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Glycine residues G338 and G342 are important determinants for serotonin transporter dimerisation and cell surface expression

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Abstract

Compelling evidence has been provided that Na^+ and Cl^--dependent neurotransmitter transporter proteins form oligomeric complexes. Specific helix–helix interactions in lipid bilayers are thought to promote the assembly of integral membrane proteins to oligomeric structures. These interactions are determined by selective transmembrane helix packing motifs one of which is the Glycophorin A motif (GxxxG). This motif is present in the sixth transmembrane domain of most transporter proteins. In order to investigate, whether this motif is important for proper expression and function of the serotonin transporter (SERT), we have analysed the effect of mutating the respective glycine residues Gly338 and Gly342 to valine upon transient expression of the respective cDNAs in HEK293 cells. As revealed by western blotting, wildtype SERT is found in monomeric and dimeric forms while both mutants are expressed as monomers solely. Confocal microscopy revealed that the wildtype SERT is expressed at the cell surface, whereas both mutant proteins are localised in intracellular compartments. Failure of integration into the cell membrane is responsible for a total loss of [^{3}H]5HT uptake capability by the mutants. These findings show that in the SERT protein the integrity of the GxxxG motif is essential for dimerisation and proper targeting of the transporter complex to the cell surface.

Keywords: Serotonin transporter; Oligomerisation; Transmembrane domain 6; Glycine residues

The action of serotonin (5-hydroxytryptamine, 5-HT) is controlled by specific, high-affinity reuptake of the neurotransmitter out of the extra cellular space into serotonergic neurons. This process is mediated by a highly selective neurotransmitter protein, the serotonin transporter (SERT; Blakely et al., 1991; Schloss and Williams, 1998). This polypeptide is a member of the Na^+–Cl^--coupled neurotransmitter transporter gene family SLC6 which also includes transporter proteins for dopamine (DAT), norepinephrine (NET), GABA (GAT) and glycine (GlyT) (Chen et al., 2004). The members of this neurotransmitter transporter family are integral transmembrane proteins and share a common putative 12 transmembrane domain (TMD) structure.

Like many other integral membrane proteins these transporter proteins have been shown to form oligomeric complexes (Klingenberg, 1981; Veenhoff et al., 2002; Sitte and Freissmuth, 2003). Evidence for oligomerisation of monoamine transporters of the SLC6 family was primarily based on radiation inactivation studies on DAT (Berger et al., 1994; Milner et al., 1994). Using cysteine cross-linking it was later demonstrated that DAT forms dimeric and tetrameric adducts (Hastrup et al., 2001, 2003). Comparably, the quaternary structure of SERT has first been investigated using a cross-linking methodology and revealed that SERT is an oligomeric protein, too (Jess et al., 1996). This finding was later corroborated by analysing heterologously expressed linear SERT–SERT and hybrid SERT–NET concatenates (Chang et al., 1998; Horschitz et al., 2003), co-immunoprecipitation of two forms of SERT tagged with different epitopes (Kilic and Rudnick, 2000) and by fluorescence resonance energy transfer (FRET) analysis (Schmid et al., 2001). The latter study also provided evidence for the oligomerisation of the rat GABA transporter GAT1. In addition, the FRET technology has been applied to visualise the oligomerisation of DAT in living cells (Sorkina et al., 2003). In most studies oligomerisation has been shown to be important for cell surface expression of the respective transporter proteins.

Two general structural motifs which are involved in protein dimerisation have been identified in the members of the SLC6

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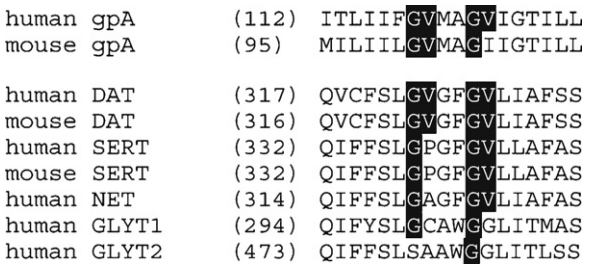

Fig. 1. Sequence alignment of the dimerisation motif of glycophorin A (gpA) and some members of the neurotransmitter transporter gene family SLC6. The number of the first residue of each sequence shown is given in parantheses. The conserved residues of the dimerisation motif are indicated by white letters on a black background.

family (Sitte and Freissmuth, 2003). One is a leucine heptad repeat (LxxxxxxL) which is located in TMD 2 of the SLC6 members, the other one is the Glycophorin A motif (GxxxG) (Brosig and Langosch, 1998), which is found in TMD 6 of the transporter proteins (for sequence alignment of TMD2 see (Sitte and Freissmuth, 2003) and TMD6 see Fig. 1). The leucine heptad repeat is a major determinant in the oligomer formation of DAT, GAT1 and SERT (Torres et al., 2003; Korkhov et al., 2004; Sato et al., 2004) and mutations within this motif disrupt oligomerisation of the rat GAT1 (Scholze et al., 2002). The GxxxG motif which has been shown to be crucial for the homodimerisation of the glycophorin A transmembrane segment (Brosig and Langosch, 1998) is a general motif for transmembrane helix–helix association (Russ and Engelman, 2000) and its integrity has also been shown to be essential for dimerisation and proper expression of the DAT protein (Hastrup et al., 2001).

Here we have analysed the effect of mutating the respective glycine residues Gly338 and Gly342 in SERT to valine. [^{3}H]5HT uptake experiments revealed that mutation of both glycine residues resulted in loss of SERT transport activity. Ligand binding studies revealed that the Gly342Val mutant did not bind the SSRI [^{3}H]citalopram, while the Gly338Val mutant still displayed about 10% [^{3}H]citalopram binding capacity, as compared to the wildtype SERT. In order to visualise the transporter also in living cells wildtype SERT and the glycine mutants have been fused to green fluorescent protein (GFP). As shown by confocal microscopy both mutant proteins are expressed in the cell interior but are not translocated to the cell surface.

Taken together our data show that in the SERT protein the integrity of the glycophorin A motif is essential for proper expression and function of the transporter protein.

1. Materials and methods

1.1. Materials

[^{3}H]-labelled citalopram (82 Ci/mmol) was purchased from Perkin-Elmer, [^{3}H]-labelled 5-HT (6.1 Ci/mmol), was obtained from GE Healthcare. Unlabelled citalopram was a gift from J. Hyttel (H. Lundbeck A/S, Copenhagen-Valby, Denmark). The antibody against the *GFP*-epitope was from Chemicon. All other chemicals were of analytical grade.

1.2. Construction of hSERT–GFP

A *Xho*I/*Hin*dIII fragment of hSERT cDNA (which was a generous gift of Dr. R.D. Blakely, Vanderbilt University, Nashville, TN) was inserted into the vector pEGFP-N1 to produce the plasmid hSERT–GFP. The GFP is in frame to the C-terminus of SERT.

1.3. Generation of G338V and G342V mutants

Mutants were generated by PCR with oligos sense 5′-CTTCTTCTC-TCTT*GTT*CCGGGCTTTGGG and antisense 5′-CCCAAAGCCCGG*AAC*AA-GAGAGAAGAAG for G338V and oligos sense 5′-GGTCCGGGCTTT-*GTG*GTCCTGCTGGC and antisense 5′-GCCAGCAGGAC*CAC*AAAGCCCG-GACC for G342V, respectively (mutant codons are underlined). Amplification was performed with the respective vector primers and hSERT–GFP as a template using *Pfu* polymerase. Conditions for all reactions were 95 °C for 1 min (initial denaturation), 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, with 10 min extension time after 34 cycles. Products were verified by sequencing.

1.4. Heterologous expression

HEK 293 cells were transiently transfected with the cDNA of hSERT–GFP, G338V, and G342V, respectively, by lipofection with the FuGENE HD reagent according to the protocol of the vendor (Roche). After transfection the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml), at 37 °C in 95% humidified air with 5% CO_2.

1.5. [^{3}H]5-HT transport measurement

For uptake measurements, HEK 293 cells expressing either hSERT–GFP, G338V or G342V, were plated into 24-well dishes, and the culture medium was replaced by 0.2 ml of TBB (125 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.5) containing 250 nM [^{3}H]5-HT. After 6 min at room temperature, the solution was rapidly removed, and the cells were washed twice with cold TBB before extraction with 0.5 ml of 10% (w/v) sodium dodecyl sulphate (SDS). Radioactivity was determined by scintillation counting using a Packard 1600TR scintillation counter. Specific [^{3}H]5-HT is defined as the difference between monoamine transporter mediated minus unspecific uptake in the presence of 50 μM citalopram.

1.6. Membrane preparation and radioligand binding

Cell membranes were prepared as described elsewhere and stored in aliquots at −80 °C (Schloss and Betz, 1995). Briefly, cells were washed twice with PBS, scraped from the cell culture dish and homogenised with 10 strokes in PBS in a teflon/glass homogeniser. Homogenisation was followed by centrifugation at 1000 × *g* for 3 min at 4 °C. Next, the supernatant was centrifuged at 17,500 × *g* for 20 min at 4 °C. The pellet was resuspended in PBS containing 5% glycerol and protein concentration was determined according to Markwell et al. (1978). Binding of [^{3}H]citalopram was performed as described previously (Schloss and Betz, 1995). Saturation binding was performed at room temperature using 30 μg membrane protein in a total volume of 200 μl assay buffer containing [^{3}H]citalopram at increasing concentrations as shown in Results.

1.7. Data analysis

All uptake data represent the means of quadruplicate determinations; binding data the means of triplicate determinations; each experiment was repeated at least three times. SEMs were routinely <5% and are indicated where larger than the symbols used. Data in Fig. 3 swere analysed by a non-linear regression analysis program (Graph Pad Prism 3.02), which fitted sigmoidal binding curves to the following equations: $B = B_{max}/(1 + [K_D/L]$, and $B/B_{max} = IC_{50}/(I + IC_{50})$. B represents binding, B_{max} maximal binding, L ligand concentration, I inhibitor concentration, IC_{50} inhibitor concentration for half maximal transport inhibition.