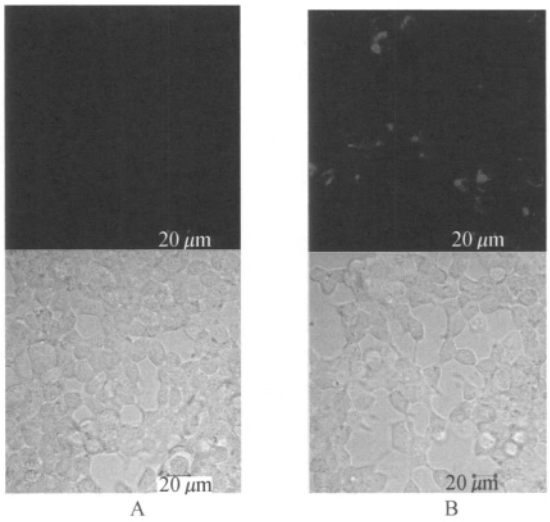


Figure 2 Map of recombinant plasmid p*BK-CMV-Δ-EGFP-SNAT*2

3.2 Confocal microscopy and Western blot

Expression and localization of SNAT2 protein were detected by the confocol microscopy method after p*BK-CMV-Δ-EGFP-SNAT*2 was transiently transfected into HEK 293T cells for 36 h. The results revealed the protein of EGFP-SNAT2 was located in both membrane and endoplasmic reticulum of the cells (Fig. 3B), while no any fluorescence can be detected in the nontransfected HEK 293T cells (Fig. 3A). These results suggested that the protein of EGFP-SNAT2 was expressed and localized normally on the HEK293 cells′ membrane.



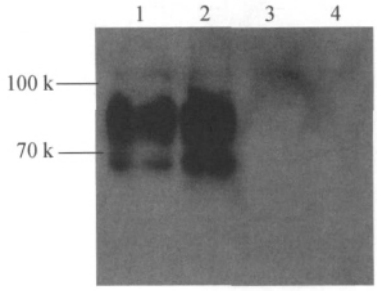
A: HEK293 cells without transfection; B: HEK293 cells transfected with p*BK-CMV Δ-SNAT*2

Figure 3 SNAT2-EGFP expression and localization detected by the confocol microscopy

After p*BK-CMV-Δ-EGFP-SNAT*2 was transiently transfected into HEK293T cells for 36 h, expression and localization of SNAT2 protein was detected using the anti-GFP polyclonal antibody by western blot. The bands at about 80 kDa were observed in p*BK-CMV-Δ-EGFP-SNAT*2-transfected cells (Fig. 4, lane 1 and 2), which is consistent with the expected size. No bands were observed in non-transfected cells (Fig. 4, lane 3 and 4). The results demonstrated further that the EGFP-SNAT2 fusion protein could express and localize in membranes normally.

4 Discussion

SNAT2 is a kind of transmembrane protein, which plays an important role in transporting some amino acids into and out of the cells. EGFP is a mutant protein from GFP, which is more sensitive than GFP. It would be convenient and commercial to detect to SNAT2 expression and localization on the cell membrane by using the anti-GFP polyclonal antibody if the EGFP-SNAT2 fusion protein is obtained. In this study, the C terminus of EGFP was successfully linked to the N terminus of SNAT2 in the mammalian expression vector p*BK-CMVΔ-SNAT2* by using PCR and single restriction endonuclease digestion techniques. The construction of p*BK-CMVΔ-SNAT2* was identified by bacteria fluid PCR and single restriction endonuclease digestion (Fig. 1). Expression and localization of the EGFP-SNAT2 fusion protein were detected by western blot using anti-GFP polyclonal antibody and LSCM. The results turned out that SNAT2 was expressed and localizated on the membrane by detecting GFP's expression (Fig. 3 and 4).



1: total protein of HEK293 cells transfected with p*BK-CMV* ⊿*-EGFP-SNAT2*; 2: membrane protein of HEK293 cells transfected with p*BK-CMV* ⊿*-EGFP-SNAT2*; 3: total protein of non-transfected HEK293 cells; 4: membrane protein of non-transfected HEK293 cells

Figure 4　SNAT2 expression detected by western blot

The successful construction of p*BK-CMV* ⊿*-EGFP-SNAT2* will be useful to study the structure and function of SNAT2 in the future, which will be of benefit to the study of structure and function properties of SNAT2.

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p*BK-CMV* ⊿*-EGFP-SNAT2* 质粒载体构建及其表达鉴定

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摘 要: SNAT2 是 SLC38 基因家族编码的 Na^+ 偶联中性氨基酸转运蛋白中属于 system A 的成员之一. 它在谷氨酸 – 谷氨酰胺循环、肝脏糖质新生等生物通路中发挥重要作用. 增强型绿色荧光蛋白 EGFP 的连接对于 SNAT2 在细胞表达中的定位以及检测都有很大的帮助. 采用 PCR 扩增和酶切技术将 EGFP 连接到质粒 p*BK-CMV* ⊿*-SNAT2* 中 SNAT2 的 N 端. 通过酶切后琼脂糖凝胶电泳和测序验证得到 p*BK-CMV* ⊿*-EGFP-SNAT2* 质粒载体,将构建好的质粒瞬时转染进人胚肾细胞(HEK293 cells) 用 Western blot 和激光共聚焦电子显微镜检测 EGFP-SNAT2 的表达与亚细胞定位. 结果表明: EGFP-SNAT2 在细胞中正确表达并定位于细胞膜上. pBK-CMV ⊿-EGFP-SNAT2 质粒载体的构建对于进一步研究 SNAT2 的结构和功能具有重要意义.

关键词: SNAT2; EGFP; 构建; 表达

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