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Expression and Purification of Human and *Saccharomyces cerevisiae* Equilibrative Nucleoside Transporters †

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Abstract

Nucleosides play an essential role in the physiology of eukaryotes by acting as metabolic precursors in *de novo* nucleic acid synthesis and energy metabolism. Nucleosides also act as ligands for purinergic receptors. Equilibrative nucleoside transporters (ENTs) are polytopic integral membrane proteins that aid in regulating plasmalemmal flux of purine and pyrimidine nucleosides and nucleobases. ENTs exhibit broad substrate selectivity across different isoforms and utilize diverse mechanisms to drive substrate flux across membranes. However, the molecular mechanisms and chemical determinants of ENT-mediated substrate recognition, binding, inhibition, and transport are poorly understood. To determine how ENT-mediated transport occurs at the molecular level, greater chemical insight and assays employing purified protein are essential. This article focuses on the expression and purification of human ENT1, human ENT2, and *Saccharomyces cerevisiae* ScENT1 using novel expression and purification strategies to isolate recombinant ENTs. ScENT1, hENT1, and hENT2 were expressed in W303 *Saccharomyces cerevisiae* cells and detergent solubilized from the membrane. After detergent extraction, these ENTs were further purified using immobilized metal affinity chromatography and size exclusion

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chromatography. This effort resulted in obtaining quantities of purified protein sufficient for future biophysical analysis.

Keywords

Nucleoside nucleotide transport; Membrane transport; membrane transporter reconstitution; Transporters; Membrane proteins

Equilibrative nucleoside transporters (ENTs) are polytopic integral membrane proteins (IMPs) found only in eukaryotic organisms that regulate the flux of purine and pyrimidine nucleosides and nucleobases, but not nucleotides across cellular membranes (1). In addition to controlling the flux of endogenous ligands, ENTs are clinically relevant drug transporters that modulate the effectiveness for a variety of FDA/EMA approved medications (e.g., antineoplastic, antiarrhythmic, antihypertensive, and antiviral medications), and ENTs are known biomarkers for therapeutic efficacy in the treatment of certain cancers (2,3). ENTs have also been associated with regulating seizure activity by regulating adenosine transport (4), and in seizures related to alcohol withdrawal syndrome (5,6). Another important functional consideration was ENT-mediated adenosine transport. Acting through distinctive GPCRs, adenosine signaling facilitates an array of physiological responses such as vasodilation, coronary blood flow, myocardial oxygen supply-demand balance, inflammation, neurotransmission, hypoxia, trauma, and ischemia (7–14). Inhibitors of ENTs also offer a protective advantage in ischemia, trauma, hypoxia, and certain types of seizure disorders by impeding adenosine uptake (4,7,8,11–13,15).

There are four human ENT isoforms (hENT 1-4, SLC29A1-4), each having 11 predicted transmembrane domains (TMDs) and large hydrophilic loops at either the N-terminus (hENT3-4) or in the region between TMD5 and TMD7 (hENT1-4) (16) – an anticipated topology that was comparable to other representatives of the Major Facilitator Superfamily (MFS) (17). ENTs are ubiquitously expressed across most tissue types. ENT1 and ENT2 are primarily located in the plasma membrane. ENT3 contains an N-terminal dileucine motif (DE)XXXL(LI) (18) (characteristic motif for endosomal/lysosomal targeting) in the hydrophilic region of the sequence that precedes the first transmembrane domain (TMD) leading to an enrichment of ENT3 in intracellular membranes of the endosome/lysosome and mitochondria.

Functionally, ENTs are bidirectional, facilitative, transporters that employ concentration gradients of transportable permeants to regulate efflux and influx across cellular membranes. Indeed, passive transport was a hallmark of the ENT family but active proton-linked transport has been identified in protozoa (19), plants (20), and the transport activity of human ENT3 and ENT4 are stimulated at acidic pH (21,22). A definitive transport mechanism has not been explicated for the ENT family, yet the working theory was that they function through an alternating access model consistent with other members of the MFS family (23,24). The presence of active co-transport suggests the molecular transport mechanism can vary for members within the ENT family. However, functional characterization of ENTs at the molecular level has been largely hampered by struggles

associated with large-scale heterologous overexpression and production of purified ENTs as a foundation for driving detailed biochemical analyses.

Initial characterization of ENTs occurred in erythrocytes (25,26), followed by mammalian tumor cell lines (27,28), then *Xenopus laevis* oocytes (1), and this ultimately led to attempts of characterizing purified transporters (29–33) – but ENTs were impervious to characterization in a purified state. Characterization of purified ENTs has faced several hurdles: 1) finding detergents that adequately solubilize and stabilize the proteins (32), 2) determining the lipid composition of artificial membranes (30,33), and 3) obtaining functionally active protein (29–32). The instability of detergent solubilized protein has been a major barrier associated with the molecular characterization of purified ENTs. Subsequently, there are currently no molecular structures for any member of the ENT family.

In an effort to thwart the difficulties associated with human ENTs, recent purification efforts have focused on orthologs from lower order species. ScENT1 from *Saccharomyces cerevisiae* (34) (previously referred to as Function Unknown Now 26) is the only known ENT in yeast. ScENT1 is ~19% identical to hENT1-3 and has a predicted topology and cellular localization (vacuolar membrane) (35,36) similar to hENT3. Unlike hENT3, ScENT1-mediated transport does not appear to be impacted by membrane potential (34). Given ScENT1's broad transport profile (34) and cellular localization, it is presumably involved in nucleoside and nucleobase recycling between the vacuole and cytoplasm in an effort to establish and maintain metabolic balance. ENTs have been identified as the sole mediators of nucleoside transport in plants, however, most plant ENTs function as substrate-proton symporters (20). *Arabidopsis thaliana* has had 5 of its 8 ENT isoforms functionally characterized. AtENT7 is ~19% identical to hENT1-2 and ScENT1, and localizes to the plasma membrane (37). Thus far, AtENT7 is the only isoform not stimulated by external pH (38). Therefore, it may be functioning as an archetypical ENT.

Recombinant ScENT1 utilized homologous overexpression and was detergent extracted, purified using membrane isolation, Immobilized Metal Affinity Chromatography (IMAC), and Size Exclusion Chromatography (SEC), and reconstituted into liposomes with non-native lipids for functional characterization (34). AtENT7 was heterologously expressed in *Pichia pastoris* followed by detergent extraction, purification using IMAC and fluorescence SEC, and functionally assessed using microscale thermophoresis (39). ScENT1 and AtENT7 are the only ENT family members to undergo detergent extraction from the lipid bilayer and retain functional characteristics in purified form - a major advancement in the path to structure determination. In the current work, we present protocols for the overexpression and purification of functional ScENT1, human ENT1, and human ENT2 proteins and compare our approaches to those previously published.

EXPERIMENTAL PROCEDURES

The composition of all buffers can be found in Table 1. The pH of Tris-HCl was determined at room temperature and proteins were kept on ice or at 4 °C unless indicated otherwise.

Molecular Cloning of ScENT1, hENT1, and hENT2

Native ScENT1 open reading frame was PCR amplified from S288C *Saccharomyces cerevisiae* genomic DNA (Novagen #69240-3) using ScENT1-FOR (GGTAGCGGCGGAGGAGGCAGCGGACTAGTCCCAAGAGGTAGTAGTACTAGTGCG GACACT) and ScENT1-REV (AATGTAAGCGTGACATAACTAATTACATGACTCGAGTCACCCCCTGATAATAAAGT CAAT) primers. The resulting PCR product was cloned into the modified yeast 2 μ plasmid "83 χ " (40,41), by homologous recombination in yeast, resulting in an N-terminal 10-histidine-thrombin-GSS-ScENT1 protein expression construct. Native hENT1 and hENT2 were PCR amplified from a human cDNA library (Invitrogen) using LIC primers FOR (CAAGGACCGAGCAGCCCCTCAACAACCAGTCACCAG) and REV (ACCACGGGGAACCAACCCTCCAGGACAAGGCCCGTTAACACACT), where the underlined nucleotides are target gene sequence without a start codon. The resulting PCR products were cloned into the modified yeast 2 μ plasmid termed "83 β 2" (Membrane Protein Expression Center, UCSF, San Francisco, CA), which contains an N-terminal 10 $\times$ histidine-MBP (maltose binding protein) tag followed by a 3C protease cleavage site. All plasmid maintenance and propagation was conducted in XL2Blue Ultracompetent *Escherichia coli* cells (Stratagene #200150).

Expression of ScENT1, hENT1, and hENT2 in *Saccharomyces cerevisiae*

expression efforts utilized W303- pep4 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 hwas3-11,15 pep4 MATa*) *S. cerevisiae* cells transformed with sheared salmon sperm DNA (Invitrogen #15632-011) using a lithium acetate transformation protocol (42). Positive yeast transformants were selected in 1 $\times$ synthetic complete histidine dropout media ("SC-HIS") to select for episomal HIS3 expression. 1 $\times$ SC-HIS growth media contains 1 $\times$ CSM-HIS (Sunrise Science #1023), 0.67% (w/v) yeast nitrogen base without amino acids (RPI #Y20040), and a final carbohydrate concentration of 2% (w/v) [ScENT1, 1% (w/v) D-glucose (Sigma-Aldrich #G8270), and 1% (w/v) D-raffinose (Carbosynth #OR06197); hENT1 and hENT2 2% (w/v) D-glucose]. ScENT1 cultures were grown in 10 L working volumes using a 12 L fermenter at 30 °C with 500 μ L of 50% antifoam 204 (Sigma-Aldrich #A6426). Agitation was set from 200 to 350 rpm based on a dissolved oxygen range of 90% to 20% with an airflow rate of 2.5 L/min. Each round of protein expression contained 7.125 L of 1 $\times$ SC-His medium and 375 ml of overnight culture. Following a 24 hr growth period, protein expression was induced by adding 2.5 L of yeast extract-peptone-galactose (YPG) containing 8% (w/v) yeast extract, 16% (w/v) peptone, and 8% (w/v) galactose. After induction, cells were grown for 16 hr at 30 °C using the same dissolved oxygen based agitation scheme described above. Airflow was increased to 5.0 L/min post-induction. hENT1 and hENT2 cultures were grown in 24 $\times$ 1L baffled shaker flasks with each containing 375 ml of 1 $\times$ SC-HIS and 10 ml of starter culture. The cultures were then grown for 24 hr (30 °C and 220 rpm) until the optical density reached ~18. Protein expression was induced by adding 125 ml of 4 $\times$ -YPG to each flask giving a final galactose concentration of 2%. Following induction, cells were grown for an additional 16 hr at 30 °C and 220 rpm. All cultures were harvested by centrifugation in 1 L volumes at 3600 X g for 30 min at 4 °C. ScENT1 cells were resuspended in 60 ml of 50 mM Tris-HCl pH 7.4, 10% (v/v) glycerol, 150 mM NaCl, 10 mM EDTA, 2 mM PMSF (phenylmethylsulfonyl fluoride), 1 $\times$ DHALT

protease inhibitor cocktail, and 5 mM β -mercaptoethanol (β -Me) ("Buffer 1a") per 80 gram of wet cell pellet. Alcohols, in particular, ethanol, have been shown to modulate the transport properties of ScENT1 (Boswell-Casteel, unpublished data) without altering protein stability. Thus, alternative purification buffers have been used that account for the removal of residual alcohols and are denoted as the Buffer X^{"b"} variation. Buffer 1b requires the removal of PMSF and DHALT protease inhibitors (suspended in 2-propanol) as well as β -mercaptoethanol, however, glycerol was retained. As an alternative, one may utilize water soluble protease inhibitors such as AEBSF (4-benzenesulfonyl fluoride). The typical yield for a 10 L culture of ScENT1 was approximately 300 g of cell paste. hENT1 and hENT2 cells were resuspended in 50 mM Tris-HCl pH 7.5, 20% (v/v) glycerol, and 1 mM PMSF ("Buffer 2"), and yield approximately 30 g of cell paste per liter of culture.

SCENT1, hENT1, and hENT2 Membrane Preparation

Suspended cells were lysed by 3 passes at ~28,000 psi in an Avestin C-3 Emulsiflex and centrifuged at $7,500 \times g$ for 1 hr at 4 °C. Supernatant was retained and total cell membranes were collected by centrifugation at $101,000 \times g$ for 1 hr at 4 °C. Pelleted cell membrane may then be collected and stored at -80 °C for future use or used immediately.

ScENT1 Solubilization and Purification

ScENT1 expression was not detected in the low-speed spin pellet by SDS-PAGE or western blotting. Pelleted membranes were resuspended in 50 mM Tris-HCl pH 7.4, 400 mM NaCl, 5% (v/v) glycerol, 2 mM PMSF, 1 $\times$ DHALT protease inhibitor cocktail and 5 mM β -mercaptoethanol ("Buffer 3a") at a ratio of 16 ml buffer per gram of cell membrane. Buffer 3b requires the removal of PMSF and DHALT protease inhibitors as well as β -mercaptoethanol with glycerol retained. Following resuspension, 150 mg of n-dodecyl- β -D-maltoside (DDM, RPI #D12000) was added per 2 grams of membrane pellet (~ 9 mM DDM). 2 mM lauryl maltose neopentyl glycol (LMNPG) may also be used for solubilization and purification. Membranes were solubilized for 1 hr at 4 °C with vigorous stirring (using a small stir bar), then the insolubilized material was removed by centrifugation at $101,000 \times g$ for 1 hr. The soluble fractions were nutated with 10 mM imidazole and 6 ml HisPur cobalt resin (Pierce #89965) slurry (50% v/v, *pre-equilibrated with Buffer 2a or 2b*) for 1 hr at 4 °C. The histidine-tagged protein was then purified via sequential washes with 10-bed volumes of Buffer 3 a or b containing 2 mM DDM and either 10 (wash 1, Buffer 3a/b1), 20 (wash 2, Buffer 3a/b2), or 200 mM (elution, Buffer 3a/b3) imidazole. Eluted protein was immediately (ScENT1 was sensitive to imidazole, histidine may be a suitable alternative for IMAC columns) exchanged into Buffer 4a/b (a: 50 mM Tris-HCl pH 7.4, 400 mM NaCl, 5% (w/v) glycerol, 2 mM DDM, 1 mM PMSF, and 5 mM β -Me; b: remove PMSF and β -Me) and concentrated in a 50,000 MWCO centrifugal concentrator (Millipore #UFC903096) to a final concentration of 0.7 – 1.0 mg / ml. The detergent-solubilized protein was further purified using size exclusion chromatography on a Superdex 200 10/300 (GE Healthcare #17-5175-01) column running at 0.5 ml / min Buffer 5a/b (a: 50 mM KH_2PO_4 at pH 7.4, 50 mM NaCl, 1 mM MgCl_2 , 1 mM PMSF, 2 mM DDM, and 5 mM β -Me; b: remove PMSF and β -Me) with 750 μ l injection volumes (Figure 1a). Peak fractions are collected in a Foxy R1 fraction collector, and can be pooled and then concentrated using a centrifugal concentrator.

Protein purity was assessed by SDS-PAGE gels and western blots using an anti-His antibody (Qiagen #35370).

hENT1 and hENT2 Solubilization and Purification

Pelleted membranes were solubilized in 25 mM Tris-HCl pH 8.0, 500 mM NaCl, 100 mM sucrose, 20 mM DDM, and 1 mM PMSF ("Buffer 6") and stirred at 4 °C for 1 hr. Insoluble material was removed by ultracentrifugation at $138,000 \times g$ at 4 °C for 1 hr. The supernatant was collected, imidazole was added to 15 mM, and 5 ml of Ni-NTA agarose (Qiagen #30250) slurry (equilibrated with Buffer 7a1: 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10% (v/v) glycerol, 1 mM DDM, and 15 mM imidazole) was added and allowed to nutate for 90 min at 4 °C. The histidine-MBP-tagged protein was then transferred to an empty gravity column and purified via sequential washes with 20 bed-volumes of Buffer 7a containing 15 (wash 1, Buffer 7a1), 30 (wash 2, Buffer 7a2), or 300 (elution, ~5 ml, Buffer7a3) mM imidazole. Following elution, protein was immediately desalted on a 10DG (Bio-Rad # 7322010) desalting column equilibrated in Buffer 8 (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10% (v/v) glycerol, and 1 mM DDM). 3C protease (MBP-His fusion, house stock) was then added at 0.6 mg per 10 OD (optical density) to catalyze tag removal, and allowed to nutate overnight at 4 °C. The next morning, 2 ml of TALON cobalt slurry was added per 1 mg of 3C protease and nutated for 1 hr at 4 °C. The suspension was then passed through an IMAC column and the flow-through was retained. The protein was then concentrated in a 50,000 MWCO centrifugal concentrator to ~1–2 OD/ml for injection (500 µl) onto a Superdex 200 10/300 GL SEC column running at 0.3 ml/min in Buffer 8. Peak fractions were collected and protein purity was assessed by SDS-PAGE gels and western blots using an anti-His antibody (Santa Cruz Biotechnology #SC-8036HRP) (Figure 1b).

ScENT1 Proteoliposome Preparation and Functional Assays

The readers are referred to the original publication (34) for a detailed protocol describing the preparation of ScENT1 containing proteoliposomes and description of functional assays. Briefly, 0.2 µm extruded liposomes consisting of 5.4% (w/v) L-α-phosphatidylcholine, 9.3% (w/v) cardiolipin, 21.9% (w/v) L-α-phosphatidylglycerol, and 63.4% (w/v) L-α-phosphatidylethanolamine were incubated with purified ScENT1 and excess DDM. LMNPG is not recommended for reconstitution purposes because it was resistant to removal. The detergent and any unincorporated protein were then removed using SEC. Tritium label substrates were monitored for uptake into the luminal volume of the proteoliposomes following filtration using liquid scintillation counting. Specific substrate transport in pmol/mg of ScENT1 was calculated by:

$$\text{Transport (pmol/mg protein)} = \frac{(\text{CPM}_{\text{sample}} - \text{CPM}_{\text{sample}})}{(\text{SR} \times \text{mg protein})}$$

where $\text{CPM}_{\text{sample}}$ (counts per minute for sample) are the counts on the filter associated with the proteoliposome, $\text{CPM}_{\text{control}}$ are the counts on the filter associated with the empty liposomes, and SR is the specific radioactivity defined as: total radioactivity (CPM) / pmol of substrate per sample, and was described previously (43). Kinetic studies of uridine transport were fit directly using nonlinear regression for each individual substrate (N=3).

Studies of bulk transport were determined by comparing mean substrate uptake (pmol substrate / mg of ScENT1) relative to [³H]-UTP by ScENT1 using one-way analysis of variance and Dunnett's multiple comparison test (UTP, N=3; Uridine, N=5; Cytidine, N=4).

RESULTS AND DISCUSSION

ScENT1, hENT1 and hENT2 were successfully expressed in *S. cerevisiae* W303 cells using a galactose-inducible system. Another system that has been successfully employed has been *Pichia pastoris* for the recombinant expression of AtENT7 (39). Additional systems have been utilized for the crude isolation of human ENTs and include other *S. cerevisiae* strains (44), BeWo cells (45), and Ehrlich cells (29,30,33). The two previously successful purification approaches employed both N-terminal 10× histidine tag (ScENT1, (34)) and C-terminal eGFP-8× histidine tag (AtENT7, (39)). We show that hENT1 and hENT2 can be successfully purified as N-terminal 10× histidine-MBP fusion constructs, demonstrating the necessity to test multiple "flavors" of recombinantly expressed ENTs to identify the fusion construct that displays optimal expression levels. Presently, we expand on the purification protocol for ScENT1 and provide insights into troubleshooting expression and purification strategies for ScENT1, hENT1, and hENT2.

The functional assessment of IMPs requires them to be removed from the plasma membrane and subsequently stabilized in an environment conducive to measuring biological activities. The purification of stable IMPs is dependent on the use of detergents, and the chemical characteristics of the detergent utilized often determine the outcome of these efforts. Nonionic detergents have been favored for the purification of ENTs. Early attempts at purification used Triton X-100 (31), but the most prevalent was octylglucoside, commonly referred to as OG or OCTG (29,30,32). One study focused on determining the most efficient detergent for the extraction of hENT1 from Ehrlich cells with the ability to preserve function and found that detergents based on the maltoside structure were the most promising (32). Specifically, decylmaltoside (DM or DCM) and cyclohexylbutylmaltoside (CYMAL-4) were more efficient at solubilizing and maintaining the stability of hENT1 (32), but each detergent was also less efficient reconstituting hENT1 in artificial membranes when compared to OG. CYMAL-4 was later determined to be inadequate for reconstitution studies due to its ability to disrupt membrane integrity as seen by the increase in non-mediated substrate uptake into control liposomes made from solubilized Ehrlich cell plasma membranes supplemented with purified lipids (30). The most recent successful efforts have also utilized a maltoside-derived detergent, DDM for the purification of stable and active ENTs (34,39). The advantageous use of DDM over DM lies in accordance with previously characterized membrane proteins; DDM has been frequently used to crystallize α-helical membrane proteins and has been most successful when used on transporters and respiratory complexes (46). This may be due to the increase in the length of the alkyl chain, which has been shown to be less deactivating (47). Additionally, we have observed that LMNPG, a newer detergent from the maltose-neopentyl glycol (MNG) class is also capable of solubilizing ScENT1, but is not suitable for reconstitution studies. MNG detergents are structured around a central quaternary carbon atom derived from neopentyl glycol, with hydrophilic groups derived from maltose (48). The development of detergents that adequately solubilize and stabilize transporters has allowed a

major hurdle associated with membrane protein purification to be overcome, and increases the likelihood of structure determination.

Once ENTs have been successfully solubilized, the next step is the development of a purification scheme. ScENT1, hENT1, hENT2, and AtENT7 were successfully purified at a pH of 7.4 (ScENT1, hENT1, and hENT2) and 6.0 (AtENT7) (34,39). Previous work involving the crude purification of hENT1 also used a pH of 7.4 (29,30,32). Salt and glycerol concentrations have varied for the reported purification schemes. Membrane solubilization ranged from 150 mM NaCl to 500 mM NaCl and 5% to 20% (v/v) glycerol. However, hENT1 and hENT2 substituted glycerol for 100 mM sucrose. IMAC conditions ranged from 150 mM NaCl to 400 mM NaCl, 5% to 10% (v/v) glycerol and were all carried out in Tris-HCl pH 7.4 – 8.0, although AtENT7 was immediately dialyzed into a MES buffer at pH 6.0. Size exclusion conditions consisted of 50 mM NaCl to 300 mM NaCl and 0% to 10% (v/v) glycerol, and the buffer systems diverged at this point with ScENT1 using potassium phosphate, AtENT7 using MES, and hENT1 and hENT2 using Tris-HCl. One important point was that ScENT1 required MgCl₂ during the purification process (Figure 1a). In the absence of MgCl₂, ScENT1 elutes in the void volume of the SEC column (Figure 1a), and shows no indication of oligomerization. However, purified AtENT7 is active in a dimeric state (39). Previous work with crude preparations of hENT1 also utilized divalent cations in the buffer conditions, namely MgCl₂ and CaCl₂, and it was recently reported that calcium-dependent regulation of nucleoside flux may be due to hENT1 regulation by calmodulin in the presence of calcium (49). Purified AtENT7 has not indicated a dependence on divalent cations for stability or activity (39). Final yield for ScENT1, hENT1, and hENT2 are 100 – 150 µg/L of culture, respectfully (Table 2). The purification steps for ScENT1, hENT1, and hENT2 are summarized in Figure 2.

ENT stability and function were previously shown to be adversely affected by extraction from cellular membranes using detergent (32). Purified ScENT1 was stable and active once reconstituted in an artificial membrane environment consisting of 5.4% (w/v) L- α -phosphatidylcholine, 9.3% (w/v) cardiolipin, 21.9% (w/v) L- α -phosphatidylglycerol, and 63.4% (w/v) L- α -phosphatidylethanolamine (Figure 3) (34). Our previous work has shown that ScENT1 is a broadly selective, high-affinity, nucleoside and nucleobase transporter (34). Additionally, the advent of new technology, microscale thermophoresis, allowed for the functional characterization of AtENT7 without the presence of an artificial lipid environment (39). While this method may be limited in its application, it represents a sound alternative for studying purified ENTs. Functional assays for ScENT1 and AtENT7 both utilized a phosphate buffer system and 100 mM NaCl, but the notable differences were AtENT7 used 10% (v/v) glycerol and ScENT1 used 1 mM MgCl₂. Additionally, ScENT1-mediated transport does not appear to be dependent on pH or salt gradients (34). Crude preparations have used ligand additives and inhibitors as an attempt to lock the transporter in a stable conformation, and found that adenosine and adenosine analogs provided a protective advantage for hENT1 (29). The addition of ligand substrates to preparation of ScENT1 did not have a noticeable effect. Phospholipids have also been shown to alter the stability of ENTs with phosphatidylserine being important for crudely prepared hENT1 (30) and phosphatidylcholine for ScENT1 (34). Given the delay in activity associated with purified and reconstituted ScENT1, it is likely that an unknown structuring of lipid

microenvironment (e.g. lipid rafts) or alternative lipids may be required for optimal activity. However, the utilization of DDM was likely one of the major contributing factors in obtaining active and stable ScENT1 and AtENT7.

The efforts outlined in this article have led to several important observations for each respective protein. The following observations were obtained during development of this ENT protein production protocol: 1) Mg^{2+} improves protein stability and enhances protein activity, 2) 2 mM DDM and 5% (v/v) glycerol were needed to stabilize the protein during concentration prior to SEC purification, 3) purified ENTs are sensitive to prolonged imidazole exposure, 4) purified ENTs are most stable in a potassium phosphate buffer, 5) once incorporated into an artificial bilayer ScENT1 is stable and active at various pH (5.5 – 8.2) and salt concentrations (0 – 300 mM NaCl) (34), and 6) the transport profile of ScENT1 is modulated by alcohols (Boswell-Casteel, unpublished data).

CONCLUSION

This work presents a novel, detailed protocol for the overexpression and purification of three equilibrative nucleoside transporters: ScENT1, hENT1, and hENT2. This work also discusses key observations and hurdles associated with previously published literature. ENTs represent high-value pharmaceutical targets that have been implicated in a variety of disease states including cardiovascular and neurological disorders, cancer, viral infections, and addiction. The ability to study ENTs at the molecular level will enhance our understanding of these disparities and offers opportunities for the growth and tuning of unique therapeutics. This work provides the foundation for the advancement of pharmaceutical development, substrate profiling and kinetic characterization, as well as opening avenues for additional biophysical analysis, such as structure determination, of the ENT family.

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The abbreviations used are

mAU	milli-absorbance units
β-Me	β-mercaptoethanol
DDM	n-docecyl-β-D-maltoside
ENT	equilibrative nucleoside transporter
IMP	integral membrane protein
MWCO	molecular weight cutoff
SC-HIS	synthetic complete histidine dropout media
SEC	size exclusion chromatography
TMD	trans-membrane domain
YPG	yeast extract-peptone-galactose
GPCR	G-protein coupled receptor
MFS	major facilitator superfamily

IMAC	immobilized metal affinity chromatography
MBP	maltose-binding protein
LMNPG	lauryl maltose neopentyl glycol
OG/OCTG	octylglucoside
DM/DCM	decylmaltoside
CYMAL-4	cyclohexylbutylmaltoside
CPM	counts per minute
FDA	Food and Drug Administration
EMA	European Medicines Agency

FIGURE 1.

Purification of ENT proteins. Size exclusion column traces with corresponding SDS-PAGE gel of the peak fraction for (a) ScENT1 and (b) hENT1 (red) and hENT2 (blue). Traces were constructed by plotting the 280 nm absorbance values on the ordinate and elution times on the abscissa. ScENT1 preparations (a) containing magnesium chloride are shown in green and red indicates the absence.

FIGURE 2.

ENT expression and purification workflow comparison. This flowchart provides a visual description of the pipeline employed for each transporter (ScENT1 - red, hENT1 - green, hENT2 - blue). Specific buffers utilized in this pipeline are referred to by number - please refer to the Materials and Methods section or Table 1 for specific buffer compositions.

FIGURE 3.

ScENT1 containing proteoliposomes are active. a) Data represents the mean uptake for [^3H]-UTP (N=3), [^3H]-uridine (N=5), and [^3H]-cytidine (N=4) tested at 100 nM concentrations over the course of 3.5 hr (34). ***, $p < 0.001$; and ****, $p < 0.0001$. b) Substrate uptake was measured at 10, 20, 50, and 60 min using substrate concentrations that ranged from 100 – 2000 nM. The slope of the linear time course for each substrate concentration was then fit directly using nonlinear regression to determine the initial uptake rates for each substrate concentration. These values in pmol/mg/min were then plotted relative to substrate

concentration and fitted directly using nonlinear regression and a simple steady-state Michaelis-Menton model (N=3).

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Table 1**Detailed Buffer Compositions**

Buffer	Composition	Location in Pipeline	Associated Protein(s)
1a	50 mM Tris-HCl, 10% (v/v) glycerol, 150 mM NaCl, 10 mM EDTA, 2 mM PMSF, 1× DHALT, and 5 mM B-mercaptoethanol	Cell Lysis	ScENT1
1b	50 mM Tris-HCl, 10% (v/v) glycerol, 150 mM NaCl, and 10 mM EDTA	Cell Lysis	ScENT1
2	50 mM Tris-HCl pH 7.5, 20% (v/v) glycerol, and 1 mM PMSF	Cell Lysis	hENT1, hENT2
3a	50 mM Tris-HCl pH 7.4, 400 mM NaCl, 5% (v/v) glycerol, 2 mM PMSF, 1× DHALT protease inhibitor cocktail and 5 mM β-mercaptoethanol	Membrane Solubilization	ScENT1
3b	50 mM Tris-HCl pH 7.4, 400 mM NaCl, and 5% (v/v) glycerol	Membrane Solubilization	ScENT1
(3×)1*	Buffer 3a or 3b plus 2mM DDM and 10 mM imidazole	IMAC Purification	ScENT1
(3×)2*	Buffer 3a or 3b plus 2mM DDM and 20 mM imidazole	IMAC Purification	ScENT1
(3×)3*	Buffer 3a or 3b plus 2mM DDM and 200 mM imidazole	IMAC Purification	ScENT1
4a	50 mM Tris-HCl pH 7.4, 400 mM NaCl, 5% (v/v) glycerol, 2 mM DDM, 1 mM PMSF, and 5 mM β-Me	Buffer Exchange	ScENT1
4b	50 mM Tris-HCl pH 7.4, 400 mM NaCl, 5% (v/v) glycerol, and 2 mM DDM	Buffer Exchange	ScENT1
5a	50 mM KH ₂ PO ₄ at pH 7.4, 50 mM NaCl, 1 mM MgCl ₂ , 1 mM PMSF, 2 mM DDM, and 5 mM β-Me	SEC Purification	ScENT1
5b	50 mM KH ₂ PO ₄ at pH 7.4, 50 mM NaCl, 1 mM MgCl ₂ , and 2 mM DDM	SEC Purification	ScENT1
6	25 mM Tris-HCl pH 8.0, 500 mM NaCl, 100 mM sucrose, 20 mM DDM, and 1 mM PMSF	Membrane Solubilization	hENT1, hENT2
7a	20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10% (v/v) glycerol, 1 mM DDM,	IMAC Purification	hENT1, hENT2
7a1	Buffer 7a plus 15 mM imidazole	IMAC Purification	hENT1, hENT2
7a2	Buffer 7a plus 30 mM imidazole	IMAC Purification	hENT1, hENT2
7a3	Buffer 7a plus 300 mM imidazole	IMAC Purification	hENT1, hENT2
8	20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10% (v/v) glycerol, and 1 mM DDM	Buffer Exchange, Tag Cleavage & Reverse IMAC, SEC Purification	hENT1, hENT2

* x denotes the use of buffer 3a or 3b as a base component.

Table 2

Purification Summary

Protein	Purification Step	Recovery (μg / L culture)	Estimated Purity (%)
ScENT1	IMAC	500	90
	SEC	150	95
hENT1	IMAC	400	70
	SEC	150	90
hENT2	IMAC	300	60
	SEC	100	85