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Cloning and Expression of Brain-Specific Anion Transporter BSAT1 and Isolation of Monoclonal Antibodies to Its Extracellular Fragment

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Brain-specific anion transporter BSAT1 extracellular fragment (451-557) cDNA was cloned in a vector for prokaryotic expression, a producer *E. coli* strain was obtained, and recombinant extracellular fragment $BSAT1_{451-557}$ was purified and used for immunization of BALB/c mice. Splenic cells from mice with verified immune response were used for hybridoma generation. Several hybridoma clones producing monoclonal antibodies to BSAT1 extracellular fragment were selected. Antibody specificity was confirmed by ELISA, immunoblotting with recombinant $BSAT1_{451-557}$, and immunofluorescent BSAT1 assay on rat brain sections and cultured HEK293 cells. It was demonstrated that the obtained antibodies specifically bind native rat and human BSAT1 and can be used in both fundamental studies of structures forming the blood-brain barrier and development of targeted transport of diagnostic preparations and drugs across the blood-brain barrier.

Key Words: *BSAT1; monoclonal antibodies; blood-brain barrier*

BSAT1 (Brain Specific Anion Transporter, Oatp14/BBB, Slc21a14) is a transporter of organic anions specific for endothelial cells of cerebral microvessels (ECM) identified by comparing transcriptomes of ECM and endothelial cells isolated from rat liver and kidneys [7]. BSAT1 cDNA (2148 b.p.) encodes a protein including 716 amino acid residues with 12 transmembrane domains. The molecular weight of BSAT1 is 90 kDa. It is thought that the function of this protein in structures of the blood-brain barrier (BBB) is transendothelial transfer of thyroxin (T4) and some other amphipathic anions. Active efflux of T4 in cells expressing BSAT1 suggests that this transporter performs two-way transfer.

Special interest to BSAT1 can be explained apart from strict specificity to ECM by its localization on the luminal surface of endotheliocytes [11] and the possibility of using this protein for targeted delivery of hydrophilic substances across BBB.

OATP-F is the closest structural and functional analog of BSAT1 in humans [12]; it is expressed in the brain and reproductive tract. The amino acid sequence of BSAT1 and OATP-F is 86%.

The identity of BSAT1 and other OATP representatives expressed in the liver, spleen, kidneys, and

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lungs ($OATP_{1-6}$) does not exceed 60%, which makes theoretically possible generation of antibodies specifically recognizing BSAT1 and not interacting with other organic anions.

Our aim was cloning and expression of cDNA for BSAT1 extracellular fragment 451-557, isolation and purification of the protein, and creation of the hybridoma producing monoclonal antibodies that recognize native extracellular fragment of BSAT1.

MATERIALS AND METHODS

Membrane topology of rat BSAT1 was evaluated using HMMTOP, TMHMM, and TMPred software (www.expasy.org). Amino acid sequence of the largest functionally-relevant extracellular fragment 451-557 was determined (Fig. 1). Since production of recombinant proteins in bacteria is usually successful in case of large polypeptides with a molecular weight of 5-10 kDa having no highly hydrophobic fragments in the amino acid sequence (*e.g.* transmembrane fragments), types of recombinant proteins were chosen for creation of monoclonal antibodies recognizing extracellular fragment of BSAT1: extracellular 451-557 fragment with N-terminal hexahistidine sequence ($BSAT1_{451-557}$, molecular weight 13 kDa, pI 8.1) and the same fragment with a larger hybrid protein abundantly produced in bacterial cells, human membrane Ca-ATPase PMCA4b (CBD) with a molecular weight of 17 kDa [5-8] ($BSAT1_{451-557}$CBD, molecular weight 30 kDa, pI 6.3).

Nucleotide sequence of the studied extracellular BSAT1 fragment were amplified from rat brain cDNA using primers agctGGATCCtgtgaaaattccagtgtggcc (forward) and agtcAAGCTTagaaatacagaaacatttggga (reverse) containing recognition sites for restriction endonucleases BamHI and HindIII. The primers were synthesized and purified by SibEnzim company. For nucleotide and protein sequences design and application, DNAStar software (Lasergene) was used.

PCR products were purified by extraction with phenol-chloroform mixture and chloroform, precipitated with ethanol, and ligated into a pGEM-T plasmid vector (Promega) using phage T4 DNA-ligase (Promega) according to manufacturer's instruction. *E. coli* JS-5 cells (Bio-Rad) were transformed with this ligase mixture and seeded onto dishes with LB agar containing 50 μg/ml carbenicillin X-Gal and isopropyl-β-thiogalactoside (IPTG). The obtained white colonies were screened by PCR for the presence of the insert. Positive clones were grown in a liquid medium and used for isolation of plasmid DNA with Wizard Plus Minipreps DNA Purification System (Promega). Purified plasmid DNA was used for sequencing.

Purified plasmid DNA containing inserts without errors were treated with the corresponding restriction endonucleases and separated by preparative electrophoresis in agarose gel; cDNA fragments of the desired length was isolated from the gel using SV Gel&PCR Clean-Up System (Promega) and ligated with plasmid DNA pQE30 (BamHI+HindIII) pCBDQ (BglII+HindIII) cleaved by the corresponding sites. *E. coli* SG13009 cells (Qiagen) transfected with ligase mixture were seeded to dishes with LB agar containing 50 μg/ml carbenicillin and 25 μg/ml kanamycin. The obtained colonies were screened by PCR for the presence of the insert in correct orientation and the positive clones were used for further experiments.

The positive clones were cultured at 37°C in liquid LB medium containing 50 μg/ml carbenicillin and 25 μg/ml kanamycin to attaining optical density of 0.7-0.9 (at 600 nm), IPTG was added to a concentration of 1 mM, and the cells were incubated for under the same conditions (4 h); after that the cells were precipitated at 3000*g* for 20 min, frozen, and stored at -70°C. The proteins were isolated under denatirating conditions on Ni-NTA-agarose according to the protocol recommended by Qiagen Company. The cells grown in 50-200 ml culture were defrosted at room temperature for 10 min, resuspended in 5 ml solution B (8 M urea, 0.1 M sodium phosphate, 0.01 M tris-HCl, pH 8.0) per 1 g wet tissue and incubated for 1 h at room temperature. The lysate was centrifuged (15,000 rpm, 15 min, 5°C, rotor JA-20), the supernatant was collected and incubated with 2 ml 50% Ni-NTA-agarose for 45 min with periodic shaking. Then the suspension was transferred to a column, washed with 15 ml solution B, 20-30 ml solution C (solution B, pH 6.3), and then eluted with 2-3 ml solution B containing 250 mM imidazole). Protein concentration was measured by the method of Bradford and depending on the results the elution was sometimes repeated. The obtained protein preparations were analyzed by electrophoresis in PAAG.

Monoclonal antibodies were obtained by the method described elsewhere [6] with some modifications. BALB/c mice were immunized with purified $BSAT1_{451-557}$ and $BSAT1_{451-557}$CBD (10 μg per immunization) with 50% complete Freund adjuvant. Immunization consisted on 4 subcutaneous injections every 10 days. The cycle was repeated after 1 month. In a month, the animals received a buster intraperitoneal injection of the corresponding antigen (10 μg) and the blood for measuring the titer of specific antibodies was taken on day 5. If the titer of antibodies to BSAT1 in indirect ELISA was high, the animal was sacrificed, the spleen was removed, and B cells were fused with Sp2/0-Ag14 myeloma cells grown in RPMI-1640 medium (Sigma) with 10% fetal calf serum (FCS, Gibco). The cells were grown in stationary liquid culture in a concentration of 10^5-10^6/ml. One day before fusion,

MDTSSKENAHLFHKNSAQPAGGPSFKAGYPSTEEARPCCGKLKVFLGALSFVYFAKALTEGYLKSTITQIERRFDIPSSL
VGIIDGSFEIGNLLVITFVSYFGAKLHRPKIIGAGCLVMGFGTMLIAVPQFFMEKYSYEKYERYSPSSNLTPNISPCYLE
SSSPSPRSIVGKSQNKINDECEVDTSSSMWVYVFLGNLLRGLGETPIQPLGIAYLDDFASEDNAAFYIGCVQTVAIIGPI
FGFLLGSLCAKLYVDIGFVNLDHITITPKDPQWVGAWWLGYLIAGFLSLLAAVPFWCLPKTLPRSQSREDSGSSSEKSKF
ITDDPVNYQMAPREESMKIMEMARDFLPSLKSLFRNPVYILYLCASTVQFNSLFGMVTYKPKYIEQQYGQSSSKANFVIG
LINIPAVALGIFSGGIVMKKFRLGICEATKLYLGSSVFGYLLFLSLFALG**CENSSVAGLTVSYQGTKPVSYHERALFSDC**
NSRCKCSDSKWEPMCGDNGITYASACLAGCQSSSRSGKNIIFSNCTCVGFAAPKSGNWSGMMGRCQKDNGCSQMFLYFLV
ISVITSYTLSLGGIPGYILLLRCIQPQLKSFALGIYTLAVRVLAGIPAPVYFGVLIDTSCLKWGFKKCGSRGSCRLYDSH
AFRHIYLGLTTLLGTVSVFLSTAVLLVLKKKYVSKRSSFITAREKIVMSSSVKKETCAARDHGLQPKYWPGKETRL

Fig. 1. Primary structure of rat BSAT1 (716 amino acid residues, pI 8.8; 10 transmembrane segments) and its membrane topology determined using HMMTOP, TMHMM, and TMPred software (www.expasy.org). Transmembrane domains are underlined; extracellular fragment cloned by us is shown in bold letters.

RPMI-1640 with 15% FCS and high glucose concentration (4.5 g/liter) was added.

During fusion, B cells were mixed 1:5 with plasmacytoma cells and PEG-DMSO (Sigma) was added in portions according to a protocol described elsewhere [10]. In 2 h, HAT (Sigma) was added to cells and the suspension was transferred to 96-well plates. The cells were incubated in HAT medium for 10 days and then it was replaced with DMEM supplemented with 20% FCS.

The first testing of the culture medium for the presence of specific antibodies to BSAT was performed when the formed clones consisted of 150-200 cells. The clones producing monoclonal antibodies to BSAT (selected by ELISA) were tested by immunoblotting on PVDF membrane (Millipore) with chemiluminescent detection using an ECL-advance kit (GE Healthcare) and by histochemical analysis on frozen sections of rat brain. Stability of antibody production by clones was verified in several successive re-cloning procedures. When 90% wells became positive after recloning, the hybrid cells were injected intraperitoneally to BALB/c mice (10^6 cells per animal).

Monoclonal antibodies from the ascitic fluid were purified by affinity chromatography on agarose with immobilized protein G (Invitrogen) according to manufacturer's protocol.

Immunohistochemical analysis of the antibodies was performed by the standard protocol of immunofluorescence on frozen brain sections of a rat perfused with 4% neutral paraformaldehyde through the aorta. Goat anti-mouse antibodies labeled with Alexa Fluor 488 (Invitrogen) were used as secondary antibodies. All dilutions and washouts were made using PBS (pH 7.4) containing 0.2% Tween-20, Triton X-100, and 1% normal goat serum. During double immunofluorescent staining, the sections at the corresponding stage were incubated with a cocktail of the specified antibodies to mouse immunoglobulins and antibodies to rabbit immunoglobulins labeled with Alexa Fluor 633 (Invitrogen). Cell nuclei were post-stained with DAPI.

RESULTS

Analysis of the primary structure, physicochemical properties, and membrane topology of BSAT1 allowed us to identify 12 transmembrane domains and extra- and intracellular fragments (Fig. 1). For further experiments, the largest extracellular fragments (amino acids 451-557) was chosen, because this fragment, apart from its size sufficient for induction of the immune response, is the most functionally important for binding to the target molecule transported across BBB.

After cloning of the nucleotide sequence encoding extracellular fragment of BSAT1 into chosen vectors, two strains producing two recombinant proteins $BSAT1_{451-557}$ and $BSAT1_{451-557}$CBD were obtained. The yield of proteins in overproduction under standard

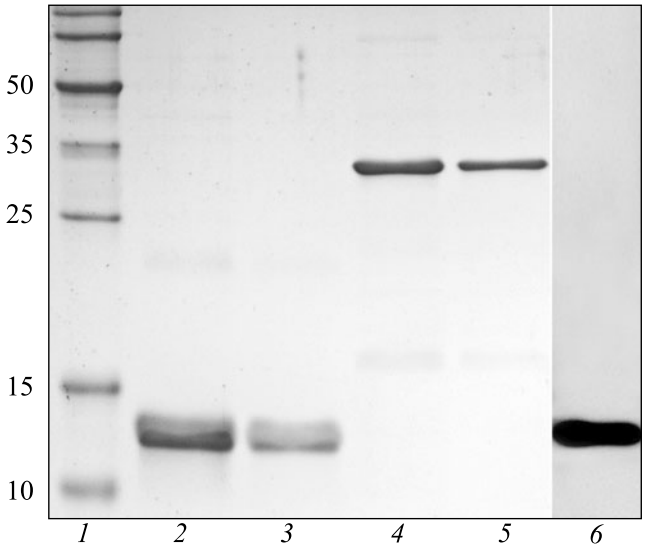

Fig. 2. Disk-electrophoresis and immunoblotting of $BSAT1_{451-557}$ and $BSAT1_{451-557}$CBD purified by affinity chromatography on Ni-NTA-agarose in 15% PAAG. *1*) molecular weight standard (Promega, V8491); *2*, *3*) $BSAT1_{451-557}$; *4*, *5*) $BSAT1_{451-557}$CBD; *6*) immunoblotting of $BSAT1_{451-557}$ using monoclonal antibodies obtained by us.

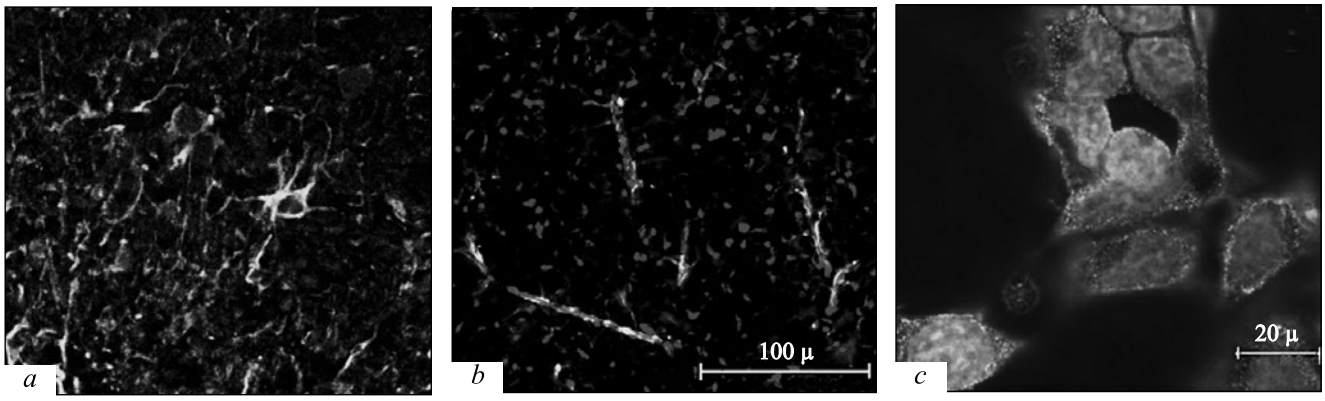

Fig. 3. Immunofluorescent analysis of BSAT1. *a*, *b*) frozen sections of rat brain: staining visualizes membrane structures of cells in brain parenchyma (*a*; ×200) and perivasal spaces (*b*; scale 100 μ); *c*) culture of HEK293 cells. *b, c*) cell nuclei are post-stained with DAPI (Invitrogen).

conditions (37°C, 4 h in the presence of 1 mM IPTG in LB medium) was 10 and 15 mg/liter culture for $BSAT1_{451-557}$ and $BSAT1_{451-557}$CBD, respectively. Protein purity according to disk-electrophoresis in SDS-PAAG followed by Coomassie R250 staining was not below 90% (Fig. 2; *1-5*). Minor band with a molecular weight of 17 kDa in rows *4* and *5* is probably an admixture of free CBD protein, which can be caused by minor proteolysis during overproduction.

The chosen scheme of immunization with purified BSAT1 preparations ensured high titer of specific antibodies after two cycles of immunization with both $BSAT1_{451-557}$ and fusion protein $BSAT1_{451-557}$CBD. B cells of immunized animals were used for creation of hybridomas. After screening hybrid cell clones, several clones producing monoclonal antibodies recognizing $BSAT1_{451-557}$ in ELISA and immunoblotting were selected (Fig. 2).

At the final stage of selection, the positive clones (according to immunoblotting data) were tested by immunofluorescent analysis on frozen brain sections and on fixed preparations of various cell cultures. Three hybridoma clones producing antibodies that allowed visualization of membrane structures on frozen brain sections and in HEK293 cell culture were selected. On brain sections, the immunofluorescent signal was primarily localized in membrane cell structures in brain parenchyma (Fig. 3, *a*) and small microvessels (Fig. 3, *b*). During immunofluorescent analysis of cell preparations, the brightest fluorescence was observed in HEK293 cells. In this case, the fluorescent signal was also localized in cell membranes (Fig. 3, *c*).

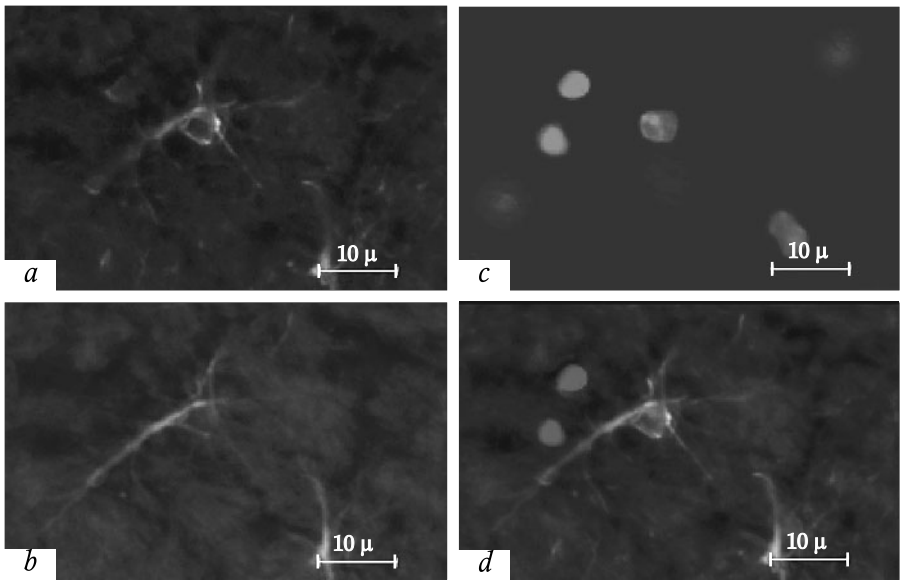

Fig. 4. Double immunofluorescent staining of frozen rat brain sections with monoclonal antibodies to BSAT1 and polyclonal antibodies to GFAP. *a*) BSAT1; *b*) GFAP; *c*) cell nuclei post-stained with DAPI; *d*) superposition: fluorescent signals of BSAT1 and GFAP do not coincide, but are localized in the same cells.

Since cells stained with the used antibodies morphologically resembled astrocytes, we performed double immunofluorescent staining with monoclonal antibodies to BSAT1 and polyclonal antibodies to GFAP obtained previously [3]. We found that immunofluorescence determined by antibodies to BSAT1 and GFAP is localized in the same cells (Fig. 4).

Taking into account immunoblotting data, the results of immunohistochemical analysis visualizing cell membranes confirm specificity of the obtained antibodies to BSAT1, because the latter in an integral membrane protein. Previous studies of the expression of this protein in HEK293 cells showed that these cells produce both tight junction proteins (ZO1, occluding, and claudin) and some neurospecific proteins [2]. Thus, the expression of brain-specific anion transporter in these cells is quite explicable. Moreover, positive staining of HEK293 cells suggests that antibodies obtained by us recognize rat and human BSAT1.

Our findings (detection of BSAT1 in astrocytes) did not contradict the previously published report [7], where nucleotide sequence of the new OATP isoform was described after analysis of cDNA obtained using total mRNA of microvascular fraction. Since astrocytes, similarly to pericytes, densely wrap microvessels, endothelial cells and astrocytes cannot be completely separated by gradient centrifugation. Therefore, cDNA BSAT1 was probably obtained from astrocyte mRNA. It should be noted that we found no published reports unambiguously demonstrating localization of 100% BSAT1 on the luminal membrane of cerebral microvascular endothelial cells.

Thus, we isolated immunogenic recombinant fragment of rat BSAT1 highly homologous to human BSAT1. After immunization of BALB/c mice with this protein and subsequent fusion of their B lymphocytes with Sp2/0 myeloma cells, monoclonal antibodies specifically recognizing native rat and human BSAT1 were obtained. These antibodies can be used for basic research of structures forming BBB and for applied studies of the targeted transport of diagnostic preparations and drugs across BBB.

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