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Characterization of mammalian equilibrative nucleoside transporters (ENTs) by mass spectrometry

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ABSTRACT

Equilibrative nucleoside transporters (ENTs) are integral membrane proteins that facilitate the movement of nucleosides and hydrophilic nucleoside analog (NA) drugs across cell membranes. ENTs are also targets for cardioprotectant drugs, which block re-uptake of the purine nucleoside adenosine, thereby enhancing purinergic receptor signaling pathways. ENTs are therefore important contributors to drug bioavailability and efficacy. Despite this important clinical role, very little is known about the structure and regulation of ENTs. Biochemical and structural studies on ENT proteins have been limited by their low endogenous expression levels, hydrophobicity and labile nature. To address these issues, we developed an approach whereby tagged mammalian ENT1 protein was over-expressed in mammalian cell lines, confirmed to be functional and isolated by affinity purification to sufficient levels to be analyzed using MALDI-TOF and tandem MS mass spectrometry. This proteomic approach will allow for a more detailed analysis of the structure, function and regulation of ENTs in the future.

Introduction

Nucleoside transporters (NTs) are important members of a complex network of proteins involved in regulating intra/extra-cellular nucleoside and nucleobase pools. Nucleotides, nucleosides and nucleobases are physiologically important for various cellular functions including nucleic acid synthesis, energy production and intra/extra-cellular signaling [1–4]. Clinically, NTs are important as targets for NT inhibitors, which are typically multi-ringed aromatic compounds that possess cardio/neuro-protective properties [5]. In addition, NTs act as molecular portals for nucleoside analog (NA) drugs that possess anti-neoplastic, anti-viral and anti-parasitic properties [1–4]. In recent years, it has been proposed that NTs can be used as biomarkers to predict the response of an individual patient to the efficacy of certain NA drug treatments [5–6]. However, since NA drug efficacy is limited by bioavailability and by effective targeting of the drug specifically to diseased cells, there is a pressing need to increase our understanding of the role of NTs in NA-drug based chemotherapies [7]. Therefore, we need a better understanding of structure, function and regulation of NTs, which, in combination with rational drug design, would allow for the development of targeted and individualized NA drug therapies [8]. To date, our understanding of NTs has been limited by the challenges associated with biochemical analyses of membrane proteins using standard methodologies [9]. While there has been some progress in developing innovative approaches to the study of some membrane proteins [10–12], ENT protein structure and mechanism of action continue to be enigmatic since some of the fundamental techniques and tools necessary for an in-depth analysis of this type have not been developed or optimized.

We hypothesized that a proteomic approach based on use of mass spectrometry would allow us to address some questions relating to ENT1 structure and regulation. Since there are no reports in the literature using mass spectrometry to study ENT1, we have developed protocols based on expression of recombinant tagged ENT1 in mammalian systems, followed by affinity based chromatography and analysis by mass spectrometry. We demonstrate in this report that this approach is feasible and can be expanded for studies on the structure, regulation and biochemistry of ENTs.[1]

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[1] *Abbreviations used:* ENTs, equilibrative nucleoside transporters; NA, nucleoside analog; NTs, nucleoside transporters; PLB, protein loading buffer; CB, Coomassie blue; HA, hemagglutinin; TAP, Tandem Affinity Purification, CBP, Calmodulin Binding Peptide; TMDs, transmembrane domains.

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Materials and methods

FLAG-tagged hENT1 protein over-expression

Recombinant hENT1 and mENT1 protein were cloned using a construct that encoded hENT1 or mENT1 cDNA (GI1845344 or AF131212 GenBank) into the plasmid p3×FLAG-CMV-7.1 (Sigma; St. Louise, MO) at the NotI/KpnI restriction sites. The resulting construct, pN-3×FLAG-hENT1/mENT1, encodes an amino-terminal 3×-FLAG-tagged hENT1/mENT1 protein under the control of a CMV promoter. Protein over-expression was conducted in the monkey kidney cell line COS-7, using transient transfections with an optimized $CaCl_2$ protocol [13]. Briefly, cells were grown to 80–90% confluence in DMEM medium (Gibco; Burlington, ON) supplemented with 10% (v/v) FBS (Wisent Inc.; ST-BRUNO, QC) on 100 mm dishes. Transfections were performed with 30 μg of pN-3×FLAG-hENT1 in 10 ml of DMEM medium and allowed to incubate at 37 °C for 18–22 h. After incubation, cells were gently rinsed twice with a Phosphate Buffered Saline solution (pH 7.4; 1× PBS) and refreshed with medium for 20–24 h at 37 °C. The transfected cells were then collected by scraping in 1× PBS followed by low-speed centrifugation (3000*g*, 4 min, 4 °C). The pellets were rinsed twice with 1xPBS and then stored as wet pellets at −80 °C. Transfection efficiencies were calculated by determining the percentage of green fluorescing cells compared to the background in plates transiently transfected with construct pEGFP-N1. This construct encodes an amino-terminal GFP-tagged hENT1 protein under the same CMV promoter as pN-3×FLAG-hENT1.

NBTI-binding assays

Binding assays (1 mL final volume) were performed at room temperature in 10 mM Tris–HCl, 100 mM KCl, 0.1 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$ (pH 7.4) with transfected cells (COS-7). N-3×FLAG-mENT1 assays were conducted in the murine cardiomyocyte cell line HL-1 [14]. Samples were incubated with increasing concentrations of [^{3}H]-NBTI (0.1–7.45 nM) in either the absence or presence of 10 μM NBTI (16.5 Ci/mmol; Moravek, Brea, CA). After incubation for 50 min, the reaction was stopped by addition of 5 mL of ice-cold binding buffer and rapidly filtered through Whatman GF/B filters (Whatman; Florham Park NJ), followed by one wash with 5 mL of the same buffer. Filters were analyzed for radioactive content by standard liquid scintillation counting.

Adenosine transport assays

Transport assays were conducted on Lipofectamine transfected COS-7 and HL-1 cells (following manufacturer protocols; Invitrogen, Burlington, ON) maintained in culture for 14 h post-transfection following established procedures [14]. Lipofectamine was utilized for transfections in this section since the $CaCl_2$ transfection method interfered with the transport assays. Cells were incubated for 15 s with sodium free Transport Buffer containing adenosine (50 μM) and radiolabeled [^{3}H]-adenosine (specific activity 54.5 Ci/mmol; Moravek). Nucleoside transport was terminated by aspiration and rapid rinses (2×) of ice-cold Transport Buffer containing 100 nM NBTI and 10 μM dipyridamole. Finally, cells were lysed (4 °C, overnight, shaking) with 1% (v/v) Triton X-100. Protein concentrations of cell lysates were determined with a modified protein Lowry Assay (BioRad; Mississauga, ON) and [^{3}H]-adenosine uptake was measured by standard liquid scintillation counting. Transport is expressed as pmol substrate per mg protein.

Na_2CO_3 membrane enrichment

This procedure was modified from an original protocol for the isolation of membranes from the endoplasmic reticulum [15]. Frozen wet pellets collected from 20 to 25 × 100 mm plates containing N-3×FLAG-hENT1 transfected cells were thawed on ice and suspended with 12 mL of ice-cold 1 mM $NaHCO_3$. A polytron homogenizer (set at level 8 with 2 min pauses to allow cooling on ice) was used to mechanically rupture cells. The number of cycles performed was determined by the percentage of cells lysed monitored with a compound microscope. Upon 90% cell lysis, 35 mL of ice-cold 130 mM Na_2CO_3 was added to the cell mixture and incubated with rotation for 15 min at 4 °C. Total membranes were pellet by ultracentrifugation (90,000*g*, 1.5 h, 4 °C). The resulting translucent gelatinous pellet was then rinsed twice with ice-cold 130 mM Na_2CO_3 and solubilized by overnight mixing at 4 °C with 4 mL of ice-cold Lysis Buffer pH 7.4 [50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1.2% (v/v) FosCholine-12 (Anatrace; Maumee, OH) and Protease Inhibitors (Roche)]. Insoluble debris was removed with centrifugation (15,000*g*, 15 min, 4 °C) and 0.2 μm filtration. The resulting membrane-enriched solubilized lysate with concentrations ranging 2.0–3.0 mg/mL (determined with RC/DC Lowry assays; BioRad) were analyzed with SDS–PAGE and anti-3×FLAG immuno-detection (described below).

FLAG-tagged hENT1 immuno-affinity purification

Membrane-enriched solubilized lysates were loaded on pre-equilibrated anti-FLAG M2 agarose resin (Sigma), at a ratio of 5 μL of resin/plate and incubated with rotation at 4 °C overnight. Following this incubation, the resin–protein complex was washed with various rounds of ice-cold Tris–Buffered Saline solution [TBS: 150 mM Tris–HCl, 150 mM NaCl, pH 7.4 cold] supplemented with 0.2% (v/v) FosCholine-12 and gentle centrifugation (8000*g*, 1 min, 4 °C). The extraction was completed by eluting the N-3×FLAG-hENT1 protein using a modified 1× Protein loading buffer (PLB: 62.5 mM Tris–HCl, pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol). This buffer lacks reducing agents and dyes to minimize the displacement of IgG from the resin and to avoid interference by the buffer in RC/DC Lowry assays. Eluted fractions with concentrations of approx. 0.6 mg/mL were stored at −80 °C for use within two weeks or were analyzed by SDS–PAGE and anti-3×FLAG immuno-blots immediately as described below.

SDS–PAGE and Immuno-detection

Proteins samples were separated (3.5 h) using a 16 × 14 cm standard Laemmli 7.5% (v/v) SDS–PAGE apparatus and later visualized through Coomassie blue (CB) staining and/or silver staining. Lower detection limits of these stains are 50–100 ng for CB and 1–10 ng for silver stain [16]. Immunoblots were prepared after SDS–PAGE by transferring protein to nitrocellulose membrane using a semi-dry blotter (BioRad). Blots were blocked at 25 °C for 30 min with a TBS solution supplemented with 0.2% (v/v) Tween-20 (TTBS) and 5% (w/v) homogenized milk. Next, the blot was incubated in a suspension of TTBS (supplemented with 1% (w/v) homogenized milk) along with HRP-labeled mouse monoclonal M2 anti-3×FLAG primary antibodies (1:60,000 dilution; Sigma) for 1.5 h at 25 °C. Various washes were performed (TTBS 4 × 10 min, TBS 1 × 10 min) and the signals were developed with the Lumi-Glo chemiluminesence substrate kit (KPL Inc.; Gaithersburg, Maryland) at short intervals (<5 min) to avoid signal over-exposure.