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The membrane-bound rat serotonin transporter, SERT1, is an oligomeric protein

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Abstract The synaptic actions of the neurotransmitter serotonin are terminated by a selective re-uptake system located in the axonal membrane. To gain information about the quaternary structure of this membrane protein, we transiently expressed the recombinant rat serotonin transporter, SERT1, in human embryonic kidney 293 cells. Treatment with sulfhydryl oxidizing agents and the homobifunctional cross-linker dimethyl suberimidate (DMS) generated adducts of 130–180 kDa and 220–270 kDa, respectively. These data indicate an oligomeric structure of SERT1.

Key words: Serotonin transporter; Quaternary structure; Cross-linking; Oligomerization; Rat

1. Introduction

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) modulates many sensory and behavioral processes in the nervous system and plays an important role in mood disorders, such as depression and anxiety [1]. Its rapid re-uptake from the synaptic cleft terminates serotoninergic signal transduction, a process mediated by the Na^+/Cl^--dependent serotonin transporter. Serotonin uptake inhibitors are clinically used as potent antidepressants and have allowed a pharmacological characterization of the serotonin transporter in membrane preparations from different species [1].

Cloning of serotonin transporter cDNAs from rat, human and *Drosophila* revealed that the predicted proteins are highly homologous to each other and represent members of a larger neurotransmitter transporter superfamily [2,3]. Analysis of the deduced amino acid sequences indicates that the encoded polypeptides form integral membrane proteins with 12 putative membrane spanning segments. Heterologous expression of the cloned rat serotonin transporter (SERT1) in *Xenopus* oocytes and mammalian cells generates Na^+-dependent 5-HT uptake, which is sensitive to antidepressants and exhibits a pharmacological profile similar to that of the native transport system [4,5]. Thus, transfection with a single cDNA confers expression of functional serotonin transport of well-defined pharmacology onto non-neuronal cells. However, little is known about the structural organization of this and other neurotransmitter transport systems in the membrane.

To investigate the quaternary structure of the membrane-bound serotonin transporter, we performed cross-linking studies on SERT1 expressed in the human embryonic kidney cell line 293 (HEK-293 cells). Here, we show that recombinant SERT1 can form dimeric and tetrameric adducts upon treatment of SERT1 containing membranes with sulfhydryl oxidizing agents. Use of the bifunctional cross-linker DMS gave similar results, indicating that SERT1 is an oligomeric membrane protein.

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Abbreviations: SERT1, serotonin transporter; DMS, dimethyl suberimidate; 5-HT, 5-hydroxytryptamine; diamide, azodicarboxylic acid [diethylamide]; DTT, dithiothreitol

2. Materials and methods

2.1. Expression of SERT1 in HEK-293 cells

Cell transfection with the SERT1 cDNA and preparation of membranes was performed as described previously [6].

2.2. Cross-linking with diamide (azodicarboxylic acid [diethylamide]) and (o-phenanthroline)$_2$-Cu^{2+}

Membrane preparations from SERT1 transfected HEK-293 cells (20–50 μg total protein) were incubated in freshly dissolved diamide or (*o*-phenanthroline)$_2$-Cu^{2+} at room temperature for different time periods. To avoid unspecific disulfide formation, free thiols were acetylated by preincubation of the membranes in 40 mM iodoacetamide at 37°C prior to oxidation. Cross-linking with (*o*-phenanthroline)$_2$-Cu^{2+} was terminated by the addition of 1 mM EDTA, and cross-linking with diamide by rapid centrifugation of the membranes and resuspension in non-reducing sample buffer prior to gel electrophoresis.

2.3. Cross-linking with dimethyl suberimidate (DMS)

DMS dissolved in 0.2 M triethanolamine (pH 8.5) was added to membrane preparations from SERT1 transfected HEK-293 cells (20–50 μg total protein) to a final concentration of 0.1 mM. After incubation for 5–60 min at room temperature, the cross-linking process was terminated by the addition of 1/10 volume of 1 M Tris-HCl, pH 7.5.

2.4. Electrophoresis and immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5%, or on 3.5–10% gradient, gels was performed according to Laemmli [7]. In all experiments, the samples were not boiled prior to SDS-PAGE, since this resulted in a loss of SERT1 immunoreactivity. For immunoblotting, the separated proteins were transferred to nitrocellulose in a horizontal semi-dry electroblotting apparatus, and the blots reacted with the affinity purified SERT1 specific antiserum AS968 followed by a horseradish peroxidase coupled secondary antibody in combination with the ECL detection system (Amersham) as described [8].

3. Results

3.1. Immunoblotting of membrane bound SERT1

Membrane preparations from transfected HEK-293 cells expressing the serotonin transporter, and from control mock-transfected cells, were subjected to SDS-PAGE under reducing (5% (v/v) β-mercaptoethanol or 100 mM dithiothreitol (DTT)) or non-reducing conditions, transferred to nitrocellulose and then reacted with a SERT1 specific antiserum [7]. Under non-reducing conditions, most of the immunoreactivity was detected in two broad bands at 130–180 kDa and 220–270 kDa (Fig. 1). In addition, a minor double band was

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seen at 62/56 kDa; the latter corresponds to the monomeric SERT1 polypeptide. None of these bands was found in membrane preparations from mock-transfected cells. Under reducing conditions, all SERT1 specific immunoreactivity was detected at 130–180 kDa and 62/56 kDa; no cross-reacting material was seen in the range of 220–270 kDa. This suggests that SERT1 can form higher molecular weight oligomers of the monomeric 62 kDa SERT1 polypeptide, which are disulfide-linked.

3.2. Cross-linking of SERT1 expressing cell membranes with (o-phenanthroline)$_2$-Cu^{2+} and diamide

To further investigate the involvement of disulfides in the formation of SERT1 adducts exhibiting reduced mobility upon SDS-PAGE, we treated membranes from SERT1 expressing cells with the specific sulfhydryl oxidizing agents (*o*-phenanthroline)$_2$-Cu^{2+} and diamide. To avoid unspecific disulfide formation, free sulfhydryl groups were blocked with iodoacetamide prior to reduction with 100 mM DTT. Under these conditions, SERT1 immunoreactivity electrophoresed at apparent molecular weights of ~62 kDa and 130–180 kDa (Fig. 2, lane 1). Oxidation of the reduced membranes with both (*o*-phenanthroline)$_2$-Cu^{2+} and diamide resulted in the appearance of 220–270 kDa adduct and increased levels of the 130–180 kDa band (Fig. 2, lanes 2, 3) concomitantly with a decrease in 62 kDa immunoreactivity. Subsequent reduction of the oxidized membrane preparation re-converted the 220–270 kDa adduct completely, and the 130–180 kDa adduct partially, into the 62 kDa SERT1 monomer (Fig. 2, lane 4). This is consistent with disulfide bridges that are poorly accessible to DTT being implicated in 130–180 kDa adduct formation. In contrast, the disulfide bridge(s) generating the 220–270 kDa complex appear(s) to be readily reduced by DTT or β-mercaptoethanol.

3.3. Cross-linking of membrane bound SERT1 with DMS

We also treated SERT1 containing membrane preparations with the homobifunctional cross-linker DMS for various time periods (Fig. 3) and at different cross-linker concentrations (not shown). The resulting SERT1 adducts then were immunodetected after resolution on SDS-PAGE under reducing conditions. Treatment with 0.1 mM DMS for up to 45 min resulted in the appearance of the SERT1 specific adduct of 220–270 kDa; its intensity increased upon prolonged incubation with the cross-linker (Fig. 3). However, in contrast to the results obtained with sulfhydryl oxidizing agents shown in Fig. 2, the amount of high molecular weight adduct was low, and no decrease in the 62 kDa monomer could be detected. At high DMS concentrations, large aggregates were formed which were retained in the stacking gel (data not shown).

These data show that a major fraction of the SERT1 protein in membrane preparations of the transfected cells exists in higher molecular weight oligomers.

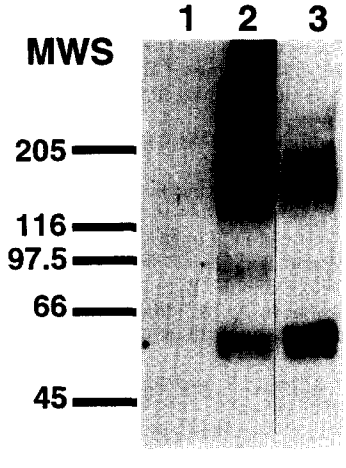

Fig. 1. Immunoblotting of membrane preparations from transfected HEK-293 cells under non-reducing (lanes 1 and 2) and reducing conditions (lane 3). Membranes (~30 μg per lane) prepared from HEK-293 cells transfected with the SERT1 cDNA (lanes 2 and 3) or from mock-transfected cells (lane 1) were subjected to SDS-PAGE without (lanes 1 and 2) or with (lane 3) prior reduction by 5% (v/v) β-mercaptoethanol. Molecular weight standards (MWS) are given in kDa.

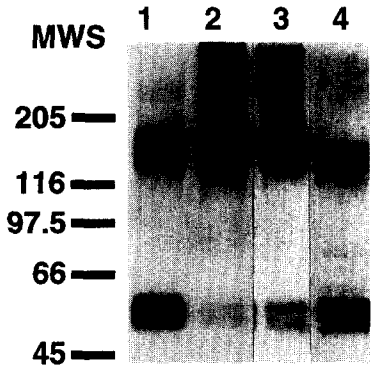

Fig. 2. Sulfhydryl specific cross-linking of SERT1 containing membranes with diamide and (*o*-phenanthroline)$_2$-Cu^{2+}. Free thiols were blocked with 40 mM iodoacetamide before reduction with 100 mM DTT (lane 1); aliquots of these membranes (~20 μg) were then oxidized with either 0.5 mM (*o*-phenanthroline)$_2$-Cu^{2+} (lane 2) or 150 μM diamide (lane 3). An aliquot of the diamide oxidized membranes was thereafter treated with 100 mM DTT (lane 4). SDS-PAGE was performed under non-reducing conditions.

4. Discussion

Chemical cross-linking has been widely used to investigate the subunit composition and the native molecular size of membrane proteins [9–11]. Here, we applied this technique to the recombinant SERT1 expressed in a mammalian cell line. Upon SDS-PAGE, the SERT1 polypeptide and derived adducts were identified by immunoblotting with the polyclonal antiserum AS 968, which has been shown to specifically recognize both the recombinant protein as well as the native transporter in brain sections [8]. Under reducing conditions, AS968 mostly revealed monomeric SERT1 as a doublet of apparent molecular weights of ~62 kDa and, to a lesser extent, ~57 kDa. The same result has also been obtained by Quian et al. [12] for SERT1 expressed in HeLa cells. These authors have shown that the immunoreactive band of higher electrophoretic mobility results from deglycosylation of the 62 kDa protein. Thus, both glycosylated and non-glycosylated forms of SERT1 seem to be present in our membrane preparations; presumably the latter reflect newly synthesized transporter, which still resides in intracellular membrane compartments.

Upon non-reducing SDS-PAGE, additional SERT1 immunoreactivity was observed in two broad bands at 130–180 kDa

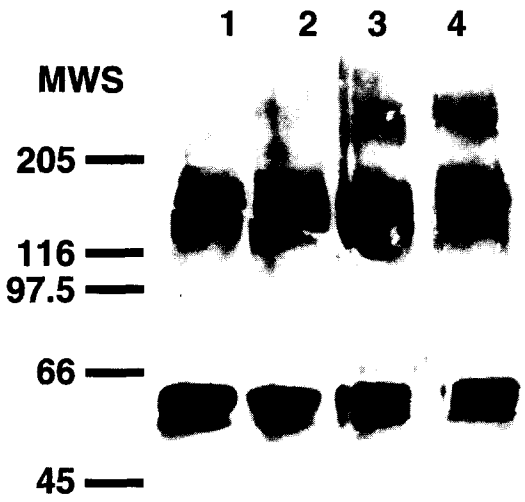

Fig. 3. Compositional analysis of SERT1 adducts produced by cross-linking with DMS. Membranes from SERT1 transfected cells (30 μg) were treated with 0.1 mM DMS for 5 min (lane 2), 25 min (lane 3) and 45 min (lane 4). In lane 1, DMS was omitted. The samples were resolved by SDS-PAGE after reduction by 5% (v/v) β-mercaptoethanol, transferred to nitrocellulose, and SERT1 adducts were immunodetected as described in Section 2.

and 220–270 kDa, respectively, the intensities of these bands varying considerably between different experiments. Due to the heterogeneity of these high molecular weight adducts, an accurate determination of their molecular sizes proved difficult. Thiol oxidation of the membranes with both (*o*-phenanthroline)$_2$-Cu^{2+} and diamide before electrophoretic analysis under non-reducing conditions increased the abundance of these higher molecular weight adducts with a parallel decrease of the 62/57 kDa antigen. Reduction of the oxidized membranes converted the 220–270 kDa band completely, and the 130–180 kDa band partially, into monomeric SERT1. These findings are consistent with the immunoreactive high-molecular weight adducts representing oligomeric complexes of SERT1. Since the 220–270 kDa band was also seen in non-reduced membrane preparations which had not been subjected to thiol oxidation, we propose that this adduct represents a tetrameric disulfide-stabilized form of the SERT1 polypeptide. In contrast, the 130–180 kDa adduct was also observed in the presence of reducing agents, although to a lesser extent than under non-reducing conditions. Interestingly, 60 kDa and 130 kDa SERT1 bands have also been found in membrane preparations from insect cells after transfection with a recombinant baculovirus [13]. In this report, the 130 kDa band was interpreted as a putative dimeric form of the SERT1 protein. Collectively, this finding and the data presented above are consistent with the 130 kDa adduct representing a disulfide linked dimer, whose disulfide bonds are poorly accessible to reducing agents. However, for a definitive compositional analysis of the higher molecular weight adducts, cross-linking experiments on the purified SERT1 protein will be required.

Our interpretation that membrane bound SERT1 forms tetramers is in good agreement with radiation inactivation studies on the dopamine transporter (DAT1), which also indicate a tetrameric structure for this membrane protein [14]. SERT1 and DAT1 belong to the same superfamily of Na^+/Cl^--coupled neurotransmitter transporters and share a high degree of amino acid identity. It therefore is reasonable to assume that both proteins have a common quaternary structure, a conclusion corroborated by the results described above. Interestingly, the hexose transporter GLUT1 also has been shown to exist as a mixture of homodimers and homotetramers, with an intramolecular disulfide bridge promoting GLUT1 tetramerization [15,16]. GLUT1 is another integral membrane protein with 12 putative membrane spanning segments; however, it displays no sequence homology to the monoamine transporters mentioned above. Thus, the similar oligomeric structure of all these transporter proteins might reflect a general structural requirement for effective substrate translocation.

Previous studies have shown that disulfides are critical determinants of serotonin transporter function. Disulfide oxidation has been reported to stimulate serotonin transport in mouse platelets [17], whereas reduction increases the affinity for imipramine, a potent serotonin uptake inhibitor [18]. These observations may indicate that the binding affinity of SERT1 is altered upon oligomerization. This again shows parallels with the hexose transporters, where the functional properties of GLUT1 have been found to depend on its state of oligomerization [19].

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