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# Analysis of Serotonin Transporter in Human Platelets by Immunoblotting Using Site-Specific Antibodies

**A. D. Dmitriev[1]*, E. V. Pavlova[2], M. I. Factor[2], O. L. Segal[1], Yu. S. Massino[1], I. V. Dobrohotov[2], M. B. Smirnova[1], D. E. Hwun[2], D. A. Yakovleva[2], G. I. Kolyaskina[2], and O. S. Brusov[2]**

*[1]Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow 117485, Russia; fax: (7-095) 338-8500; E-mail: dmitr@rcmh.msk.ru*
*[2]Mental Health Center, Russian Academy of Medical Sciences, Zagorodnoe Shosse 2, Moscow 113152, Russia; fax: (7-095) 952-8940; E-mail: oleg_brusov@hotmail.com*

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**Abstract**—We have produced a panel of site-specific antibodies recognizing different regions of the human serotonin transporter (SERT). This panel included: 1) monoclonal antibodies 23C5 (mAbs 23C5) to the C-terminal region (amino acid residues 597-630); 2) polyclonal antibodies (pAbs) to the N-terminal region (amino acid residues 69-83); 3) pAbs to the region (amino acid residues 86-100) in the beginning of the first transmembrane domain (TMD). The antibodies were produced using recombinant proteins and synthetic peptides (containing certain sequences of SERT) as antigens. These antibodies were purified by affinity chromatography, conjugated to horseradish peroxidase (HRP), and used for immunoblotting analysis of SERT in extracts of human platelets. Sodium dodecyl sulfate extracts were prepared under conditions preventing non-specific proteolytic degradation of the proteins. In platelet extracts, all antibodies were able to detect the 67 kD protein, apparently corresponding to full-length SERT molecule (its theoretical mass is about 70 kD). These antibodies also detected several polypeptides of smaller size (56, 37, 35, 32, 22, and 14 kD), apparently corresponding to N-terminal, C-terminal, and non-terminal SERT fragments. Specificity of immunostaining was confirmed by preincubation of HRP-labeled anti-SERT antibodies with excess of corresponding antigen, which resulted in disappearance of protein band staining. It is suggested that SERT undergoes a programmed proteolytic cleavage (processing) resulting in formation of several SERT-derived polypeptides of smaller size. It is possible that one of the cleaved SERT species is required for serotonin transport activity. Possible sites for specific proteolysis may be located in the region near TMD1 and in the intracellular loop between TMD4 and TMD5.

*Key words*: serotonin transporter, proteolytic processing, human platelets, immunoblotting, site-specific antibodies, monoclonal antibodies

In man and animals, the activity of the serotonergic system is significantly influenced (and determined) by the serotonin transporter (SERT), an integral protein of the presynaptic membrane, which is responsible for synaptic serotonin reuptake [1-5]. During the last decade genes encoding SERT in man, some mammals, and *Drosophila melanogaster* have been cloned [6-13]. SERT belongs to a family of $Na^+$ and $Cl^-$-dependent transport proteins involved in transmembrane transport of neurotransmitter and neuromodulator biogenic amines and amino acids: serotonin (5-hydroxytryptamine, 5-HT), dopamine, noradrenaline, gamma-aminobutyric acid, glycine, etc. (see for review [14]). Genes encoding SERT and protein transporters of other neurotransmitters share significant homology [14]; high homology also exists among genes encoding SERT in different species [6-13]. Taking into consideration the primary structure of the *SERT* gene, the following structural organization of this transporter protein has been proposed (Fig. 1a) [6, 7]. According to this model, human SERT consists of 630 amino acid residues; its molecular mass is about 70 kD. SERT is suggested to contain 12 transmembrane domains (TMD) and a large hydrophilic loop between the third and the fourth TMDs; this loop has putative glycosylation sites. The N- and C-terminal fragments of the SERT molecule are faced to the intracellular space (Fig. 1a).

*Abbreviations*: DAT) dopamine transporter; GFP) green fluorescence protein; GT) glutathione transferase; 5-HT) 5-hydroxytryptamine (serotonin); mAbs) monoclonal antibodies; pAbs) polyclonal antibodies; PB) phosphate buffer; PBS) phosphate buffered saline; SERT) serotonin transporter; TH) thioredoxin-6His; TMD) transmembrane domain.

* To whom correspondence should be addressed.

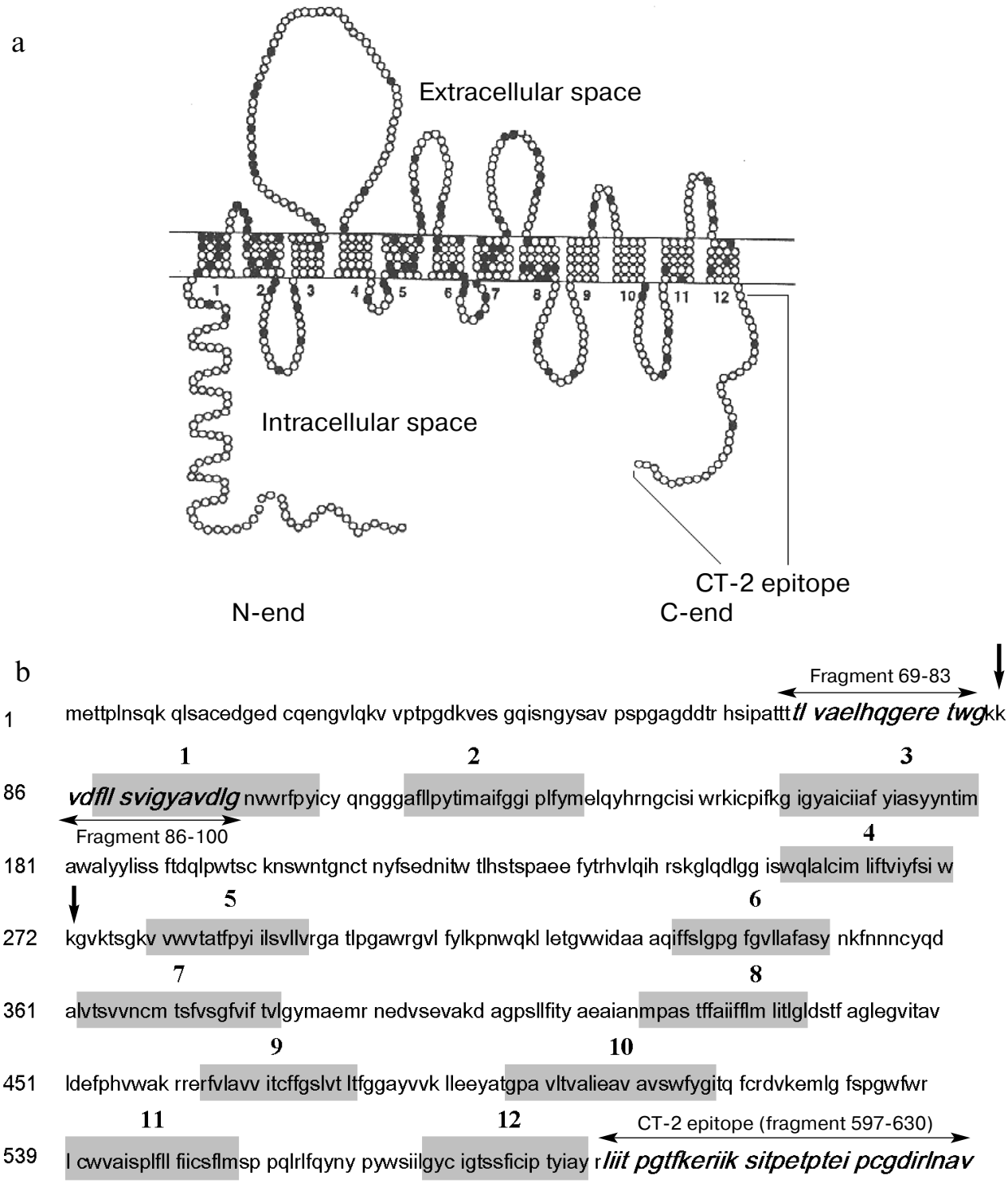


**Fig. 1.** Structure of serotonin transporter: a) topographic model of SERT [16]. Numbers indicate transmembrane domains. Closed circles indicate amino acid residues preserved in all members of the family of $Na^+/Cl^-$-dependent neurotransmitter transporters; b) primary structure of human serotonin transporter. Numbered gray quadrants mark amino acid sequences corresponding to transmembrane domains. Bold italic and horizontal arrows indicate SERT fragments for which antibodies have been obtained. Vertical arrows show putative cleavage sites in the SERT molecule, which are consistent with the hypothesis of the proteolytic SERT processing proposed in this study (see "Discussion").

Similar structure has been proposed for other members of this family [14].

SERT attracts much attention not only as an important research object for neurobiology but also for design of pharmacological drugs. This protein is a target for tricyclic antidepressants, selective serotonin reuptake inhibitors, and also for cocaine and narcotic amphetamines [6-15]. However, in spite of significant progress in molecular genetic studies, many important details of the protein biochemistry of SERT remain unclear. In particular, pathways of posttranslational processing of this protein require further investigation. One of the most effective approaches of SERT study involves the use of specific antibodies. There are reports on immunoblot analysis of SERT [16-18]. However, their results are rather contradictive. For example, there is significant variability in molecular masses of SERT immunoreactive polypeptides detected in various laboratories [16-18].

In this study, we have analyzed human platelet SERT by the immunoblotting method. A panel of site-specific anti-SERT antibodies has been obtained, characterized, and evaluated as a tool for SERT study. They include: 1) monoclonal antibodies (mAbs) to the C-terminal fragment of rat SERT fragment 597-630 (these antibodies also recognize human SERT); 2) polyclonal antibodies (pAbs) to the 69-83 fragment of N-terminal part of human SERT; 3) pAbs to the 86-100 fragment of TMD1 of human SERT. It is known that SERT is localized in the platelet plasma membrane, where it is involved in maintenance of hemostasis [19]. Neuronal and platelet SERT molecules are synthesized from expression of the same gene, and it is believed that they share identical pharmacological properties [20]. Thus, platelets represent a convenient extracerebral model for studies of SERT [21, 22]. However, it should be noted that platelet SERT has not been analyzed by the method of immunoblotting by using a panel of antibodies produced against various sites of the transporter molecule. Results of this study suggest that in platelets the SERT molecule undergoes endoproteolysis with the formation of several polypeptides.

## MATERIALS AND METHODS

**Construction of recombinant proteins carrying rat SERT fragment 597-630 (CT-2 epitope).** The recombinant protein denominated in this study as P1 was a kind gift of Professor R. Blakely (School of Medicine, Vanderbilt University, Nashville, Tennessee, USA). For construction of the plasmid for expression of this protein, a segment of rat *SERT* encoding 34 C-terminal residues (listpgtlkeriiksitpetpteipcgddirmnav) was cloned into the pGEX2T plasmid [16]. In P1 protein, modified glutathione transferase (GT) was fused to rat SERT fragment 597-630 (denominated as CT-2 epitope) [16]. The rat SERT fragment 597-630 differs from the corresponding region of human SERT (Fig. 1b) by three amino acid substitutions [16]. In the recombinant protein denominated in this study as P2, modified thioredoxin-6His (TH) was fused with rat SERT fragment 597-630. The plasmid carrying nucleotide sequence encoding P2 was obtained in the Laboratory of Gene Chemistry (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow). For preparation of P2, the nucleotide sequence encoding SERT fragment 597-630 was cloned from pGEX2T plasmid (supplied by Prof. Blakely) into plasmid pEC32. Protein products P1 and P2 purified by affinity chromatography and migrating on SDS-PAGE as single bands were used.

**Synthesis of peptide fragments of SERT.** Peptides corresponding to fragments 69-83 (tlvaelhqgeretwg) and 86-100 (vdfllsvigyavdlg) of human SERT (Fig. 1b) were synthesized in the Laboratory of Peptide Chemistry Synthesis (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow).

**Preparation of mAbs to C-terminal SERT fragment 597-630.** For hybridoma preparation BALB/c mice were immunized (by six subcutaneous injections with interval of 14-21 days) with the recombinant P2 (fused 597-630–TH) protein, emulsified in complete (first immunization) or incomplete (subsequent immunizations) Freund's adjuvant (15 μg per mouse). During four days before hybridization, mice received daily intraperitoneal injections of the recombinant P1 (fused 597-630–GT) protein (100-200 μg) in 0.15 M NaCl. Hybridomas were obtained by the method of Koller and Milstein [23] using hybridization protocol accepted in our laboratory [24, 25]. Cultural medium from each hybridoma was tested for the presence of antibodies against P1 and P2 by the ELISA method [26, 27]. Plastic immunologic plates (GOSNIIMEDPOLYMER) were saturated with P1 or P2 (2 μg/ml in 0.05 M carbonate buffer, pH 9.5, 100 μl per well) overnight at room temperature. Immune complexes between immobilized antigens and hybridoma antibodies were registered using peroxidase-labeled rabbit antibodies against mouse IgG and tetramethylbenzidine as substrate. Color reaction was read at 450 nm using a Titertek Multiskan MC plate spectrophotometer. For preparation of ascites liquid, cells of cloned (at least three times) hybridomas were inoculated into peritoneum of BALB/c mice. The isotype of mAbs was determined using a hybridoma subisotyping kit (Calbiochem, USA). Antibodies from culture supernatants were also tested in immunoblotting with platelet extracts (see "Immunoblotting" paragraph).

**Purification of ascites liquid mAbs against SERT.** Monoclonal antibodies were isolated from ascites liquid by chromatography on DEAE-Sepharose 4B (Pharmacia, Sweden) or by affinity chromatography on BrCN-Sepharose 4B (Pharmacia) conjugated with antigen (P2). Chromatographic conditions were described earlier [26, 28, 29]. Purified mAbs were precipitated by addition of

ammonium sulfate (0.5 g/ml) followed by subsequent centrifugation (10,000*g*, 20 min, 5°C). The precipitate was dissolved in 0.05 M sodium carbonate buffer, pH 9.5. After dialysis in the cold against the same buffer, the antibodies were stored at −30°C.

**Preparation of pAbs to SERT peptide fragments 69-83 and 86-100.** Rabbits were immunized with the human SERT peptide fragments conjugated with BSA using glutaraldehyde [30]. The immunization scheme was the same as in [30]. Antibodies to peptide fragments were detected in rabbit blood serum using standard ELISA procedures [25, 26]. Immunologic plates were saturated with peptides overnight at room temperature (5 μg/ml in 0.05 M sodium carbonate buffer, pH 9.5; 100 μl per well). Immune complexes were detected by donkey anti-rabbit IgG conjugated with horseradish peroxidase using tetramethylbenzidine as substrate for color reaction.

**Purification of pAbs to SERT peptide fragments by affinity chromatography.** For preparation of sorbents for affinity chromatography SERT fragments (69-83 and 86-100) were covalently bound to BrCN-Sepharose 4B (Pharmacia) following supplier recommendations (0.4 mg peptide was conjugated to 0.2 g activated BrCN-Sepharose 4B). For each antibody purification procedure, pooled sera from three rabbits were used. The pooled serum sample (5 ml) was diluted three times with 0.025 M sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl (PBS, phosphate buffered saline) and passed three times through the column (5 × 15 mm) with the immunosorbent. The column was washed with the same buffer until the baseline was reached at 280 nm; absorbance was registered using a Uvicord 2138 flow spectrophotometer (LKB, Sweden). Antibodies were eluted with 0.1 M acetic acid, pH 2.2 (pH was adjusted with HCl). The eluate was neutralized with concentrated ammonia. Yield of antibodies was 50-210 μg per ml of immune serum. Purified pAbs were precipitated by addition of ammonium sulfate (0.5 g/ml) followed by centrifugation (10,000*g*, 20 min, 5°C). The precipitate was dissolved in 0.05 M sodium carbonate buffer, pH 9.5. After dialysis in the cold against the same buffer, the antibodies were stored at −30°C.

**Protein determination.** Protein concentration was determined by the Lowry method [31] using BSA as standard, which was prepared using the ratio of $A^{1\,cm}_{280\,nm} = 7.6$ for 1% BSA [32]. Concentration of purified antibodies and peroxidase was determined by the optical method using the ratios of $A^{1\,cm}_{280\,nm} = 14$ [32] for 1% antibody solution and $A^{1\,cm}_{403\,nm} = 22.75$ and $A^{1\,cm}_{280\,nm} = 7.3$ for 1% purified peroxidase, respectively.

**Isolation of human platelets.** Fifty milliliters of blood was collected into tubes containing 5 ml of an anticoagulant (0.09 M sodium citrate, 0.07 M citric acid, 0.110 M glucose, pH 5.7). Erythrocytes were removed by centrifugation (200*g* for 15 min). Platelets pelleted from the supernatant by subsequent centrifugation (3000*g* for 20 min) were washed with the citrate buffer and used for preparation of extracts.

**Preparation of platelet extracts for electrophoresis.** Platelet extracts were prepared by the following methods.

*Method A.* Freshly isolated platelets were suspended in the lysing buffer containing 0.01 M Tris-HCl, pH 7.4, 0.1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and a mixture of protease inhibitors (1 mg/ml soybean trypsin inhibitor, 50 μM leupeptin, 1 mM phenanthroline, 1 mM iodoacetamide, 250 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 μM tosylphenylalanine chloromethylketone, and 50 μM tosyllysine chloromethylketone). The lysate stored for 1-2 h (in various experiments) at room temperature was then treated with reducing buffer (1/5 of the lysate volume): 0.025 M Tris-HCl, pH 7.4, 10% SDS, 5% 2-mercaptoethanol. After the treatment for 30 min at room temperature, the lysate was used for electrophoresis.

*Method B.* Freshly isolated platelet were suspended in the cold in five volumes of 0.025 M Tris-HCl, pH 6.8, containing 8 M urea (Merck, USA) and 2% 2-mercaptoethanol. Extracts were then mixed with equal volume of 10% (w/v) aqueous SDS, incubated at room temperature for 1 h, and then used for electrophoresis.

*Method C.* Platelet lysate prepared by method B was immediately heated (after adding SDS) on a boiling water bath for 1.5 min and used for electrophoresis.

*Method D.* Freshly isolated platelets were suspended in five volumes (w/v) of 0.025 M Tris-HCl buffer, pH 6.8, containing 20% glycerol and 2% 2-mercaptoethanol. After addition of equal volume of 10% aqueous SDS, the mixture was immediately heated on a boiling water bath for 1.5-2 min. The resultant lysate was used for electrophoresis.

**SDS-PAGE of platelet extract proteins.** Electrophoresis in 10-15% polyacrylamide gel in the presence of SDS was carried out by the method of Laemmli [34]. Protein molecular mass markers (Pharmacia) were used as standards.

**Antibody conjugation with horseradish peroxidase.** Mono- and polyclonal antibodies (8 mg) purified by affinity chromatography were conjugated with horseradish peroxidase (4 mg; $R_z = 3$) by the periodate method [35].

**Immunoblotting.** Proteins were electrotransferred onto a nitrocellulose membrane after electrophoresis by the method of Towbin et al. [36] using Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, USA, Sweden). The electrotransfer was carried out at 4°C and voltage 6 V/cm for 16-18 h. Subsequent steps varied depending on the antibodies to SERT (pAbs or mAbs). In the case of immunoblotting with anti-SERT pAbs, the nitrocellulose membrane was washed with PBS after the electrotransfer and incubated in 20% neutral rabbit serum in PBS (1 h for 37°C). The membrane was washed with PBS containing 0.05% Tween-20 and incu-

bated with the anti-SERT pAbs conjugated with horseradish peroxidase (1000-1500 ng/ml in PBS containing 20% neutral rabbit serum and 0.05% Tween-20). The sample was incubated at room temperature for 2.5 h.

In the case of immunoblotting with anti-SERT mAbs, the membrane with electrotransferred proteins was washed with PBS and then incubated in 5% aqueous solution of dry milk at 37°C for 1 h. After washing with PBS containing 0.05% Tween-20, the membrane was incubated with the anti-SERT mAbs conjugated with horseradish peroxidase (50-150 ng/ml in PBS containing 0.5% BSA and 0.05% Tween-20). The sample was incubated at room temperature for 2.5 h. Immunoreactive polypeptides were stained by the chemiluminescence-based method using corresponding kits (Amersham, USA).

To control for specificity of staining, one identical strip of the transfer nitrocellulose membrane representing the electrophoresis of individual protein sample (applied across the entire width of a gel) was incubated with peroxidase-labeled anti-SERT antibodies pretreated with excess of antigen (5-10 μg/ml in PBS with 0.5% BSA and 0.05% Tween-20) for 1 h at room temperature.

In the case of anti-SERT antibody testing in supernatants of hybridoma cultures (see above paragraph "Preparation of mAbs for C-terminal SERT fragment 597-630"), nitrocellulose membrane representing the electrophoresis of an individual sample (human platelet extract), applied across the entire width of a gel, was cut into vertical strips. Each strip was incubated with individual hybridoma culture supernatant diluted 20-fold with PBS. The sample was incubated at room temperature for 2.5 h. Immune complexes were detected by peroxidase-labeled rabbit antibodies against mouse IgG.

**Purification of human platelet extracts by affinity chromatography.** Antibodies (2 mg) to SERT C-terminal fragment 597-630 (clone 23C5) purified by chromatography on DEAE-Sepharose were conjugated with BrCN-Sepharose 4B (0.2 g) following the supplier's recommendations. Human platelets were extracted (in the cold) with five volumes of PBS containing 8 M urea. The extract mixed with an equal volume of PBS containing 2% Triton X-100 and 2% sodium deoxycholate was incubated at room temperature for 40 min. Supernatant obtained after centrifugation of extract (15,000*g*, 20 min, 0°C) was dialyzed against PBS containing 0.05% Triton X-100 and 0.5% sodium deoxycholate. The dialyzed supernatant was then used for the affinity chromatography procedure, which was carried out at room temperature. The platelet extract was passed three times through a small column (2 × 15 mm) packed with the affinity sorbent (Sepharose conjugated with mAbs 23C5). The column was washed with PBS containing 0.05% Tween-20 until reaching baseline absorbance. A fraction bound to antibodies was eluted with 0.1 M acetic acid, pH 2.2 (the pH value was adjusted with concentrated HCl). The eluate was neutralized with concentrated ammonia, frozen, and lyophilized. The lyophilized sample obtained after affinity purification of extracts from 200 mg of platelets was prepared for electrophoresis as described (see method B in the paragraph "Preparation of platelet extracts") by preparing 200 μl of solution, and 10-15 μl aliquot was loaded onto each lane during electrophoresis.

## RESULTS

**Preparation of hybridomas secreting mAbs to SERT C-terminal fragment including amino acid residues 597-630.** For hybridoma production, mice were immunized with the recombinant protein P1 and P2 carrying rat SERT fragment 597-630. Fusion of myeloma cells with mouse splenocytes resulted in growth of more than 2000 primary hybrid cultures. Using ELISA assay, potent anti-P1 and anti-P2 activity was detected in 21 wells. (Color reaction of ELISA assay exceeded the scale at dilution 1 : 100.) This suggested the presence of hybridomas producing antibodies specific to SERT fragment 597-630 in both recombinant proteins. (In P1 and P2, this fragment was fused with GT and TH, respectively.) Culture supernatants obtained from these hybridomas were additionally tested in immunoblotting with human platelet extracts (prepared by method A described in the paragraph "Preparation of platelet extracts for electrophoresis" of the "Materials and Methods" section). Antibodies of 18 hybridomas (of 21 tested) exhibited a typical immunoblotting pattern: total staining in the zone of proteins of molecular mass >50 kD and two strongly stained protein bands of molecular masses of 35 and 37 kD (Fig. 2). For hybridomas producing antibodies selected for further study, additional controls for specificity were carried out. We tested antibody-binding ability using recombinant proteins, which included the same protein carriers, but different sense sequences. In these experiments, we employed two proteins: modified thioredoxin-6His fused with human interleukin-13 and modified GT fused with α-7 subunit of human acetylcholine receptor[1]. Antibodies produced by four hybridomas selected for further experiments did not interact with these recombinant proteins in the ELISA test. Control recombinant proteins also were not stained in the immunoblotting with antibodies produced by these selected hybridomas. Taken together, results of these experiments provided convincing evidence for the absence of antibody interaction with GT and TH (data not shown). These hybridomas were subjected to triple cloning; during these clonings only single clones exhibiting a strongly positive result in ELISA tests on the presence of anti-P1 and anti-P2 activities were

[1] Control recombinant proteins were kindly supplied by A. F. Shevalie (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow).

selected. Finally, we obtained a panel of four cloned hybridomas producing antibodies putatively specific to SERT fragment 597-630.

**Immunoreactivity of mAbs to SERT fragment 597-630 in optimized immunoblotting of human platelet extract.** Antibodies from four various cloned hybridomas were studied in more detail for their ability to detect SERT in human platelet extracts by immunoblotting. All selected mAbs exhibited a similar immunoblotting pattern (the only differences were found in intensity of polypeptide staining). In the present report, we demonstrate results obtained using antibodies produced by the hybridoma denominated in Fig. 2 as No. 15 (mAbs 23C5). These antibodies were the most effective as evaluated in the first testing. During optimization of conditions for detection of polypeptides in chemiluminescence immunoblotting, we had to reject the conventional procedure of immunoreactive peptide staining (detection of antigen–antibody complex by peroxidase-labeled antibodies against mouse immunoglobulins). Use of such methods was accompanied by potent nonspecific staining of proteins with molecular masses of >50 kD (Fig. 2). So, we selected a single step immunoblotting variant employing anti-SERT mAbs (purified by affinity chromatography), which were conjugated with peroxidase. This approach eliminated nonspecific staining of proteins with molecular mass exceeding 50 kD (where additional protein bands appeared, see below). Special attention was given to preparation of platelet extracts for SDS-PAGE. Use of some methods of lysate preparation caused artifacts originated from nonspecific degradation of proteins or their aggregation [37, 38]. At the first stage of this study (testing of hybridomas), we used method A of extract preparation. This method included treatment of the platelets with the lysing buffer containing a mixture of detergents and a cocktail of protease inhibitors. However, we suggested that this method usually employed for SERT analysis [16] could not exclude the possibility of nonspecific proteolysis of extracts even in the presence of protease inhibitors. So, we also used some other known approaches preventing proteins from degradation and possible aggregation [37]. The main characteristic feature of these methods consists of immediate treatment of platelet proteins with harsh factors causing irreversible denaturation of all cell proteins including cell proteases. The extraction was carried out in the presence of 8 M urea and 5% SDS (method B); other treatment also included heating of extracts at 90°C for 1.5-2 min in the presence of 8 M urea and 5% SDS (method C) or in the presence of 5% SDS only (method D) (see paragraph "Preparation of platelet extracts for electrophoresis" in the "Materials and Methods" section).

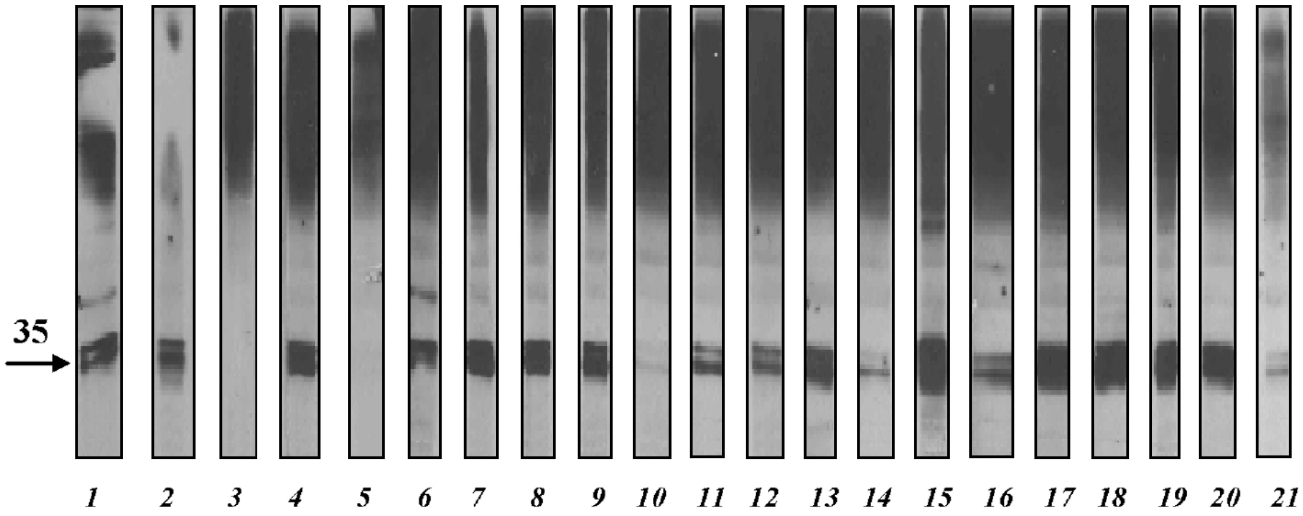


**Fig. 2.** Testing of antibody against rat SERT fragment 597-630 in culture medium obtained from primary hybridomas by means of immunoblotting of human platelet extract. Supernatants of culture media of various primary hybridomas exhibiting strong positive reaction in ELISA test during binding to recombinant protein carrying the 597-630 fragment of rat SERT were used for immunoblot analysis. Human platelet extract was prepared by method A (see paragraph "Preparation of platelet extracts" in the "Materials and Methods" section) in the presence of protease inhibitors. After SDS-PAGE and electroblotting of the extract, the nitrocellulose membrane was cut into strips. Each strip was used for testing with culture medium antibodies (diluted by 1 : 20) from each separate hybridoma defined in the figure by the corresponding number. Formation of immune complexes was detected by peroxidase-labeled rabbit antibodies against mouse Ig (preadsorbed with human IgG) using the chemiluminescence-based system. All details are given in the "Materials and Methods" section. Each lane corresponds to a sample of culture medium from a separate primary hybridoma. (The total number of analyzed primary hybridomas was 21). The arrow indicates the position of molecular mass (kD).

Figure 3 shows results of immunoblotting of human platelet extracts prepared by the above-mentioned methods. In all extracts, peroxidase-labeled mAbs 23C5 detected a protein with molecular mass of 67 kD. It is relevant to suggest that this 67 kD protein represents the full-length SERT molecule. Some underestimation of molecular mass compared with the deduced value (70.5 kD) obtained on the basis of human SERT cDNA [20] may be attributed to frequently observed abnormal mobility of highly hydrophobic membrane proteins in polyacrylamide gels [16]. This antibody also detected two polypeptides of lower molecular mass (37 and 35 kD). In all lysates obtained by using harsh methods (B, C, and D, see "Materials and Methods"), mAbs 23C5 detected an additional band corresponding to a protein of molecular mass of 56 kD. All protein bands disappeared if the antibody was pretreated with recombinant proteins P1 or P2 (Fig. 3, lanes *3* and *4* in variant A, lanes *1* and *2* in variant B). The latter suggests specificity of staining. The most intensive staining was observed for protein bands of 35 and 37 kD, whereas 67 and 56 kD polypeptides were stained less intensively. It should noted that 67, 37, and 35 kD proteins were detected in lysates prepared by mild treatment of platelets in the presence of protease inhibitors (method A) and by harsh methods B, C, and D. A duplex of polypeptides with molecular mass of 35 and 37 kD was detected as intensive bands in all platelets obtained from various donors irrespectively to methods used for extract preparation. This suggests significant stability of these polypeptides. In contrast to this duplex, the polypeptide of 56 kD was detected only in extracts prepared using harsh methods B, C, and D. It should be noted that on loading of equal amounts of proteins onto each lane, intensity of staining of all immunoreactive bands including the 67 kD protein was higher when preparation of samples employed harsh methods (B, C, and D) than the mild treatment (method A). This suggests that the harsh methods used for platelet preparation cause almost immediate inactivation of all cellular proteases in the lysate and such treatments protected SERT from nonspecific degradation better than mild treatment of platelets in the presence of protease inhibitors. Thus, immunoblotting patterns obtained after use of harsh methods for extract preparation reflect the actual state of SERT in platelets; they obviously do not represent artifacts related to the preparation of samples. It is possible that harsh conditions favor maintenance of unstable 56 kD protein in lysates. The lysates prepared by using harsh conditions gave better reproducibility of the immunoblotting pattern (cf. variants B, C, and D in Fig. 3). Use of the mild treatment for lysate preparation revealed additional polypeptides. For example, lanes *1*, *2*, and *3* (Fig. 3a) corresponding to electrophoresis of various lysates prepared under mild conditions are characterized by the presence of additional bands (e.g., of ~200 kD on lane *2* and ~41 and 27 kD on lane *3*). It should be noted that an increase in exposure time resulted in the appearance of 200 kD protein (putative aggregated form of SERT) often detected as a thin band in extracts prepared by harsh methods (data not shown). In subsequent studies of platelet SERT, we always employed method B

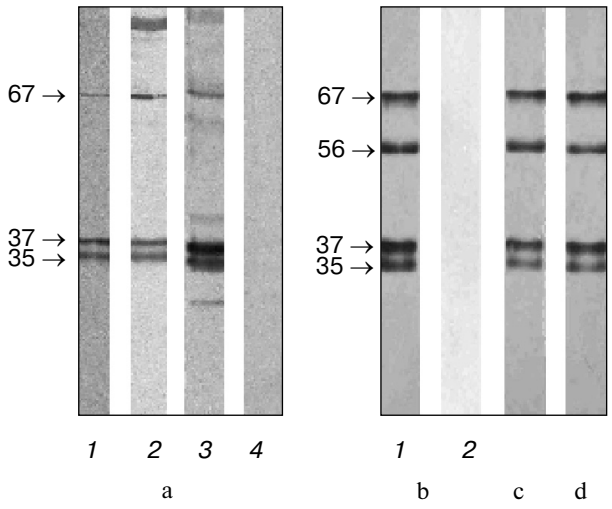


**Fig. 3.** Use of peroxidase-labeled mAbs to 597-630 SERT fragment (mAb 23C5) for SERT detection in human platelet extracts prepared by various methods. After electrophoresis and electroblotting of extracts, prepared by four different methods (see methods A, B, C, and D in the paragraph "Preparation of platelet extract" of the "Materials and Methods" section), the nitrocellulose membrane was incubated with mAb 23C5 labeled horseradish peroxidase and proteins were developed by the chemiluminescence-based method. To control specificity of staining the peroxidase labeled mAb 23C5 was preincubated (before immunoblotting) with excess of the antigen, recombinant P2 protein. Experimental details are given in corresponding paragraphs of the "Materials and Methods" section. a) Immunoblotting of platelet extracts prepared by method A: platelets were suspended in the lysing buffer containing the mixture of protease inhibitors. After 2 h, samples were treated with the reducing buffer (5% 2-mercaptoethanol and SDS) for 1-2 h at room temperature. *1*, *2*, *3*) Immunoblotting of lysates of platelets obtained from various donors; *4*) control for specificity of protein staining. b) Immunoblotting of platelet extract prepared by method B: platelets suspended in Tris-HCl buffer, pH 6.8, containing 8 M urea and 2% 2-mercaptoethanol were treated with 5% SDS for 1 h at room temperature. A nitrocellulose strip representing the pattern of one electrophoretic lane was incubated with mAb 23C5 (*1*) or with mAb 23C5 preincubated with excess of the antigen (*2*) (control for specificity of staining). c) Immunoblotting of platelet extract prepared by method C: platelets suspended in Tris-HCl buffer, pH 6.8, containing 8 M urea and 5% 2-mercaptoethanol, were mixed with SDS (final concentration 5%) and immediately heated on a boiling water bath for 1.5 min. d) Immunoblotting of platelet extract prepared by method D: platelets suspended in Tris-HCl buffer, pH 6.8, containing 20% glycerol and 2% 2-mercaptoethanol were mixed with SDS (final concentration 5%) and immediately heated on a boiling water bath for 1.5-2 min. In variants b, c, and d, lysates were prepared using platelets from one donor; isolated platelets were separated into three equal portions and lysed as described. Arrows indicate positions of molecular masses of peptides (kD).

(platelet extraction with buffer containing 8 M urea followed by subsequent treatment with SDS at room temperature) because it gave the most reproducible results.

**Preparation of pAbs to SERT fragments 69-83 and 86-100 and their use for analysis of human SERT.** For better analysis of SERT, we also obtained polyclonal rabbit antibodies to human SERT. Epitope specificity of these antibodies differed from that of mAbs 23C5. For preparation of one type of serum, we used a synthetic polypeptide corresponding to the fragment of N-terminal region including residues 69-83 (Fig. 1). For preparation of the other type of serum, we used the neighboring sequence 86-100 positioned in the beginning of TMD1 (Fig. 1). We denominated these antibodies as pAbs 69-83 and pAbs 86-100. In immunoblotting, pAbs 69-83 and pAbs 86-100 purified by affinity chromatography (on Sepharose covalently bound to the peptides) and conjugated with horseradish peroxidase were used. For demonstration of specific binding of these antibodies to the SERT molecule, we used the following approach. The antibody mAbs 23C5 (to the SERT C-terminus) was covalently bound to BrCN-Sepharose, and this affinity sorbent was used for purification of platelet extracts as described in "Materials and Methods". Protein fraction of platelet extract bound to the affinity sorbent was analyzed further by immunoblotting. Nitrocellulose strips belonging to the same electrophoretic lane were incubated with peroxidase labeled mAbs 23C5 (i.e., with the antibody used for affinity purification) or with peroxidase labeled pAbs 69-83 or pAbs 86-100. Figure 4 shows representative results of these experiments. In the fraction of platelet extract bound to mAbs 23C5-Sepharose, immunoblotting with the same antibody revealed three polypeptides (Fig. 4, variant *1*): 67 kD protein and duplex of polypeptides of 37 and 35 kD. Use of mAbs 23C5 did not reveal the 56 kD protein in the fraction purified by affinity chromatography. This may suggest its high instability. Immunoblotting with pAbs 69-83 and pAbs 86-100 detected only the 67 kD polypeptide in the platelet fraction purified by affinity chromatography. Again, this is consistent with molecular mass of the whole SERT molecule (Fig. 4, variants *2* and *3*). These antibodies did not reveal the polypeptide duplex 37-35 kD that was recognizable by mAbs 23C5. This suggests lack of N-terminal part of the SERT sequence in these proteins. Thus, in immunoblotting both mAbs 23C5 (to C-terminal SERT fragment 597-630) and antibodies to SERT fragments 69-83 and 86-100 (located in N-terminal part of SERT molecule) detect the 67 kD protein purified from human platelet extracts by affinity chromatography on mAbs 23C5-Sepharose. This is consistent with our suggestion that the 67 kD protein revealed by mAbs 23C5 during immunoblotting of human platelet lysates (Fig. 3) represents the full sized SERT molecule (including both N- and C-terminal parts). These results also indicate that pAbs to SERT fragments 69-83 and 86-100 as well as mAbs to SERT fragment 597-630 can reveal the SERT molecule during immunoblotting.

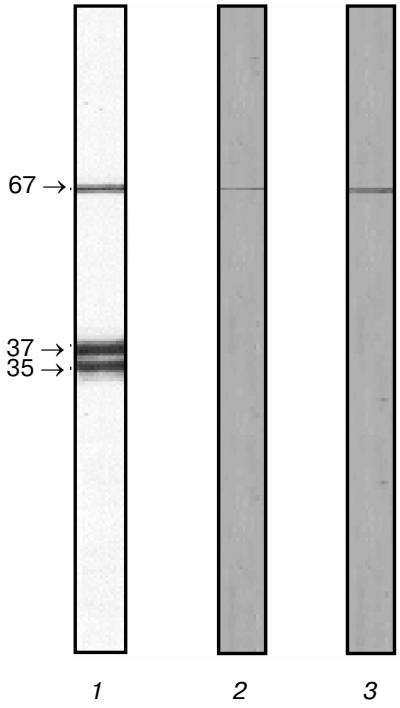


**Fig. 4.** Immunoblot identification of SERT in human platelet extracts purified by affinity chromatography on Sepharose conjugated with mAb 23C5 (to SERT fragment 597-630). All details are given in the paragraph "Purification of platelet extracts by affinity chromatography" of the "Materials and Methods" section. After SDS-electrophoresis and electroblotting of purified extracts (applied across the entire width of a gel), the nitrocellulose membrane was cut into strips, and immunostaining with peroxidase-labeled antibodies to various sites of SERT molecule was carried out. Immunoblotting was developed by the chemiluminescence-based method. *1*) Immunoblotting with mAb 23C5 (to SERT fragment 597-630) conjugated with peroxidase; *2*) immunoblotting with polyclonal antibodies to human SERT fragment 69-83 (which were purified by affinity chromatography and conjugated with peroxidase); *3*) immunoblotting with polyclonal antibodies to human SERT fragment 86-100 (which were purified by affinity chromatography and conjugated with peroxidase). Arrows indicate positions of molecular masses of polypeptides (kD).

Figure 5 shows results of SERT comparative analysis in platelet lysates by immunoblotting with pAbs 69-83, pAbs 86-100, and mAbs 23C5. Polyclonal antibodies to SERT fragment 69-83 revealed 2 protein bands of 14 and 32 kD (Fig. 5, variant *2*). These antibodies did not detect 67 kD protein; its presence in the same lysate was demonstrated using mAbs 23C5 (Fig. 5, variant *1*). However, pAbs 69-83 revealed 67 kD protein during immunoblotting of platelet extracts purified by affinity chromatography (Fig. 4). It is possible that the affinity of these antibodies to this protein is insufficient for detection of 67 kD protein in crude platelet extracts. In fact, we found that

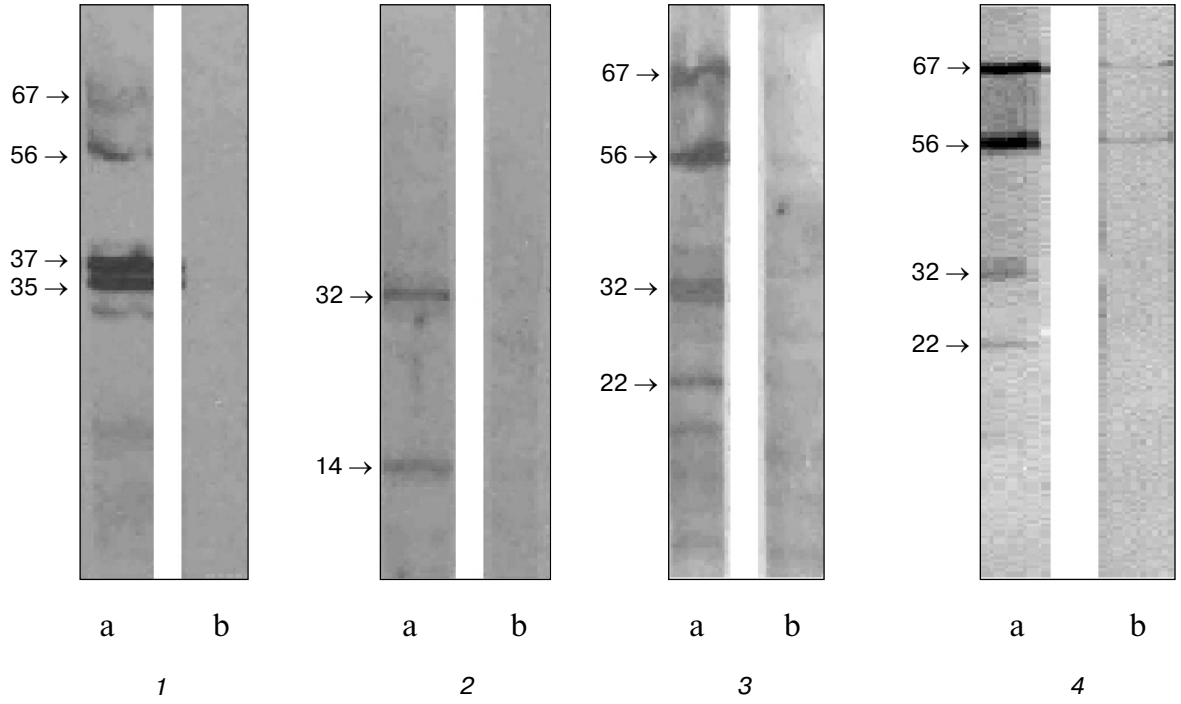


**Fig. 5.** Immunoblot detection of SERT in human platelet extracts using antibodies to various fragments of its molecule. After SDS electrophoresis of human platelet extracts in 15% polyacrylamide and electroblotting, the nitrocellulose membranes were cut into strips, each of which represented the pattern of one electrophoretic lane; these strips were then incubated with either peroxidase-labeled antibodies against SERT (a) or with the same antibodies preincubated with antigen excess (b). Immunoblotting was developed by the chemiluminescence-based method. *1*, *2*, *3*) These strips represent the patterns of the same electrophoretic lane. *1*) Immunoblotting with mAb 23C5 (to SERT fragment 597-630) conjugated with peroxidase; *2*) immunoblotting with polyclonal antibodies to SERT fragment 69-83, which were purified by affinity chromatography and conjugated with peroxidase; *3*, *4*) immunoblotting with polyclonal antibodies to SERT fragment 86-100, which were purified by affinity chromatography and conjugated with peroxidase (immunoblotting of two various lysates from independent experiments). Arrows indicate positions of molecular masses (kD).

affinity of anti-SERT pAbs was significantly lower than that of mAbs 23C5. For example, effective immunoblotting required 50-150 ng/ml concentrations of peroxidase-labeled mAbs 23C5, whereas staining with pAbs 69-83 and 86-100 required much higher antibody concentrations (1000-1500 ng/ml). In the case of pAbs, polypeptide staining was less intensive than in the case of mAbs. Polyclonal antibodies pAbs 86-100 reliably revealed in the same lysate four polypeptides of 67, 56, 32, and 22 kD (Fig. 5, variant *3*). Figure 5 shows results of analysis of two various platelet lysates obtained using pAbs 86-100 (Fig. 5, variants *3* and *4*). In both cases, pAbs 86-100 revealed polypeptides of 67, 56, 32, and 22 kD, whereas the presence of polypeptides of 35 and 17 kD was not obligatory. These results were well reproduced in various experiments. All the bands in Fig. 5 disappeared (or their staining sharply decreased) when the antibodies were preincubated with excess of antigen (corresponding synthetic peptide) (Fig. 5, variants *1b*, *2b*, *3b*, and *4b*).

Thus, under conditions preventing nonspecific protein degradation in lysates, immunoblotting of human platelet extracts with mAbs 23C5 (to SERT C-terminus), pAbs 69-83 (to SERT N-terminus), and pAbs 86-100 (to SERT TMD1) revealed seven polypeptides of 14, 22, 32, 35, 37, 56, and 67 kD.

## DISCUSSION

In the present study, we prepared mAbs and pAbs to three fragments of the SERT molecule, including amino acid sequences 69-83, 86-100, and 597-630. These antibodies provide an effective tool for immunoblot analysis of SERT in human platelet extracts. We compared our results with results obtained by other authors. Antibodies to SERT were originally obtained and described by Qian et al. [16]. They used pAbs to the C-terminal fragment of rat SERT 597-630 (defined as CT-2 epitope) for SERT immunoblot analysis. SERT was detected in rat tissues and transfected HeLa cells expressing rat SERT. It should be noted that the SERT region including residues 597-630 is high conservative. For example, this region in rat and human SERT differs by three amino acid substitutions [16]. The C-terminal region of SERT is characterized by high divergence to highly homologous proteins, i.e., transporters of other neurotransmitters. For exam-

ple, the C-terminal regions of SERT and noradrenaline transporter (the closest SERT homolog) share only 29% homology [39]. Thus, antibodies to SERT fragment 597-630 represent a convenient tool for identification and characterization of SERT in human and animal tissues. Qian et al. [16] found that after short term exposure of immunoblots, SERT was detected in transfected cell preparations as a 61 kD protein; prolonged exposure also revealed additional bands with molecular masses of 90 and 200 kD. The appearance of high molecular weight forms of >200 kD was attributed to protein aggregation [16]. In membrane fraction of rat brain tissue SERT was detected as a 76 kD protein. Treatment of membranes with glycosidase also resulted in appearance of 56 and 58 kD SERT immunoreactive protein. The authors [16] suggested that these proteins represent deglycosylated SERT molecules. Underestimated values of molecular mass of these proteins compared with molecular mass of rat SERT deduced by its cDNA structure (68 kD) were explained by abnormal migration of hydrophobic transport proteins in polyacrylamide gels. According to these authors, after short term exposure of immunoblots, SERT was revealed in rat platelet preparations as a polypeptide of 94 kD. Thus, Qian et al. employing pAbs to SERT fragment 597-630 did not find any evidence for the existence of low molecular weight forms of SERT. In our study mAbs to the same SERT fragment always detected a duplex of proteins of 37 and 35 kD (besides higher molecular weight proteins of 67 and 56 kD). Belous et al. [40, 41] also used pAbs to three SERT regions including other sequences (596-614, 596-622, and the whole C-terminus) for analysis of SERT in platelet extracts. Besides diffuse staining of proteins of 68-105 kD, these authors found immunoreactive protein of 43 kD. We failed to detect 43 kD protein using mAbs to SERT C-terminus. Reasons for this discrepancy remain unclear. We believe that results of previous studies on SERT analysis in platelets obtained by traditional variants of immunoblotting (using anti-SERT pAbs and the second peroxidase-labeled antibodies) require re-evaluation because of strong nonspecific staining of platelet proteins, which complicates SERT analysis. It should be noted that immunoblotting patterns of platelet extracts obtained in the present study using peroxidase labeled mAbs to SERT C-terminus and mild conditions of extract preparation (method A) reproduced immunoblotting patterns obtained using mAbs 23C5 earlier [42]. In the previous report, we demonstrated results of semi-quantitative analysis of SERT in platelet extracts prepared under mild conditions[1]. Inazu et al. [18] detected SERT as 73 and 120 kD polypeptide. However, the results of our study are consistent with earlier study by Gulesserian et al. also detecting low molecular weight forms of SERT [17]. These authors used the immunoblotting method for analysis of SERT in postmortem brains of normal subjects, patients with Down's syndrome, and patients with Alzheimer's disease. Using commercially available pAbs to rat SERT fragment 579-599 (PC177L, Oncogene, Cambridge, MA), these authors detected SERT as a 31 kD polypeptide in immunoblotting analysis [17]. Some observations consistent with our data were found in other laboratories studying another homologous protein, dopamine transporter (DAT); the latter also belongs to a family of neurotransmitter transporters. Daniels and Amara [43] expressed DAT in dog kidney cell line as a chimeric protein. The construct included DAT fused with green fluorescence protein (GFP) "attached" to the N-terminus of the transporter. An immunoblotting test employing antibodies to GFP detected two forms of the chimeric proteins of 108 and 66 kD in the transfected cells. Since the molecular mass of GFP is about 27 kD, these chimeric proteins may include DAT forms with molecular masses of 81 and 39 kD, respectively. These results obtained with closely related transport protein suggest that low-molecular-weight forms of SERT, detected in the present article, are an interesting phenomenon rather than an artifact. All the observations considered here may reflect common elements of metabolism of homologous transport proteins.

The comparison of results obtained by various groups who have studied SERT by immunoblotting methods is complicated by the use of different experimental conditions and various antibodies and cell models. Evidently, these factors can influence results. For example, in transfected cells processing of newly synthesized proteins may be altered [44]. Correct use of immunoblotting for SERT analysis (and for any other similar study) requires fixation of the transporter (and possible products of its posttranslational modification) at the moment of lysis of cells or tissues and specificity of immunoreactive peptide staining. We believe that our method of platelet lysis (extraction with 8 M urea followed by immediate treatment with SDS and 2-mercaptoethanol at room temperature) avoids nonspecific proteolysis of SERT. Specificity of immunoblot protein band staining was confirmed for all anti-SERT antibodies employed in this study in control experiments with antibodies preincubated with 100-fold molar excess of the antigen. It is also important that we eliminated nonspecific staining and increased resolution of this method by using single step immunoblotting and peroxidase-labeled anti-SERT antibodies. Using these approaches, we have demonstrated that antibodies to three various fragments of SERT reliably detect seven polypeptides of various molecular masses (14, 22, 32, 35, 37, 56, and 67 kD) in human platelet lysates prepared under harsh conditions. The largest of these proteins (67 kD) detected by all antibodies (to the C-end and to

[1] We apologize for the error in the previous report [42]: protein duplex of 35-37 kD detected by mAbs was wrongly defined as "protein duplex of 40-43 kD".

the N-end of SERT) apparently corresponds to the full-length SERT molecule. Other authors (Qian et al. [16]) also indicated existence of various immunoreactive forms of SERT. These authors [16] suggested that the existence of several immunoreactive forms of SERT detected in their study may be attributed to various glycosylation degrees of these molecules (immature proteins are detected as proteins of lower molecular mass). Thus, it is implied that SERT forms differing by electrophoretic mobility represent full-length SERT molecules of identical primary structure. Existence of different immunoreactive forms of dopamine and noradrenaline transporters found in several studies also was attributed to various glycosylation [43, 45]. However, these findings and viewpoints cannot explain our results. It should be noted that in this study antibodies to different SERT fragments detected different sets of SERT-immunoreactive proteins in the same platelet lysate (Fig. 5). Even mAbs 23C5 of high affinity did not detect the whole spectrum of SERT-immunoreactive proteins (seven variants) detected using the panel of three antibodies to various regions of the SERT molecule. Taking into consideration the significant differences in molecular mass of these polypeptides (14-67 kD), it seems unlikely that all SERT immunoreactive proteins have identical primary structure and the only difference consists in glycosylation degree. On the other hand, it is known that if a protein undergoes intracellular endoproteolysis (proteolytic processing) this may yield several immunoreactive polypeptides of various size, which could be also detected during immunoblotting analysis of cell extracts for this full-length protein [46]. It is relevant to suggest that SERT immunoreactive proteins of molecular masses 14, 22, 32, 35-37, and 56 kD detected in the present study represent polypeptide fragments formed during endoproteolysis of the precursor protein (of deduced molecular mass of 70.5 kD) which has been detected as a 67 kD polypeptide. The average mass of one amino acid residue in the SERT molecule is 106 daltons. Let us assume that the SERT molecule undergoes cleavage by trypsin-like proteolytic enzymes at Lys84-Lys85 and Lys272-Gly273-Val274-Lys275-Tre276-Ser277-Gly278-Lys279 (within the region of intracellular loop between TMD4 and TMD5) as shown at Fig. 1b. In this case two N-terminal fragments with molecular masses of 10 and 30 kD (putatively including amino acid sequences 1-84 and 1-272) and non-terminal 20 kD polypeptide (fragment formed by 85-272 residues) including TMD1-4 should be formed. Cleavage of fragments including 1-84 and 1-272 residues should also yield two C-terminal fragments with molecular masses of 56 and 37 kD; these fragments may include residues 85-630 and 273-630. The molecular masses of polypeptides detected by mAbs to SERT C-terminus (35-37, 56, and 67 kD), pAbs to SERT fragment 69-83 (14, 32, and 67 kD), and pAbs to SERT fragment 86-100 (22, 32, 56, and 67 kD) (Figs. 3-5) are very close to the calculated molecular masses of polypeptides that would be detected by antibodies of given epitope specificity provided that the SERT molecule undergoes endoproteolysis at sites indicated in Fig. 1b. Some discrepancies between calculated and experimentally determined molecular masses of proteolytic products may be related to the large charge of polypeptide in SDS-PAGE. For example, SERT fragment 1-84 (hypothetic molecular mass ≈10 kD, experimentally determine value ≈14 kD) includes five lysine and two arginine residues. Thus, the fragment 1-84, like other positively charged proteins (e.g., histones), may give overestimated molecular mass values on SDS-PAGE [38]. The presence of a protein duplex of 35-37 kD (instead of expected one band in this region) may be attributed to subsequent proteolysis of the 37 kD SERT fragment (due to cleavage of N-terminal fragment including ≈25 residues) or some other posttranslational events leading to changes in the electrophoretic mobility. It is also possible that several neighboring cleavage points exist; a similar situation was proposed for endoproteolysis of presenilin-1 [47]. It should be mentioned that we obtained pAbs to SERT fragments 69-83 and 86-100 located on both sides from the first putative cleavage site (Fig. 1b) to validate correctness of our suggestion about endoproteolysis of SERT. The suggested existence of a proteolytic site at Lys84-Lys85 appeared during SERT analysis employing mAbs to the C-terminus; those experiments revealed the presence of 56 kD polypeptide, which could be formed during N-terminal cleavage of polypeptide of 11 kD from the SERT molecule.

In the context of interpretation of our results, it is important to emphasize that our method of preparation of platelet SDS lysates almost excludes nonspecific degradation of SERT (and related artifacts) at the stage of extract preparation and subsequent electrophoresis. Therefore, our data may be considered as evidence for endoproteolysis of SERT. However, we cannot rule out the possibility of nonspecific degradation of SERT molecules at preceding stages such as isolation of platelets from blood. However, such possibility, which is not even considered by other authors, is unlikely. We always used only freshly prepared platelets: extracts for electrophoresis were prepared within 1.5-2.5 h from blood collection. Evaluation of results of immunoblotting also includes evaluation of probability of unexpected cross-reactions. It is known that mAbs may demonstrate cross-reactivity with antigens that differ from the antigen used for their elaboration [48]. However, it seems unlikely that such coincidence between calculated and experimental data (including number and sizes of products of putative proteolysis) might stem from accidental cross-reactivity of antibodies with polypeptides unrelated to SERT fragments. In this context, it should be also mentioned that some of the detected immunoreactive proteins (67, 56, and 32 kD) could be also detected in the same experiments by using antibodies to different fragments of SERT.

For example, 56 kD protein was detected by mAbs 23C5 and pAbs to 86-100 (Fig. 5). This also supports that detected polypeptides belong to SERT. The polypeptide duplex of 35-37 kD was also detected not only by mAbs 23C5, but also by another 17 antibodies produced by different primary hybridomas (Fig. 2). Recently, we obtained clear evidence that some of these antibodies differ in epitope specificity from mAbs within the sequence including 597-630 residues. (This will be a subject for another publication.) In general, we do believe that our hypothesis on endoproteolysis of SERT requires further investigation.

In the context of this problem, it should be mentioned that proteolytic processing has been demonstrated for several transmembrane proteins. These include presenilins [44, 47], Notch protein [49], and *Drosophila melanogaster* ods/ten-m protein involved in segment formation [50]. Endoproteolytic processing was also demonstrated for recently discovered seven-domain receptor proteins of the PAR family (Protease Activating Receptor); thrombin and trypsin receptors also belong to this family (see for review [51]). After proteolytic cleavage of an intact molecule at one or many points (different transmembrane proteins undergo different modes of endoproteolytic processing), the resultant subunits are assembled into functionally active heterodimer aggregates. Thus, proteolytic cleavage is often used in "natural technologies" (the term proposed by Ugolev [52]) for assembly of molecular machineries inserted into cell membranes. We speculate that specific proteolysis of SERT results in excision of a subunit (or subunits) playing a key role in 5-HT transport. These putative subunits might form functionally active transport aggregate. (There is evidence that SERT exists as oligomer rather than monomer [53].) However, it is also possible that putative endoproteolysis represents a process of programmed protein degradation regulating SERT activity.

We have compared results of the present study with results obtained in the early 1980s using photoaffinity labeling of SERT. Tritiated specific 5-HT reuptake inhibitors were covalently bound to the transporter molecules by ultraviolet irradiation (binding occurred only when irradiated agents interacted as a receptor and a ligand). Using [$^3$H]azidoimipramine as the photoaffinity probe and 2D-electrophoresis procedure, Rotman and Pribluda [54] found several labeled proteins (65, 60, 45, 35 and 34 kD) in human platelet lysates. However, these authors questioned the possible relation of all of these proteins to 5-HT reuptake because imipramine could bind not only to SERT but also to other receptor molecules (although with lower affinity). Wennogle et al. [55] analyzed binding of the photoaffinity probe [$^3$H]2-nitroimipramine to membrane proteins of human platelets. In that study, specific binding was controlled by label displacement by various selective 5-HT reuptake inhibitors. Use of SDS-PAGE revealed several proteins of 21, 23, 30, 35, and 37 kD, which specifically bound the photoaffinity probe in platelet membranes. The highest amount of label (30%) was found in a 30 kD protein. It was suggested that the 30 kD protein is a component of SERT. However, certain discrepancies in results of these groups exist. For example, Wennogle et al. [55] did not find the 45 kD protein detected by Rotman and Pribluda [54]. (We also have not detected such a protein in this study.) Previous works and our study employed different experimental approaches for SERT detection (functional methods and antibodies to various fragments of primary structure, respectively) and also different procedures of cell extract preparation obviously influencing SERT detection. Nevertheless, results of previous studies are generally consistent with our data on existence of SERT-immunoreactive proteins of low molecular mass.

It should be noted that our hypothesis on endoproteolysis of SERT does not exclude possible involvement of glycosylation in heterogeneity of SERT as suggested by others [16]. It is possible that these processes are coupled (e.g., glycosylation might alter SERT sensitivity to endoproteolysis). Some authors also demonstrated alternative splicing of primary transcript of *SERT* [56]. However, it remains unclear whether this mechanism is responsible for formation of various forms of SERT. Thus, elucidation of possible reasons for heterogeneity of immunoreactive SERT forms observed by us and other authors requires further investigations.

In conclusion, we are confident that results of the present study can be useful both in terms of methodology and study of structure and function of SERT.

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