Demonstration of Equilibrative Nucleoside Transporters (hENT1 and hENT2) in Nuclear Envelopes of Cultured Human Choriocarcinoma (BeWo) Cells by Functional Reconstitution in Proteoliposomes

Rajam S. Mani‡§§, James R. Hammond‡**, Jihan M. Marjan‡§§, Kathryn A. Graham‡§§, James D. Young§§§§§, Stephen A. Baldwin¶¶, and Carol E. Cass‡§§¶

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The equilibrative nucleoside transporters (ENTs) are a newly recognized family of membrane proteins of which hENT1 is the nitrobenzylmercaptopurine ribonucleoside (NBMPR)-sensitive (es) and hENT2 the NBMPR-insensitive (ei) transporter of human cells. BeWo cells exhibit large numbers (>10⁷/cell) of NBMPR-binding sites and high es and ei nucleoside transport activities relative to other cell types. In this work, we have demonstrated that proliferating BeWo cells possess (i) mRNA encoding hENT1 and hENT2 and (ii) hENT1-specific immunoprecipitates. We examined NBMPR binding and its inhibition of uridine transport in various BeWo membrane fractions and proteoliposomes derived therefrom to determine if NBMPR binding to intracellular membranes represented interaction with functional es transporters. Unfractionated membranes and fractions enriched 5-fold in plasma membranes relative to postnuclear supernatants exhibited high NBMPR binding activity. Intact nuclei and nuclear envelopes also exhibited abundant quantities of NBMPR-binding sites with affinities similar to those of enriched plasma membranes (Kd = 0.4–0.9 nM). When proteoliposomes were made from octyl glucoside-solubilized membranes, high affinity NBMPR-binding sites were not only observed in crude membrane preparations and plasma membrane-enriched fractions but also in nuclear envelope fractions. Proteoliposomes prepared from either unfractionated membranes or nuclear envelopes exhibited both hENT1-mediated (82–85%) and hENT2-mediated (15–18%) transport of [³H]uridine. These results provided evidence for the presence of functional es and ei transporters in nuclear membranes and endoplasmic reticulum, suggesting that hENT1 and hENT2 may function in the translocation of nucleosides between the cytosol and the luminal compartments of one or both of these membrane types.

Multiple nucleoside transport (NT) mediated processes exist in mammalian cells and are divided into two groups, depending on whether they are equilibrative or concentrative in nature (for recent reviews, see Refs. 1–3). The equilibrative transporters accept both purine and pyrimidine nucleosides as permeants and are found in most, possibly all, cell types, whereas the concentrative transporters have relatively narrow selectivities for nucleoside permeants and are limited to specialized cell types. The transporters are low abundance proteins, and the equilibrative transporters of human and pig erythrocytes are the only ones that have been purified to homogeneity (4, 5). The equilibrative transporters have been subdivided on the basis of sensitivity to nitrobenzylthioinosine (NBMPR); one subtype (es) is inhibited by ≈1 nM NBMPR, as a result of high affinity (Kd < 5 nM) binding of NBMPR at or near the permeant binding site (6–8), whereas the other subtype (ei) is unaffected by low concentrations (<1 µM) of NBMPR. The concentrative nucleoside transporters comprise several functional subtypes (1) that differ in their substrate selectivities and tissue distributions and, with some exceptions (9), are unaffected by high concentrations (>10 µM) of NBMPR.

Understanding of relationships among nucleoside transporters has been greatly advanced by the recent isolation and functional expression from rat and human cells of cDNAs encoding NT proteins (Refs. 10–16; reviewed in Ref. 3). These NT proteins comprise two structurally unrelated protein families that are designated ENT and CNT, depending on whether they mediate, respectively, equilibrative (E) or concentrative (C) NT processes. The mammalian ENT proteins identified thus far have approximately 450 amino acid residues (10, 11, 16) and 11 predicted transmembrane domains, whereas the mammalian CNT proteins have approximately 650 amino acid residues (12–14) and 14 predicted transmembrane domains. Members of the ENT family (10, 11, 16) exhibit functional characteristics that are typical of es-mediated (e.g. hENT1, rENT1) or ei-

### Notes

1. The abbreviations used are: NT, nucleoside transporter; CNT1 and -2, concentrative nucleoside transporter 1 and 2, respectively; ENT1 and -2, human ENT1 and -2, respectively; rCNT1 and -2, rat CNT1 and -2, respectively; NBMPR, nitrobenzylmercaptopurine ribonucleoside (also known as nitrobenzylthioinosine); ei, equilibrative NBMPR-insensitive; es, equilibrative NBMPR-sensitive; FITC, fluorescein isothiocyanate; NBGPR, nitrobenzylthioguanosine; OCTG, octylglucopyranoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

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** A visiting scientist of the Alberta Heritage Foundation for Medical Research.

†† A Heritage Medical Scientist.

‡‡ A Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada. To whom correspondence should be addressed: Dept. of Oncology, University of Alberta, 11560 University Ave., Edmonton, Alberta T6G 2J2, Canada. Tel.: 403-432-8320; Fax: 403-432-8425; E-mail: carol.cass@cancerboard.ab.ca.
mediated (e.g. hENT2, rENT2) transport processes. Members of the CNT family (11–14) are Na⁺-nucleoside symporters that catalyze inward transport of pyrimidine nucleosides plus adenosine (e.g. hCNT1, rCNT1) and purine nucleosides plus uridine (e.g. hCNT2, rCNT2).

Cultured human choriocarcinoma (BeWo) cells have been studied mainly for their ability to undergo differentiation from proliferating cytotrophoblast-like cells to larger syncytiotrophoblast-like cells when exposed to methotrexate in a process that resembles normal in utero development (17, 18). Previous studies with proliferating BeWo cells (19) revealed unusual NBMPR-binding characteristics in comparison with other cell types. BeWo cells have extraordinarily large numbers of high affinity sites (>2 × 10⁶/cell) of two apparent classes (K_d = 0.6 and 14.5 nM), whereas most cultured cell types have far fewer high affinity sites of a single apparent class (e.g. HeLa cells possess 4.1 × 10⁶/cell with K_d = 0.7 nM). NBMPR-binding proteins have been shown in reconstitution studies to mediate es NT activity in human erythrocytes (20, 21), cultured leukemia (CEM) cells (22), and Ehrlich ascites tumor cells (23). The existence of two classes of binding sites, together with the lack of proportionality between NBMPR-binding sites and maximal transport activities of BeWo and HeLa cells, led to the suggestion (19) that the second class of NBMPR-binding sites in BeWo cells may be es transporters associated with intracellular membranes.

In the present study, we established that BeWo cells, which possess es- and ei-mediated nucleoside transport activities, express hENT1 and hENT2 mRNAs. We quantified binding of NBMPR to various BeWo membrane fractions by analysis of isolated and detergent-solubilized membranes and of reconstituted proteoliposomes. A single class of high affinity NBMPR-binding sites was observed in all membrane preparations, including intact nuclei and nuclear envelopes. There was no evidence of the lower affinity NBMPR-binding sites previously observed in intact BeWo cells in any of the membrane preparations, suggesting loss of these sites during cell disruption.

Functional reconstitution of both NBMPR-sensitive (es) and NBMPR-insensitive (ei) uridine transport activities was demonstrated in proteoliposomes derived from both crude membrane and nuclear envelope-enriched fractions. Our results suggest that hENT1 and hENT2 may play a role in the translocation of nucleosides between the cytosol and the lumen of nuclear envelopes and/or endoplasmic reticulum and provide the first evidence for functional ENTs in intracellular membranes of mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**[G-³H]NBMPR (22.5 Ci/mmol) and [5,6-³H]uridine (40 Ci/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA). [¹⁴C]Cholesteryl oleate (0.1 mCi/ml) was obtained from Amersham Canada Ltd. (Oakville, Canada). Radiolabeled nucleosides were purified by high pressure liquid chromatography using water-methanol gradients on a C18 reverse phase column. Asolectin (soybean phospholipids) was purchased from Associated Concentrates (Woodside, NY) and stored under N₂. Other phospholipids and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). OCTG, adenosine, uridine, NBMPR, and dipryridamide were purchased from Sigma, and Sephadex G-50 (fine and medium) was from Amersham Pharmacia Biotech Inc., Baie d’Urfe, Canada. Dilazep (N,N’-bis[3,4,5-trimethoxybenzoxyl-oxy]propyl)homopiperazine) was a gift from Hoffmann-La Roche.

**Cell Culture—**The origin and characteristics of BeWo cells have been described (24, 25). Cultures were initiated from microlumps of frozen stock cells (26). The cultures are known to be at least 95% CNT positive, as described previously (25). Cells were harvested at weekly intervals from proliferating cultures by trypsin treatment and maintained in plastic culture flasks in Roswell Park Memorial Institute (RPMI) 1640 basal medium supplemented with 5% fetal bovine serum and 5% NUC serum type IV. Cell numbers were determined with an electronic particle counter after dissociation with trypsin/EDTA and resuspension in 0.15 M NaCl solution. For preparation of membranes, cells (5 × 10⁶/flask) were grown in 15 Corning disposable tissue culture flasks (15-cm² surface area), and actively proliferating cells were harvested 10–14 days later, yielding from 8 × 10⁶ to 1 × 10⁸ cells.

**Cell Lysates—** Cultures were lysed with 1 ml of 1.0 × 10⁶ cells with a Fast Track 2.0 kit (Invitrogen, Carlsbad, CA) and used on RNA blots and in reverse transcription polymerase chain reaction (RT-PCR) amplification. To prepare the RNA blots, BeWo mRNA was subjected to electrophoresis on 1% denaturing agarose-formaldehyde gels and transferred to Hybond-N⁺ nylon filters (Amersham). The resulting RNA blots were probed with ³²P-labeled cDNA specific to hENT1 (10) or using Express cDNA LONTECT (Palo Alto, CA), conforming to the manufacturer’s recommendations. The blots were washed to high stringency and exposed to x-ray film for autoradiography. To perform the RT-PCR reactions, BeWo cDNA was made using the SuperScript Preamplification System (Life Technologies, Inc.) and subjected to PCR amplification using Taq polymerase (Life Technologies) and primers specific to hENT1 (5’-CCTGTGTCCTGTTCTCTGAC and 5’-TGTTGCA-CACAATTGGCCGGCGAGAG) or hENT2 (5’-GCGTGCGTTCCTGTT-CGTCG and 5’-GCGACGTCTCGACGAGCCCTTGGAAG). The resulting PCR products were resolved on 0.8% agarose gels and either (i) stained with ethidium bromide for visualization using a 1-kilobase pair ladder (Life Technologies) as a size standard or (ii) purified and sequenced using the PCR primers, a dRhodamine cycle sequencing kit (PE Applied Biosystems) and MacVector sequence analysis software (Oxford Molecular, Oxford, UK).

**Isolation of BeWo Membranes—** Membrane fractions were prepared from trypsin-treated cultures of BeWo cells by sucrose gradient centrifugation (26, 27). Cells were resuspended in phosphate-buffered saline (PBS) composed of 20 mM phosphate and 0.15 M NaCl at pH 7.4 and centrifuged (10 min, 1000 × g). Packed cells were resuspended in 1 ml of ZnCl₂ and fragmented with a Polytron homogenizer (Brinkmann Instruments). Nuclei and unbroken cells were removed by gentle centrifugation (2 min, 900 × g), after which the supernatants were centrifuged (30 min, 11,000 × g) and the resulting membrane pellets were resuspended in 9.25% (w/w) sucrose. The membrane fractions were then separated using a 15–45% sucrose gradient and stored at −80 °C in 15% (v/v) dimethyl sulfoxide. Sucrose solutions were in 0.5 mM ZnCl₂, 5 mM K₃HPO₄, pH 7.4. The fractions collected at 25% sucrose had 4–5 times the specific activity of crude nuclei-free membranes when assayed for alkaline phosphatase (EC 3.1.3.1) and 5’-nucleotidase (EC 3.1.3.5) and thus were enriched in plasma membranes (Sigma Diagnostics 5’-nucleotidase assay kit). Nearly 40% of the total protein applied to the sucrose gradient was recovered in this sucroes fraction. Nuclear membranes were parsed from the nuclei collected during the isolation stage of membrane preparation according to Kay et al. (28), whereby nuclei were treated with a low level of DNase I in the presence of Mg²⁺ at slightly alkaline pH, after which membranes were collected by centrifugation (30 min, 11,000 × g) and stored at −80 °C in 15% (v/v) dimethyl sulfoxide.

**Solubilization and Reconstitution of BeWo Membranes—** Membranes were thawed at room temperature, washed extensively to remove dimethyl sulfoxide, and resuspended in ice-cold reconstitution buffer (pH 7.4) composed of 100 mM KCl, 10 mM Tris, 0.1 mM MgCl₂, and 0.1 mM CaCl₂. Membranes were then mixed with a solution of OCTG (1% final concentration) and asolectin (0.15% final concentration) prepared in reconstitution buffer and incubated on ice for 1 h with frequent mixing. The insoluble material was removed by centrifugation (80 min, 100,000 × g, 4 °C), and the supernatant was retained on ice until use. Because solubilization efficiency of detergents is often more dependent on the ratio of detergent to protein than on the actual detergent concentration used (29, 30), the membrane protein concentrations were monitored using a modified Bradford assay (23) and adjusted to approximately 200 μg of protein/ml. The reconstitution technique was based on a method used previously (23). The solubilized membranes (2 ml) were supplemented with a sonicated preparation of lipids (0.3 ml) consisting of phosphatidylcholine (bovine brain), cholesterol, phosphatidylethanolamine (egg) and phosphatidylserine (bovine brain) in molar ratios of 33:33:28:8, respectively, plus a trace (10⁻⁶ dpm/ml) of [¹⁴C]cholesteryl oleate. The detergent was removed by gel filtration (Sephadex G-50 medium, 1.5 × 38-cm column) at a flow rate of 1 ml/min; this step removed any remaining detergent and formation of vesicles (31). The void volume fractions containing proteoliposomes were pooled, frozen in ethanol and dry ice, and stored at −80 °C for up to 1 month. For functional assays, proteoliposomes were thawed at room temperature, centrifuged (40,000 × g, 20 min), resuspended in approximately 1 ml of reconstitution buffer, sonicated for 15 s in a cylindrical tank sonicator (Laboratory Supplies Company Inc., Hicksville, NY), and then
stored on ice until use. Brief sonication after the freeze/thaw cycle is known to enhance the activity of the reconstituted erythrocyte nucleoside transporter (21).

**Equilibrium Binding of \([3H]NBMPR—High affinity binding of \([3H]NBMPR, which has been used extensively in the characterization of the nucleoside transport proteins (1, 8, 32, 33), was performed as follows. Membrane preparations, proteoliposomes (10 μg of protein/ml final concentration), and intact nuclei and nuclear membranes (at ~40 μg of protein/ml) were incubated for 45 min at room temperature with a range of concentrations (0.24–24 nM) of \([3H]NBMPR in either the absence (total binding) or presence (nonspecific binding) of 10 μM NBMPR (1-ml final assay volume). Proteins were precipitated by incubation with a mixture of 3-drop ethanol (1.65 ml) and polyethylene glycol (M, 8000, 10% (w/v) final concentration) and then filtered under vacuum through Whatman GF/B filters. The filters were washed twice with reconstitution buffer containing 8% (w/v) polyethylene glycol at 4 °C and analyzed for radioactive content by standard liquid scintillation counting techniques as described previously (23).**

**Uptake of \([3H]Uridine by Proteoliposomes—**The uptake assays were initiated by the rapid addition of 100 μl of proteoliposome suspension (~5 μg of protein, with or without inhibitors) to 25 μl of reconstitution buffer with \([3H]uridine (20 μM final concentration) and mixed by repeated pipetting. After specific incubation times, 100 μl of the reaction mixtures were layered on ice-cold Sephadex G-50 fine minicolumns (see below) and centrifuged (45 s, 700 × g). The effluents were collected in preweighed tubes from which portions were removed for the determination of protein content and \(^3H\) and \(^14C\) contents (dual label counting). The \([3H]uridine content of each sample was normalized for intrarexperiment variation in eluate volume and phospholipid content based on the \(^14C\)-labeled lipid concentration of the minicolumn effluent. Approximately 50% of the volume added to the minicolumns was recovered in the effluents. The minicolumns used were prepared by filling 1-ml syringes (fitted with a polyethylene filter) with Sephadex G-50 equilibrated in reconstitution buffer containing 10 μM dilaurel and nitrogen-dithioguanosine (NBTGR). The gel-filled minicolumns were centrifuged (45 s, 700 × g) and placed on ice for at least 30 min prior to use. Estimates of zero time uptake values were obtained by measuring the uptake of \([3H]uridine at ~2 s in the presence of ice-cold solutions containing 10 mM adenosine, 10 μM dipyridamole, and 10 μM NBTGR to inhibit all transporter-mediated influx. The zero-time uptake values were subtracted from all other data points, and the accumulation of \([3H]uridine was expressed as picomol taken up/mg of proteoliposomal protein. Mediated influx of \([3H]uridine was defined as the total uptake minus uptake observed in the presence of 1 mM adenosine, 10 μM dipyridamole, and 10 μM NBTGR (nondetermined). For inhibition assays, proteoliposomes were incubated with test compounds for 10 min before initiating transport reactions. Protein was measured using bovine serum albumin as the standard with appropriate corrections for detergent interference (34, 35).

**Flow Cytometry—**Nuclei (obtained by cell disruption as described above) were collected by centrifugation (5 min, 500 × g), washed twice in PBS, and resuspended at a density of 10⁶ nuclei/ml. The nuclei were then incubated for 1 h at 4 °C with either (i) fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies directed against nuclear pore complex proteins (M4414-FITC from Babo (Richmond, CA)) at 0.01 mg/ml or (ii) with rabbit polyclonal antibodies (1 μg/ml) raised against a synthetic peptide corresponding to amino acid residues 55–64 (EL-SDKAQASA) of the predicted hENT1 sequence (designated hENT1 (55–64)) and immediately thereafter with goat anti-rabbit FITC-conjugated antibodies. The nuclei were then washed twice with PBS and resuspended in 500 μl of 1% paraformaldehyde-PBS solution prior to being analyzed on a FACSFlow cytometer (Becton Dickinson Immunochemistry Systems, San Jose, CA). Rabbit anti-mouse FITC-conjugated antibodies were used as the negative control for the M4414-FITC, and goat anti-rabbit FITC-conjugated antibodies were used as a negative control for the hENT1 (55–64) antibodies. The synthetic peptide (hENT1 (55–64)) was prepared and conjugated to keyhole limpet hemocyanin (Alberta Peptide Institute, Edmonton, Canada) from an analog peptide sequence with the keyhole limpet hemocyanin (Surfit) matrix (Matrix Science, London) that predicts exposed, potentially immunogenic residues. The antibodies that recognized hENT1 (55–64) were purified by first passing rabbit sera through a Protein G (Amersham Pharmacia Biotech Inc.) column to collect the IgG fraction, followed by dot blot affinity purification using the synthetic peptide. The specificity of the hENT1 (55–64) antibodies was assessed by comparing fluorescence intensity of hENT1-containing cells in the presence and absence of excess hENT1 (55–64).

**NBMPR Binding to BeWo Membrane Fractions—**Because \([3H]NBMPR binds specifically and with high affinity to functional nucleoside transporters, equilibrium binding analysis has been used extensively as a surrogate marker for the presence of the \(e\) transporter protein (1, 8, 32, 33). BeWo membrane fractions collected at different sucrose concentrations were first assayed for NBMPR binding activity at a single concentration (5 mM) under conditions previously shown in studies with intact cells (19) to be sufficient to label over 40% of the high affinity sites. Crude membranes (the ‘postnuclear’ supernatant) collected at 45 min at room temperature. The BeWo membrane fractions were collected at different sucrose concentrations after density gradient fractionation and different specific binding activities (data not shown), with the maximum activity observed in the fraction isolated from the 25% sucrose layer (26 pmol/mg of protein). Thus, there was an approximate 4-fold increase in binding activity relative to that observed in the crude membranes, and the protein recovery in this fraction amounted to roughly 40% of the total protein present in the various mem-
brane fractions. Subsequent experiments with “plasma membrane” utilized the membranes collected from the 25% sucrose layer, since this fraction was enriched 4–5-fold in plasma membranes relative to crude membranes when assayed for the activities of the plasma membrane marker enzymes alkaline phosphatase and 5’-nucleotidase (Table I).

Crude membranes and plasma membranes were subjected to detailed analyses of NBMPR binding to quantify the number of binding sites and their relative affinities for NBMPR (see Fig. 2 for results of a typical experiment with each preparation and Table II for a summary). Since the Scatchard plots were linear (Fig. 2, insets), a one-site binding model was used to estimate $K_d$ and $B_{\text{max}}$ values. Plasma membranes (Fig. 2B, Table II) exhibited $K_d$ and $B_{\text{max}}$ values of 0.9 ± 0.2 nM and 75.0 ± 5 pmol/mg of protein, respectively (mean ± S.E., $n = 4$). The $K_d$ value was essentially the same as that determined previously (0.6 nM) for the high affinity binding sites in intact BeWo cells (19), and there was no indication of a second set of lower affinity binding sites. There was a nearly 4-fold increase in specific binding activity relative to that of the crude membrane preparations (Fig. 2A; Table II), indicating that high affinity binding of NBMPR represents a specific interaction with the transporter, that density gradient centrifugation in 25% sucrose yielded a plasma membrane fraction that was enriched in $es$ transporter protein.

NBMPR Binding to Detergent-solubilized Plasma Membranes and Proteoliposomes—Achieving efficient solubilization while maintaining functional integrity is a prerequisite for reconstitution studies, since the solubilized proteins will be used in making proteoliposomes. Solubilization of [3H]NBMPR binding activity from the 25% sucrose fraction was achieved using 1% OCTG, and the concentration of membrane protein was maintained at ~200 μg/ml. Under these conditions, nearly 70% of total protein was solubilized. The resulting mixed micelles exhibited a single class of high affinity binding sites ($K_d$, 3.5 nM) and contained the same number of NBMPR-binding sites/mg of protein as the nonsolubilized plasma membrane preparations (Table II). The slight decrease in affinity for [3H]NBMPR after solubilization with OCTG could have been due to the presence of the detergent; similar reductions in binding affinity have been observed with micellar preparations of solubilized murine es transporters (23).

Proteoliposomes prepared from solubilized plasma membranes also exhibited only one class of NBMPR-binding sites (Table II). The proteoliposomes bound 35 pmol of [3H]NBMPR/mg of protein with an affinity ($K_d$, 1.1 nM) that was identical to that observed for binding of NBMPR to unsolubilized plasma membranes. The efficiency of incorporation of NBMPR-binding sites from the plasma membranes into proteoliposomes was approximately 45% based on a comparison of the observed $B_{\text{max}}$ values for NBMPR binding.

**NBMPR Binding to Nuclei**—In an earlier study conducted with intact BeWo cells (19), the slow time courses of association of NBMPR and the presence of two classes of high affinity binding sites were interpreted as evidence for intracellular NBMPR-binding sites. In the present study, only one class of sites ($K_d = 1$ nM) was observed with the various membrane fractions. It was possible that the second class of sites ($K_d = 14.5$ nM) seen previously in intact cells was associated with internal components (e.g. nuclei) that were discarded in the preparation of membranes. Thus, intact nuclei and nuclear envelopes were isolated from BeWo cells for analysis of site-specific binding of [3H]NBMPR.

The nuclei and nuclear membranes were first tested for the presence of plasma membranes and organelles such as mitochondria and lysosomes, since these organelles have been reported previously (36, 37) to exhibit either NBMPR binding or
nucleoside transport activities. The nuclei and the nuclear envelope preparations contained relatively few contaminating plasma membranes (2–3%), as indicated by the low activities of marker enzymes relative to those observed in crude and plasma membrane fractions (Table I). The nuclear preparations were not contaminated with mitochondria and lysosomes, based on the absence of (>1%) detectable succinate-INT reductase and β-galactosidase activity, respectively. For comparison, the crude membranes had an approximate mitochondrial content of 9 ± 1% and lysosomal content of 7 ± 1%.

The nuclear preparations were analyzed by flow cytometry using murine monoclonal antibodies (M414-FITC) directed against a related family of nuclear pore complex proteins (38) and polyclonal antibodies directed against a synthetic peptide derived from the predicted extracellular loop between transmembrane domains 1 and 2 of hENT1 (Fig. 3). The preparations consisted almost completely of nuclei, since 99% of the population reacted with the M414-FITC antibodies, with a substantial increase in fluorescence intensity relative to that of nuclei stained with FITC-conjugated antibodies against murine IgG (the negative control). When polyclonal antibodies directed against hENT1 were used, 92% of the nuclei reacted positively, suggesting that most nuclei possessed immunoreactive epitopes (39) recognized by the hENT1- (55–64) antibodies (Fig. 3B). These results, although consistent with the presence of the hENT1 protein in nuclei, could also reflect the presence of cross-reactivity with other proteins.

Equilibrium analysis of site-specific binding of [3H]NBMPR to nuclei and nuclear membranes was undertaken in the experiments of Fig. 4 (see also Table II). The nuclei bound 7.0 pmol of [3H]NBMPR/mg of protein with an affinity \( K_d = 0.7 \) nM that was virtually identical to that observed with plasma membranes enriched in NBMPR binding activity. The NBMPR binding capacity of the nuclear envelopes was nearly one-fifth of the binding capacity of plasma membranes in the 25% sucrose fractions. The observed NBMPR binding activities associated with the nuclei and the nuclear envelopes were not due to plasma membrane contamination, since the alkaline phosphatase and 5′-nucleotidase activities were only 2–3% of that observed with the plasma membranes recovered from the 25% sucrose fractions (Table I).

[3H]Uridine Transport by Proteoliposomes Prepared from Detergent-solubilized Membranes—Although the demonstration of high affinity binding of [3H]NBMPR to various membrane fractions provided strong evidence for the presence of \( e_s \) transporter proteins, the ultimate proof of functionality is the demonstration of transmembrane fluxes of [3H]nucleoside. A centrifugal gel filtration method was employed to assess uptake of [3H]uridine by proteoliposomes. Uridine was selected because it is a recognized substrate for all of the known nucleoside transporters (1), and the passive permeability of liposomal membranes to uridine is lower than that for adenosine (39). Mediated uptake was determined by subtracting uptake of [3H]uridine in the presence of a mixture of known inhibitors of nucleoside transport (10 mM adenosine, 10 μM NBMPR, 10 μM dipyridamole) from uptake in its absence.

Reconstituted proteoliposomes prepared from solubilized crude membranes (all fractions except nuclei) were capable of transporting [3H]uridine. The initial rate computed from time courses of mediated uptake of 30 μM [3H]uridine (data not shown) was 12.0 ± 2.0 pmol/mg of protein/s (mean ± S.E., n = 4). The ability of NBMPR to inhibit transporter-mediated influx of [3H]uridine in proteoliposomes prepared from the OCTG/ASO-solubilized crude membranes is shown in the concentration-effect relationship of Fig. 5. Maximal inhibition (about 82%) of mediated transport of [3H]uridine was observed at 40 nM NBMPR, and the remaining component (about 18%) was resistant to 5 μM NBMPR. These results demonstrated the operation of both \( e_s \) and \( e_i \) transport processes in proteoliposomes prepared from crude membrane fractions.

The representative experiment shown in Fig. 6 examined the initial rates of uptake of 20 μM [3H]uridine by proteoliposomes prepared from the plasma membrane-enriched fractions. The value (mean ± S.E.) from six separate experiments for the uninhibited initial rate was 55.0 ± 5.0 pmol/mg of protein/s, and preincubation of proteoliposome with a mixture of nucleoside transport inhibitors (adenosine, NBMPR, dipyridamole) decreased this rate to 10.0 ± 2.5 pmol/mg of protein/s, or 27% of total uptake. The mediated component of uridine uptake, which was obtained by subtracting the inhibited value from the total value, was 45.0 ± 5.0 pmol/mg of protein/s.

The presence of NBMPR binding activity in nuclear membrane fractions and of the hENT1 immunoreactive protein on intact nuclei suggested that nuclear membranes and/or the associ-
FIG. 4. Binding of [3H]NBMPR to intact nuclei (A) and nuclear membranes (B) prepared from BeWo cells. Nuclei and nuclear membranes were incubated for 45 min with graded concentrations of [3H]NBMPR (0.24–24 nM) at room temperature in the absence (total) and presence (nonspecific) of 30 μM dilazep. Specific binding (defined as total minus nonspecific binding) is plotted as a function of the free [3H]NBMPR concentration when equilibrium was achieved. Each point represents the mean ± S.E. from four independent experiments conducted in duplicate; error bars are not shown where values were the same or smaller than those represented by the symbols. Inset, mass law analysis (Scatchard plot) of the relationship between specific binding of the [3H]NBMPR and the equilibrium concentration of free [3H]NBMPR. Ordinate, ratio of bound to free ligand concentration (pmol bound/mg of protein/nM); abscissa, membrane-associated [3H]NBMPR (pmol bound/mg of protein). The experimental data fit best to a one-site binding model, and the r² values obtained from the linear relationship shown (A and B) were 0.9 and 0.96, respectively.

FIG. 5. Inhibition of [3H]uridine influx by NBMPR in proteoliposomes prepared from detergent-solubilized crude membranes. Proteoliposomes were prepared from OCTG-solubilized crude membranes (without nuclei) as described under “Experimental Procedures.” The proteoliposomes were incubated at room temperature with the indicated concentrations of NBMPR for 10 min and then with 20 μM [3H]uridine for 20 s in the presence (nonmediated uptake) and absence (total uptake) of 30 μM adenosine, 10 μM NBTPG, and 10 μM dipyridamole, and values for mediated uptake were calculated from the difference between total and nonmediated uptake (data not shown). Influx in the presence of NBMPR is expressed as a percentage of the amount of [3H]uridine accumulated by the proteoliposomes in the absence of NBMPR (control uptake). Each point represents the mean ± S.E. of values from four independent experiments conducted in duplicate; error bars are not shown where values were the same or smaller than those represented by the symbols.

DISCUSSION

Cultured BeWo cells exhibit high levels of es and ei transport activities and unusually large numbers (>10⁷/cell) of NBMPR-binding sites (19). Because of these features, BeWo cells have been a useful model system for analysis of relationships between NBMPR binding and es-mediated transport capability. In this work, we have established that BeWo cells possess both hENT1 and hENT2 mRNA, and we conclude that the transporter proteins responsible for mediating the high levels of es and ei activity previously reported (19) in BeWo cells are hENT1 (es) and hENT2 (ei).

NBMPR is a tightly binding and specific inhibitor of equilibrative transport of nucleosides in mammalian cells and, in its radioactive form, has been used to identify transporter proteins in several cell lines (23, 40, 41). NBMPR-binding proteins purified from human erythrocytes exhibit both uridine transport and NBMPR-binding activities when reconstituted in unilamellar phospholipid vesicles (20), leading to the conclusion that NBMPR-binding proteins represent es transporters. NBMPR-binding site abundance has frequently been taken as a measure of functional es transporters, since the total number of NBMPR-binding sites was shown to be proportional to Vₘₐₓ values for uridine influx in erythrocytes of several mammalian species (42). However, earlier studies with BeWo cells (19), which possess an elevated number of NBMPR-binding sites and high thymidine transport activity, revealed (i) two classes of high affinity NBMPR-binding sites (Kᵣ = 0.6 and 14.5 nM) and (ii) a lack of proportionality between NBMPR binding site densities and thymidine transport activity when compared with other cell types. These observations, which suggested that BeWo cells may possess NBMPR-binding proteins that are not...
FIG. 7. Time course of [3H]uridine uptake by proteoliposomes prepared using nuclear membranes (A) and the inhibition of [3H]uridine influx by NBMPR in nuclear membrane proteoliposome (B). Proteoliposomes prepared from the OCTG-solubilized nuclear membranes were incubated with 20 μM [3H]uridine for the indicated times in the presence (●, nonmediated uptake) or absence (□, total uptake) of 10 mM adenosine and 10 μM dipyridamole. Values for mediated uptake (☆) were calculated from the difference between total and nonmediated uptake. A shows representative plots from one of three independent experiments conducted in duplicate. B shows inhibition studies in which proteoliposomes were preincubated with the indicated concentrations of NBMPR at room temperature for 10 min and then with 20 μM [3H]uridine for 20 s in the presence (●, nonmediated uptake) or absence (□, total uptake) of 10 mM adenosine and 10 μM dipyridamole. Influx in the presence of NBMPR is expressed as a percentage of the amount of [3H]uridine accumulated by the proteoliposomes in the absence of NBMPR (control uptake). Each point represents the mean ± S.E. from three independent experiments conducted in duplicate; error bars are not shown where values were the same or smaller than those represented by the symbols.
teins retained the NBMPR binding characteristics observed in studies with isolated membranes. Reconstituted proteoliposomes, which retained approximately 45% of NBMPR binding activity, were capable of accumulating [3H]uridine by processes that were inhibited by NBMPR and dipiridamole with characteristics that indicated the presence of both es and ei-mediated transport processes. hENT1 immunoreactivity, NBMPR binding activities in the nuclear envelope preparations, providing the first indication that the ENT transporters are associated with intracellular membranes in a functional state.

Lysosomes and mitochondria exhibit nucleoside transport activity (3, 36, 37), and our finding of es and ei activities in nuclear envelope preparations, which are comprised of nuclear membranes and endoplasmic reticulum, provides further evidence for the occurrence of nucleoside transporters in intracellular membranes of mammalian cells. The finding that both hENT1 and hENT2 are functionally active when isolated from nuclear envelope preparations raises two possibilities. Endoplasmic reticulum contains many enzymes of nucleotide metabolism, and it is possible that hENT1 and hENT2, which are bidirectional exchangers, may play a role in translocation of nucleosides between the cytosolic and luminal compartments. It is also possible that the nuclear envelope-associated hENT1 and hENT2 proteins serve as a reserve pool for translocation to the plasma membrane when additional transport capability is required. Studies are in progress to determine turnover rates of hENT1 and hENT2 in cells under different growth conditions and to determine more precisely the subcellular location of the hENT1 and hENT2 proteins.

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