



Expression of neurotransmitter transporters for structural and biochemical studies

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ABSTRACT

Neurotransmitter transporters play essential roles in the process of neurotransmission. Vesicular neurotransmitter transporters mediate storage inside secretory vesicles in a process that involves the exchange of luminal H⁺ for cytoplasmic transmitter. Retrieval of the neurotransmitter from the synaptic cleft catalyzed by sodium-coupled transporters is critical for the termination of the synaptic actions of the released neurotransmitter. Our current understanding of the mechanism of these transporters is based on functional and biochemical characterization but is lacking high-resolution structural information. Very few structures of membrane transport systems from mammalian origin have been solved to atomic resolution, mainly because of the difficulty in obtaining large amounts of purified protein. Development of high yield heterologous expression systems suitable for mammalian neurotransmitter transporters is essential to enable the production of purified protein for structural studies. Such a system makes possible also the production of mutants that can be used in biochemical and biophysical studies.

We describe here a screen for the expression of the vesicular monoamine transporter 2 (VMAT2) in cell-free and baculovirus expression systems and discuss the expression of VMAT2 in other systems as well (bacterial, yeast and mammalian cell lines). After screening and optimization, we achieved high yield (2–2.5 mg/l) expression of functional VMAT2 in insect cells. The system was also used for the expression of three additional plasma membrane neurotransmitter transporters. All were functional and expressed to high levels. Our results demonstrate the advantages of the baculovirus expression system for the expression of mammalian neurotransmitter transporters in a functional state.

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Introduction

Synaptic transmission involves the concerted function of two classes of neurotransmitter transporters: the plasma membrane sodium-coupled neurotransmitter transporters and the proton-gradient dependent vesicular transporters (SLC18 family). Crystal structures of prokaryotic homologues of the neurotransmitters had a major impact on the field. Notably in the case of the plasma membrane transporters they have served as structural paradigms for interpretation of the wealth of functional data available on the eukaryotic counterparts from biochemical and electrophysiological experiments [1–3]. However, the work with the prokaryotic homologues, while informative at the mechanistic level can provide only an incomplete view of the mammalian protein [4]. Despite their importance and clinical relevance, no high-resolution structures of mammalian neurotransmitter transporter are yet

available. A basic reason for the lack of high-resolution atomic data for mammalian integral membrane proteins in general is the difficulty of establishing high yield heterologous expression systems and obtaining large amounts of functional purified protein [5]. Some of the structures of mammalian membrane proteins have been solved to high-resolution with proteins purified from native source [6,7] while some others from heterologous expression systems [8–12], but these are still hardly a handful of examples.

The vesicular storage of monoamines, namely serotonin, dopamine, norepinephrine, epinephrine, and histamine, is mediated by the vesicular monoamine transporter family (VMATs²) that operate by exploiting the proton-gradient formed by the V-type ATPase [13]. Two mammalian genes encode for two vesicular monoamine transporters that share 62% identity. Both proteins exchange two protons per substrate molecule, but they display different pharmacological profile and tissue distribution [14–16]. Immunohistochemistry analysis showed that VMAT2 is the more common isoform in most tissues, and it is the only one expressed in neuronal

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² Abbreviations used: VMATs, vesicular monoamine transporter family; MDMA, 3,4-methylenedioxymethamphetamine; HA, hemagglutinin; v-cath, v-cathepsin; FCS, fetal calf serum.

cells, while VMAT1 is found only in some types of endocrine cells. Both genes are expressed in chromaffin cells of the human adrenal medulla [14].

VMAT2 has been shown to be an essential protein and homozygous VMAT2 knockout mice die shortly after birth [17,18]. VMAT2 displays higher affinities towards all the native substrates and also to the inhibitors reserpine and tetrabenazine. In addition to the native substrates and the above-mentioned inhibitors, the VMATs interact with many clinically relevant drugs, including the psychostimulants 3,4-methylenedioxymethamphetamine (MDMA) and amphetamines and the parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP⁺). Expression of VMAT confers to mammalian and yeast cells resistance to MPP⁺, a process accomplished by compartmentalization of the drug in intracellular acidic compartments, thus removing it from its presumed target [19,20].

In the present study, the vesicular monoamine transporter VMAT2 served as a case study for the heterologous over-expression of neurotransmitter transporters in quality and amounts high enough for structural analysis. We present a systematic study of various expression platforms including two cell-free systems and three baculovirus vectors used for the expression of VMAT2 in insect cells. After optimization for VMAT2 of the baculovirus system, the same conditions were shown appropriate for expression of three plasma membrane sodium-coupled neurotransmitter transporters.

Materials and methods

Materials

RTS 100 *Escherichia coli* HY and RTS 100 WG HY kits, ProteoExpert software license, pIVEX vectors, mouse anti His6 monoclonal antibody were purchased from Roche Diagnostics GmbH (Mannheim, Germany). *flashBAC*TM baculovirus vectors were purchased from Oxford Expression Technologies Ltd (Oxford, UK). ESF 921 protein-free Insect Cell Culture Medium from Expression Systems LLC (Woodland, CA), Sf9 insect cells from Invitrogen (Carlsbad, CA), pVL1393 vector from BD Biosciences (San Jose, CA).

EscortTM transfection reagent, protease inhibitors, DNaseI, Concanavalin A Sepharose 4B and Methyl- α -D-Manno-Pyranoside were from Sigma (St. Louis, MO). Texas Red-conjugated anti-mouse secondary IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Detergents used, *n*-dodecyl β -maltoside (DDM) and *n*-octyl β -D-glucopyranoside (OG) were purchased from Glycon Biochemicals GmbH (Luckenwalde, Germany). Antibiotic solution of Penicillin–Streptomycin–Amphotericin B was purchased from Biological Industries (Beit Ha'emek, Israel).

Methods

Expression of neurotransmitter transporters using cell-free expression systems

The cDNAs coding for rVMAT2 (a rat VMAT2 gene with hemagglutinin (HA) tag in the first loop between positions 96 and 105, a generous gift from RH Edwards, UCSF), GAT1 and EAAC1 were cloned into the appropriate pIVEX vectors using the NdeI and XhoI restriction sites. The vectors used were pIVEX 1.3, 1.4, 2.3d and 2.4d. Protein was synthesized using the rapid-translation system RTS 100 *E. coli* HY and rapid-translation system RTS 100 WG HY kits, according to the manufacturer instruction manual. Radiolabeling of VMAT2 was achieved by addition of 1–3 μ Ci of [³⁵S] methionine (>1000 Ci/mmol, Amersham Life Sciences, Arlington Heights, IL).

Expression of VMAT2 in insect cells

cDNAs coding for rVMAT2 with or without a His tag at the C-terminus were cloned into a pVL1393 transfer vector (BD Pharmi-

gen) using the EcoRI and NotI restriction sites. Top10 *E. coli* cells (Invitrogen) were used throughout cloning and mutagenesis processes of VMAT2. The same procedure was applied also for the VMAT2 mutants (termed GlyD and GlyQ) and for the cDNAs coding for rat GAT1, rat GLT1 and rabbit EAAC1. GlyD is a VMAT2 mutant in which 5 putative glycosylation sites have been canceled by mutating Asn residues at positions: 56, 80, 81, 89 and 111 to Asp. In the mutant GlyQ Asn residues in the same positions were changed to Gln. Mutations in VMAT2 were introduced using the QuikChange[®] II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Sequences of all constructs were verified by DNA sequencing.

Recombinant virus was produced by co-transfection of the transfer vector DNA together with the *flashBAC*TM DNA into Sf9 insect cells, as instructed by manufacturer. Recombinant baculovirus stocks were subjected to three rounds of amplification in Sf9 cells to produce high-titer viral stock. For assessing virus amounts suitable for expression, we performed calibration infections in suspension cultures using 40–50 ml of $\sim 2.5 \times 10^6$ Sf9 cells/ml per assay.

VMAT2 expression in Sf9 cells and purification

Sf9 cells grown in suspension were infected with rVMAT2 recombinant baculovirus. Sf9 cells were grown at 27 °C in a serum-free protein-free medium supplemented with antibiotics in sterile shaker flasks. Cells expressing VMAT2 were harvested 72–76 h post-infection, re-suspended in lysis buffer (0.3 M sucrose, 15 mM HEPES pH 7.4, 5 mM MgCl₂, 15 μ g/mL DNase I (Sigma) and protease inhibitor cocktail (Sigma)) and disrupted using a Sonics Vibra Cell probe sonicator. The membrane fraction was collected by ultracentrifugation at 213,500g for 1 h at 4 °C and re-suspended in buffer containing 150 mM NaCl and 15 mM Tris pH 7.5. Membranes were solubilized with *n*-dodecyl β -maltoside (DDM) at a final concentration of 2%. After 1.5 h incubation at 4 °C, unsolubilized material was removed by centrifugation, imidazole was added to 40 mM, and the sample was loaded onto HiTrapTM chelating HP column (GE Healthcare, Fairfield, CT) mounted on ÄktaTM Explorer (Amersham Biosciences) and washed with 0.08% DDM, 150 mM NaCl, 15 mM Tris–HCl, and 40 mM imidazole, pH 7.5, till A₂₈₀ of the flow-through decreased below 0.05. VMAT2 was eluted with a gradient of up to 400 mM imidazole. Major peak fractions were pooled, MnCl₂ and CaCl₂ were added to 1 mM final concentration and the protein solution was bound to Concanavalin A Sepharose 4B (Sigma) for 1 h at 4 °C. The column was washed with a buffer containing 0.08% DDM, 150 mM NaCl, 15 mM Tris–HCl, 1 mM MnCl₂ and 1 mM CaCl₂ and protein was eluted in the same buffer supplemented with 0.5 M Methyl- α -D-Manno-Pyranoside (Sigma).

Identity of the purified protein was verified by mass spectrometry performed at the Smoler Proteomics Center, Technion, Israel. The sample was cleaved with trypsin and chymotrypsin in parallel and analyzed by LC-MS/MS on the Orbitrap mass spectrometer (Thermo). The fragments were identified by Sequest 3.31 software against the IPI mouse database and against a decoy database. The results were filtered according to the Xcore value.

Western blot analysis

Samples were separated by SDS–PAGE on 12.5% Laemmli gels [21] and Western blot analysis was performed essentially as described in [22].

Immunocytochemistry

Cells grown on coverslips at 50% confluency were infected with recombinant baculovirus. Two days post-infection, cells were fixed with methanol at –20 °C. Cover slips were incubated with mouse anti His6 monoclonal antibody at a 1:300 dilution followed by Texas Red-conjugated anti-mouse secondary IgG at a 1:500 dilu-

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