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Identification and reconstitution of the nucleoside transporter of CEM human leukemia cells

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The major nucleoside transporter of the human T leukemia cell line CEM has been identified by photoaffinity labeling with the transport inhibitor nitrobenzylmercaptopurine riboside (NBMPR). The photolabeled protein migrates on SDS-PAGE gels as a broad band with a mean apparent molecular weight (75 000 ± 3000) significantly higher than that reported for the nucleoside transporter in human erythrocytes (55 000) (Young et al. (1983) J. Biol. Chem. 258, 2202–2208). However, after treatment with endoglycosidase F to remove carbohydrate, the NBMPR-binding protein in CEM cells migrates as a sharp peak with an apparent molecular weight (47 000 ± 3000) identical to that reported for the deglycosylated protein in human erythrocytes (Kwong et al. (1986) Biochem. J. 240, 349–356). It therefore appears that the difference in the apparent molecular weight of the NBMPR-sensitive nucleoside transporter between the CEM cell line and human erythrocytes is a result of differences in glycosylation. The NBMPR-binding protein from CEM cells has been solubilized with 1% octyl glucoside and reconstituted into phospholipid vesicles by a freeze-thaw sonication technique. Optimal reconstitution of uridine transport activity was achieved using a sonication interval of 5 to 10 s and lipid to protein ratios of 60:1 or greater. Under these conditions transport activity in the reconstituted vesicles was proportional to the protein concentration and was inhibited by NBMPR. Omission of lipid or protein, or substitution of a protein extract prepared from a nucleoside transport deficient mutant of the CEM cell line resulted in vesicles with no uridine transport activity. The initial rate of uridine transport, in the vesicles prepared with CEM protein, was saturable with a K_m of 103 ± 11 μM and was inhibited by adenosine, thymidine and cytidine. The K_m for uridine and the potency of the other nucleosides as inhibitors of uridine transport (adenosine > thymidine > cytidine) were similar to intact cells. Thus, although the nucleoside transporter of CEM cells has a higher molecular weight than the human erythrocyte transporter, it exhibits typical NBMPR-sensitive nucleoside transport activity both in the intact cell and when reconstituted into phospholipid vesicles.

Introduction

At least four distinct carriers are involved in the transport of nucleosides across the plasma membrane of mammalian cells. Two of these are facilitated diffusion mechanisms that can be distinguished on the basis of their 1000-fold difference in sensitivity to the transport inhibitor NBMPR [1]. Both of these carriers have been found in a number of normal and neoplastic cells and have a broad and similar substrate specificity (for reviews see Refs. 2–5). In addition, concentrative, Na^+-dependent nucleoside transport has been described in several normal tissues [6–15] and the mouse leukemia cell line L1210 [16]. It has recently been demonstrated in mouse intestinal epithelial cells [13] that there are two Na^+-dependent nucleoside carriers, neither of which is inhibited by NBMPR. These Na^+-dependent transporters have narrower substrate specificities than the two facilitated diffusion carriers and differ from one another in substrate specificity.

Abbreviations: NBMPR, (nitrobenzylthioinosine) 6-(4-nitrobenzyl)-thio-9-β-D-ribofuranosylpurine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

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The most extensively characterized nucleoside transporter is the NBMPR-sensitive carrier of human erythrocytes. Experiments utilizing [^{3}H]NBMPR as a photoaffinity label have implicated a 55 kDa membrane protein in nucleoside permeation in these cells [17]. The partially purified protein [18], and more recently a highly purified preparation [19], have been reconstituted into phospholipid vesicles and exhibit NBMPR-sensitive nucleoside transport with properties similar to that of the native protein in erythrocytes. Like human erythrocytes, the cultured human leukemia cell line CEM has NBMPR-sensitive nucleoside transport activity [20] with very little ($< 0.5\%$) NBMPR-insensitive nucleoside transport activity (J.A. Belt, unpublished data). In the present study we demonstrate, however, that the molecular weight of the NBMPR-binding protein in CEM cells is significantly higher than that of the erythrocyte transporter. This protein has been solubilized and reconstituted into phospholipid vesicles and exhibits typical NBMPR-sensitive nucleoside transport activity. Portions of this work have been presented in a preliminary report [21].

Materials and Methods

Cells and growth conditions. CCRF-CEM is a human T lymphoblast cell line originally derived from a patient with acute lymphocytic leukemia [22]. The Tub4C line was isolated from a hypoxanthine-guanine phosphoribosyltransferase deficient parent CEM cell line (referred to as CEM). The isolation and selection conditions were identical to those described for the nucleoside transport deficient mutant Tub4D [20]. Like Tub4D, the Tub4C cell line is deficient in nucleoside transport activity and high affinity NBMPR-binding sites (Belt, J.A. and Ullman, B., unpublished data). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated defined calf serum. Stock cultures were kept in a humidified incubator with an atmosphere of 5% CO_2 in air and were maintained as stationary suspension cultures by dilution to a density of $(0.5 \text{ to } 1.0) \cdot 10^5$ cells per ml with fresh medium three times per week. Cells for transport and binding studies and membrane preparations were grown to a density of $5 \cdot 10^5$ cells per ml in roller bottles gassed with 5% CO_2 in air and turned at 1.5 rpm.

Membrane preparation. Membrane fractions were prepared from CEM and Tub4C cells by a modification [23] of the method described by Ross et al. [24]. All steps were performed at 0 to 4° C. Washed cells ($3 \cdot 10^9$) were suspended in 100 ml cavitation buffer (150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 20 mM Hepes, pH 7.4 at 22° C) and disrupted by nitrogen cavitation after equilibration with N_2 at 400 psi for 20 min. The lysate was then centrifuged for 5 min at $1080 \times g$ and the resulting supernatant collected and centrifuged for 20 min at $43000 \times g$. The membrane pellet was resuspended in cavitation buffer at $5 \cdot 10^8$ cell equivalents/ml (8–12 mg protein/ml) and stored at −74° C prior to solubilization.

Photoaffinity labeling. Photoaffinity labeling of membrane proteins with [^{3}H]NBMPR and sample analysis by SDS-PAGE were done as described previously [23]. Briefly, mixtures (0.3 ml) containing 0.7–0.9 mg membrane protein, 50 mM DTT and 100 nM [^{3}H]NBMPR in cavitation buffer were prepared with or without 10 μM NBMPR as a competing nonradioactive ligand. The mixtures were equilibrated at room temperature for 10 min and then cooled to 4° C and transferred to quartz spectrophotometric cuvettes (1 mm light path). Cuvettes were illuminated with a 450 W mercury arc lamp (Conrad Hanovia, Newark, NJ) for 1 min at a distance of 4 cm from the outside of the quartz cooling sleeve (5.5 cm from the lamp). Samples were diluted 60-fold with cavitation buffer containing 1 mM PMSF and 10 μM NBMPR, incubated for 20 min at room temperature and the membranes collected by centrifugation ($40000 \times g$ for 20 min). The membrane pellet was dissolved in 0.2 ml of SDS sample buffer (SDS (2% w/v), glycerol (10% w/v), Bromophenol blue (0.1 mg/ml), 10 mM DTT, 1 mM PMSF and 62.5 mM Tris-HCl (pH 6.8, 22° C)). Portions (40 μl) of the solubilized preparation were subjected to SDS-PAGE in 1.5 mm thick slab gels as described previously [23]. Gels were either cut into 2 mm slices for assay of ^{3}H content or stained with Coomassie blue for comparison with molecular weight standards.

Treatment of photolabeled membranes with endoglycosidase F. After photolabeling and washing, membranes were resuspended (3 mg protein/ml) in 100 mM sodium phosphate buffer (pH 6.1) containing 50 mM EDTA, 75 mM β-mercaptoethanol, 0.5% Triton X-100 and 0.05% SDS. The preparation was divided in half and endoglycosidase F (7 units/ml) added to one portion. The membranes were incubated 19 h at room temperature and then analyzed by SDS-PAGE as described above.

Octyl glucoside solubilization. A mixture containing membranes (5.5 mg protein/ml), 1% octyl glucoside, 150 mM NaCl, 1 mM DTT and 20 mM Hepes (pH 7.4 at 22° C) was stirred for 30 min at 4° C followed by centrifugation at $130000 \times g$ for 60 min. The pellet (octyl glucoside insoluble fraction) was resuspended in a volume of buffer (150 mM NaCl, 1 mM DTT and 20 mM Hepes) equal to that of the solubilization mixture. Both the resuspended pellet and supernatant (octyl glucoside soluble fraction) were dialyzed overnight in the cold against 600 vols. of buffer (20 mM Tris-HCl, pH 7.4 at 22° C and 1 mM DTT). Protein was determined by the method of Lowry [25], as modified by Bensadoun and Weinstein [26], using bovine serum albumin as a standard. The dialyzed octyl glucoside soluble material was stored at −74° C.

NBMPR-binding measurements. Equilibrium [^{3}H]NBMPR binding activity of membranes and octyl glucoside soluble and insoluble fractions was measured at saturating concentrations (10 nM) of [^{3}H]NBMPR in the presence and absence of excess (10 μM) nonradioactive NBMPR. Membranes (10 μg protein) and octyl glucoside soluble (5 μg protein) and insoluble (30 μg protein) fractions were incubated for 30 min at 22° C in 0.2 ml volume containing 10 nM [^{3}H]NBMPR, 150 mM NaCl and 20 mM Hepes (pH 7.4). Assays with membranes and octyl glucoside insoluble material were terminated by addition of 1 ml ice-cold buffer (same as for incubation) and filtration through glass-fiber filters (Whatman GF/C) under suction. Filters were rapidly washed twice with 10 ml volumes of ice-cold buffer, placed in scintillation vials containing 1 ml of 5% Triton and shaken for 30 min prior to liquid scintillation counting in 10 ml of ACS. Assays with octyl glucoside soluble material were terminated by a centrifuged gel filtration method [27]. A 75 μl portion of the incubation mixture was loaded onto a 1 ml G-50 Fine Sephadex column (in a tuberculin syringe) and centrifuged at $100 \times g$ for 2 min in a swinging bucket rotor. Prior to use, the columns had been equilibrated with a buffer containing 150 mM NaCl, 20 mM Hepes (pH 7.4), centrifuged at $100 \times g$ for 2 min and kept on ice. Radioactivity of the column eluant was determined by liquid scintillation counting in 1 ml H_2O plus 10 ml ACS scintillation cocktail.

NBMPR-binding assays for whole cells were initiated by mixing washed cells ($7.5 \cdot 10^5$ cells in a 10 ml final volume) with increasing concentrations (0.15 to 6 nM final) of [^{3}H]NBMPR in the presence and absence of 10 μM nonradioactive NBMPR. After incubation for 30 min at room temperature the assays were terminated by filtration and washing on glass-fiber filters (Whatman CF/A) as described above.

For all preparations specific NBMPR-binding was calculated as the difference between radioactivity in the absence and presence of excess nonradioactive NBMPR. For whole cells the dissociation constant (K_d) for NBMPR-binding and number of binding sites per cell (B_{max}) were determined by the method of Scatchard [28].

Reconstitution of nucleoside transport. The octyl glucoside soluble fraction was reconstituted into vesicles by the method of Kasahara and Hinkle [29] as described previously for the erythrocyte nucleoside transporter [18]. Solubilized protein (0.04–0.16 mg) was added to sonicated phospholipid vesicles (9.6 to 14 mg) in a final volume of 0.4 ml and the mixture frozen rapidly in a solid CO_2/methanol bath. The mixture was allowed to thaw slowly at room temperature and then sonicated for 10 s in a bath type sonicator (Laboratory Supply, Hicksville, N.Y., Model G112–SP1T), unless otherwise stated. Vesicle preparations were kept on ice until assayed for uridine uptake activity.

Uridine uptake by vesicles. Uptake of [^{3}H]uridine by reconstituted vesicles was measured at 22° C using the centrifuged gel-filtration method described above. Uptake was initiated by adding 25 μl of 60 μM [^{3}H]uridine (30 μCi/ml in 10 mM Tris-HCl (pH 7.4)) to 50 μl of vesicles. Uptake was terminated by the rapid addition of 25 μl of ice-cold 40 μM NBMPR in 10 mM Tris-HCl. A 75 μl portion of the reaction mixture was rapidly loaded onto a chilled 1 ml pre-centrifuged Sephadex G-50 column, equilibrated with 10 mM Tris-HCl containing 10 μM NBMPR and centrifuged at $100 \times g$ for 2 min. Radioactivity of the column eluant was determined by liquid scintillation counting in 1 ml H_2O plus 10 ml ACS scintillation cocktail. In those cases where the inhibition of uridine uptake by other nucleosides was examined, the inhibitory nucleoside was added to the [^{3}H]uridine solution before mixing with the vesicles.

Uridine uptake by cells. Uridine uptake by intact cells was determined by an inhibitor-stop method as described previously [30]. In preliminary experiments, it was determined that uridine uptake was linear for at least 3 s over a uridine concentration range from 30 to 600 μM. Thus an assay interval of 3 s was used in subsequent experiments. Uptake values were corrected for the amount of [^{3}H]nucleoside trapped in the extracellular space of the cell pellet and the intracellular volume of cells was determined using ^3H_2O and [^{14}C]sucrose as described previously [1]. Again, when the inhibition of uridine uptake by other nucleosides was evaluated, the inhibitory nucleoside was present in the [^{3}H]uridine solution.

Materials. RPMI 1640 and glutamine were obtained from K.C. Biologicals (Lenexa, KS) and defined calf serum from Hyclone Laboratories (Logan, UT). [5-^{3}H]Uridine, ^3H_2O, [^{14}C]sucrose and endoglycosidase F were obtained from New England Nuclear (Boston, MA) and [^{3}H]NBMPR from Moravek Biochemicals (Brea, CA). ACS (aqueous counting scintillant) was obtained from Amersham (Arlington Heights, IL). NBMPR was prepared from 6-mercaptopurine riboside as described [31]. Other nucleosides and fine chemicals were purchased from Sigma (St. Louis, MO). Soybean phospholipid (asolectin) was obtained from Associated Concentrates (Woodside, NY).

Results

Photoaffinity labeling. Exposure of CEM membrane preparations to ultraviolet light in the presence of [^{3}H]NBMPR under equilibrium binding conditions resulted in substantial labeling of membrane protein. As shown in Fig. 1A, the labeled protein migrated in SDS-

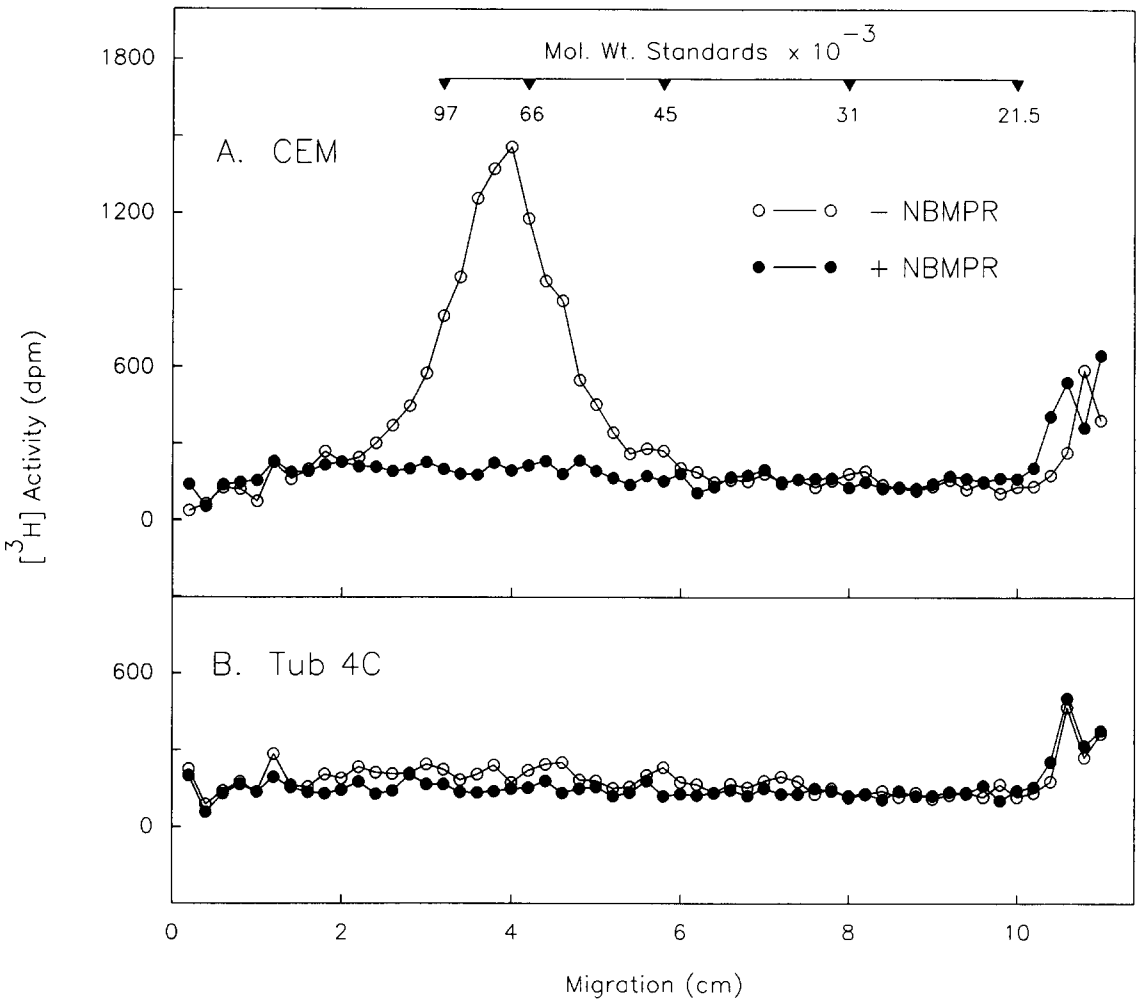

Fig. 1. Photoaffinity labeling of membrane preparations from CEM and Tub4C cells with [^{3}H]NBMPR. Plasma membrane preparations of CEM (A) and Tub4C cells (B) were labeled with [^{3}H]NBMPR in the presence and absence of 10 μM nonradioactive NBMPR and analyzed by SDS-PAGE as described in Materials and Methods. ^{3}H profiles for CEM (0.23 mg protein) and Tub4C (0.19 mg protein) membranes and the position of molecular weight standards are from the same gel.

polyacrylamide gels as a single broad band with an average apparent molecular weight of 75 000 ± 3000 (mean ± S.D. of four experiments). Radiolabeling of membrane protein was abolished when photoactivation was performed in the presence of 10 μM nonradioactive NBMPR. An identical photolabeling experiment (Fig. 1B) was performed with membranes from the Tub4C mutant which is devoid of both nucleoside transport and NBMPR binding activities (Belt, J.A. and Ullman, B., unpublished data). The broad peak of radiolabeled protein seen with CEM membranes was absent in the Tub4C membrane preparation. The small degree of radiolabeling was similar in the presence and absence of 10 μM nonradioactive NBMPR and represented nonspecific radiolabeling. These results strongly suggest that the 75 kDa peak of radiolabeled protein on electrophoretograms of CEM membrane preparations represents the covalent labeling of the plasma membrane nucleoside transporter. Treatment of NBMPR-labeled CEM membranes with endoglycosidase F (Fig. 2) converted the broad band to a sharp peak with an apparent molecular weight of 47 000 ± 3000 (mean ± S.D. of three experiments).

Solubilization of the nucleoside transporter. The nucleoside transporter was solubilized from CEM membranes using the nonionic detergent octyl glucoside. Extraction and recovery of the transporter was monitored by equilibrium binding of [^{3}H]NBMPR. As observed by others [18], no binding of NBMPR to the membranes could be detected in the presence of the detergent and thus all fractions were dialyzed to remove the octyl glucoside before determining the NBMPR-binding activity. In five experiments 91 ± 14% of the initial NBMPR-binding activity of the membranes was recovered in the soluble fraction with a 3–6-fold increase in the specific activity (8.5 to 17 pmol/mg protein).

Uridine uptake by reconstituted vesicles. Fig. 3 shows a time-course of uridine uptake by vesicles reconstituted with the dialyzed octyl glucoside soluble fraction and

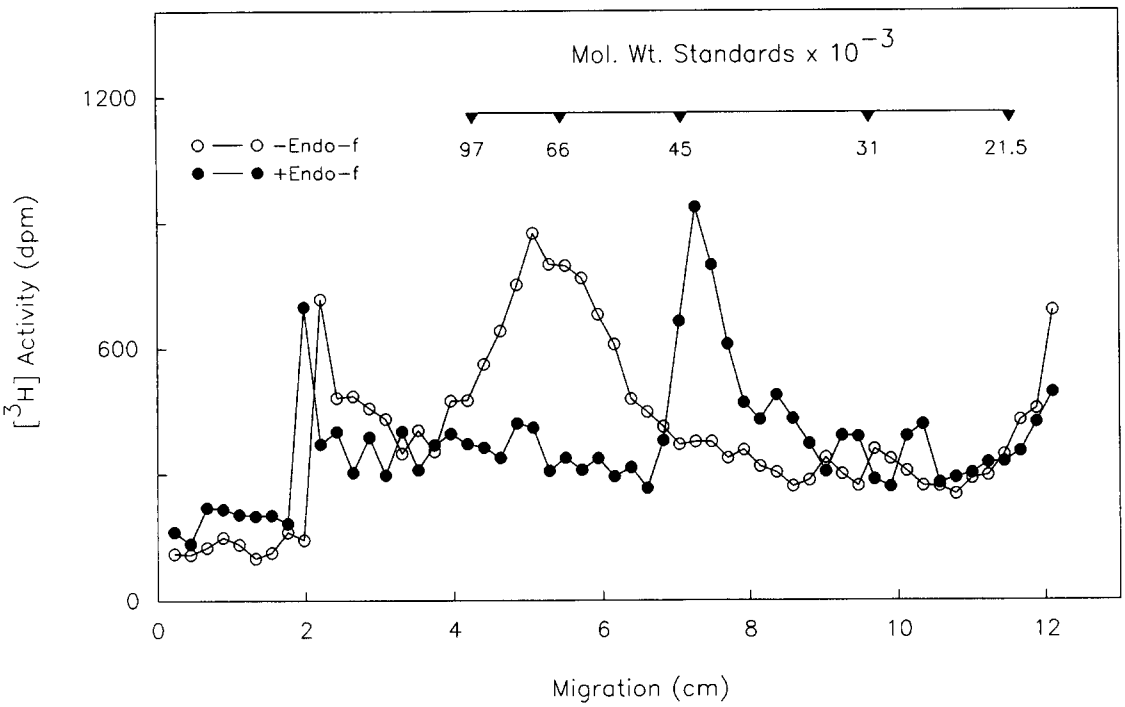

Fig. 2. Effect of endoglycosidase F on the apparent molecular weight of the NBMPR-binding protein. CEM membranes were labeled with [^{3}H]NBMPR and then incubated with or without endoglycosidase F for 19 h as described in Materials and Methods. Labeled membranes were then analyzed by SDS-PAGE as in Fig. 1.

soybean phospholipids. There was a rapid, initial rate of uridine uptake into vesicles that was inhibited by NBMPR. The slow uptake of uridine by the vesicles in the presence of NBMPR can be attributed to simple diffusion of the nucleoside across the lipid bilayer. Evidence to support this is shown in Table I. The rate of uridine uptake in the presence of NBMPR by vesicles reconstituted with the CEM membrane extract was similar to the rate of uridine uptake (±NBMPR) by vesicles prepared without membrane extract (i.e., liposomes), or vesicles made with membrane extracts from the nucleoside transport deficient mutant Tub4C. It is also shown in Table I that the membrane extract from CEM cells had insufficient endogenous phospholipid for reconstitution of uridine transport activity. Thus, reconstitution of uridine transport activity required the addition of both exogenous phospholipid and an active protein extract, and transport activity can be defined as the

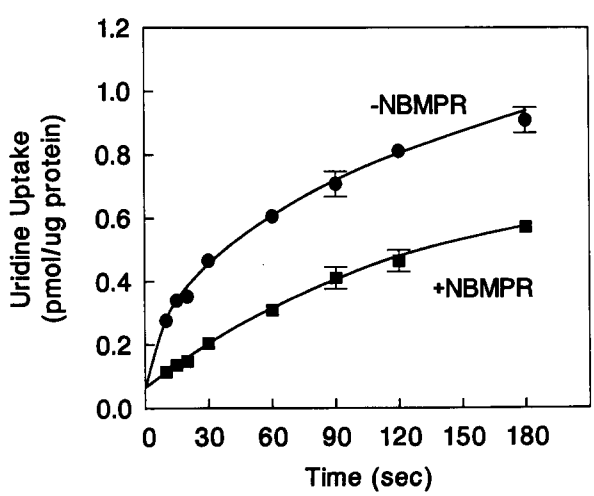

Fig. 3. Time-course of uridine uptake into reconstituted vesicles. The uptake of 20 μM [^{3}H]uridine (10 μCi/ml) in the absence and presence of 10 μM NBMPR was measured at 22°C as described in Materials and Methods. The reconstituted preparation contained 24 mg phospholipid/ml and 0.4 mg protein/ml. Values are mean±S.D. of triplicate determinations.

TABLE I

Reconstitution of membrane extracts from CEM and Tub4C cells in liposomes

[^{3}H]Uridine (20 μM, 10 μCi/ml) uptake was measured for 10 s intervals at 22°C as described in Materials and Methods. The reconstituted vesicles contained 24 mg/ml phospholipid and 0.4 mg/ml extract protein (CEM or Tub4C). Controls without exogenous lipid or without membrane extract (liposomes) were subjected to the same freeze-thaw and sonication steps as the reconstituted vesicles. Values are the mean±S.D. for two experiments performed with different membrane extracts. Δ is the difference in uridine uptake in the absence and presence of 10 μM NBMPR.

Membrane extract	Uridine uptake (pmol/10 s)		Δ
	−NBMPR	+NBMPR	
CEM	3.0±0.3	1.1±0.2	1.9±0.3
CEM without exogenous lipid	0	0	0
Tub4C	1.0±0.1	1.0±0.1	0
None (liposomes)	1.2±0.1	1.1±0.1	0

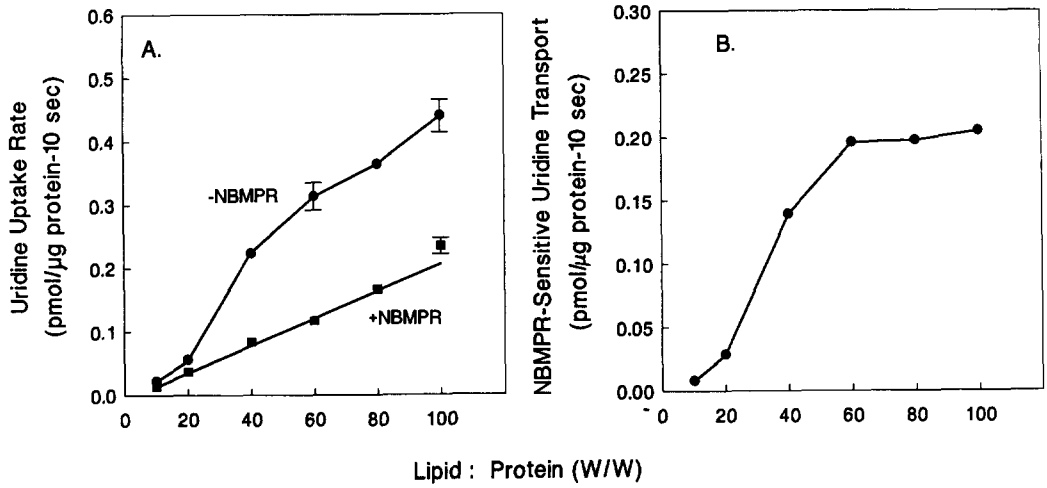

Fig. 4. Effect of exogenous phospholipid concentration on reconstitution of uridine transport activity. Membrane extract was reconstituted into vesicles at a constant protein concentration (0.35 mg/ml) and increasing concentrations of sonicated phospholipid (3.5 to 35 mg/ml) resulting in lipid to protein ratios of 10 to 100:1. The uptake of 20 μM [^{3}H]uridine (10 μCi/ml) in the absence and presence of 10 μM NBMPR was measured at 22°C as described in Materials and Methods. Values are mean ± S.D. of triplicate determinations. Panel B shows the rate of NBMPR-sensitive uridine transport as a function of phospholipid : protein ratio.

difference in uridine uptake by the vesicles in the absence and presence of NBMPR.

Effects of sonication time and lipid to protein ratios on reconstitution of nucleoside transport activity. Two important parameters affecting the extent to which nucleoside transport activity was reconstituted by the freeze-thaw sonication method were the duration of sonication after the freeze-thaw step and the lipid to protein ratio during reconstitution. Uridine uptake was determined with batches of vesicles sonicated for durations of 0 to 60 s after the freeze-thaw step. The sonication time had little effect on the diffusion of uridine into the vesicles (+NBMPR), but did have a significant effect on the NBMPR-sensitive transport of uridine into the vesicles. Sonication times of 5 to 10 s resulted in optimal reconstitution of nucleoside transport activity (data not shown). Since a sonication time of 10 s gave the most reproducible results, vesicles were sonicated for 10 s in all subsequent experiments.

The lipid to protein ratio during reconstitution also had a significant effect on the degree to which transport activity was reconstituted. Batches of vesicles prepared with a constant protein concentration (0.35 mg/ml) and final lipid to protein ratios from 10:1 to 100:1 were assayed for uridine uptake (Fig. 4). As expected, the diffusional component of uridine uptake (+NBMPR) increased in a linear fashion with increasing lipid to protein ratios, i.e., increasing amounts of total vesicles in the assay. The NBMPR-sensitive transport of uridine by the vesicles, however, was clearly saturable with increasing lipid (Fig. 4B). Maximal reconstitution of transport activity was achieved at lipid to protein ratios of 60:1 or greater. As shown in Fig. 5, when optimal conditions were used, the rate of uridine transport by the reconstituted vesicles was a linear function of the protein concentration during reconstitution.

Comparison of uridine transport by reconstituted vesicles and CEM cells. Uridine transport in both reconstituted vesicles and intact cells exhibited typical Michaelis-Menten kinetics with similar K_m values (Fig. 6). In five experiments with intact cells and three experiments with vesicles reconstituted with different membrane extracts the apparent K_m values were (mean ± S.D.) 107 ± 9 μM and 103 ± 11 μM, respectively. To compare the efficiency of the reconstituted transporter to that of intact cells the turnover number was calculated for each preparation from the ratio of the V_{max} for uridine transport to the maximum number of NBMPR

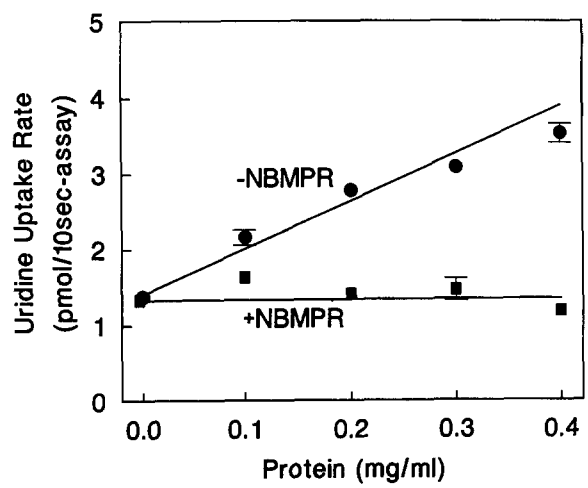

Fig. 5. Relationship between protein concentration and uridine transport in vesicles. Membrane extract was reconstituted into vesicles at varying concentrations (0 to 0.4 mg protein/ml) at a constant concentration of sonicated phospholipid (24 mg/ml) resulting in lipid : protein ratios of 60:1 or greater. The uptake of 20 μM [^{3}H]uridine (10 μCi/ml) in the absence and presence of 10 μM NBMPR was measured at 22°C as described in Materials and Methods. Values are mean ± S.D. of triplicate determinations.

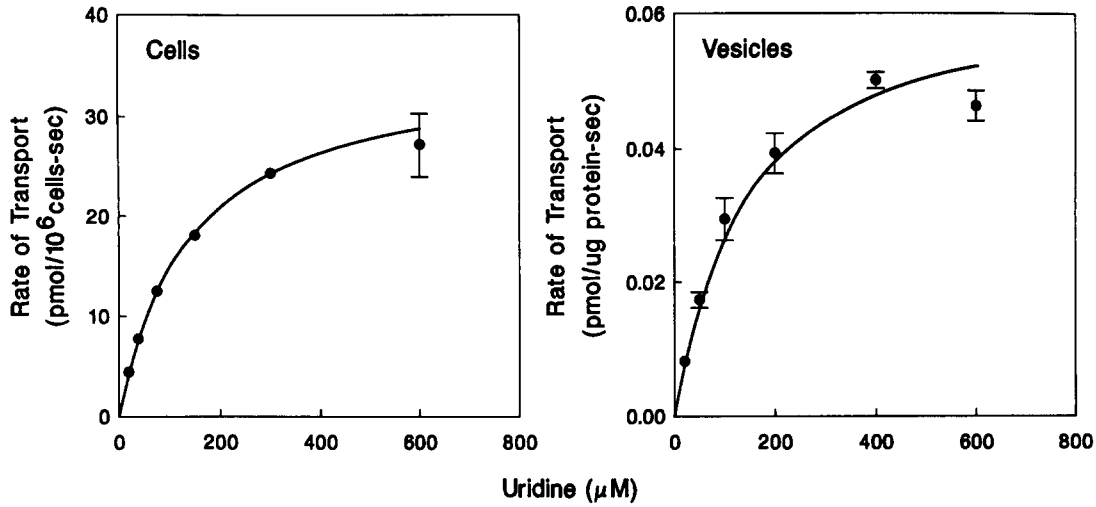

Fig. 6. Kinetics of uridine transport in CEM cells and vesicles. The uptake by cells of graded concentrations of [^{3}H]uridine (2.5 μCi/ml) was measured for 3-s intervals as described in Materials and Methods. The reconstituted vesicle preparation contained 24 mg phospholipid/ml and 0.4 mg protein/ml. Uptake by vesicles of graded concentrations of [^{3}H]uridine (10 μCi/ml) was measured for 10-s intervals in the absence and presence of 10 μM NBMPR as described in Materials and Methods. Transport rates for vesicles were calculated from the difference between uptake in the absence and presence of NBMPR. Transport rates for both cells and vesicles are mean $\pm$ S.D. of triplicate determinations. The apparent K_m and V_{max} values were determined by a direct computer fit of the nonlinear rate equation as described by Cleland [38].

binding sites (B_{max}), assuming that each transporter molecule has a single NBMPR binding site. The V_{max} and B_{max} (mean $\pm$ S.D.) for intact cells were 33 ± 4 pmol/10^6 cells per s ($n = 5$) and $2.8 \pm 0.4 \cdot 10^5$ NBMPR-binding sites/cell ($n = 3$), respectively, for a turnover number of approx. 70 molecules/transporter per s. In contrast, the corresponding values in the reconstituted vesicles were 40 ± 10 pmol/mg protein per s ($n = 3$) and 14 ± 4 pmol of NBMPR-binding sites/mg protein ($n = 3$), respectively, for a turnover number of approx. 3 molecules/transporter per s. Thus, the efficiency of the reconstituted transporter is only about 4% of that of the protein in the native membrane of the cell. Low levels of efficiency have also been reported for the reconstituted glucose [29] and nucleoside [18] transporters from human erythrocytes, and it has been suggested that the decreased efficiency of the reconstituted transporters is a result of incomplete incorporation of proteins into the lipid bilayer or a reflection of changing the lipid environment of the proteins.

Inhibition of uridine transport by other nucleosides in reconstituted vesicles and CEM cells. As shown in Fig. 7, the transport of [^{3}H]uridine in intact cells and reconstituted vesicles was inhibited in a similar manner by the nucleosides tested, with the order of potency being adenosine > thymidine > cytidine in both cases. In three

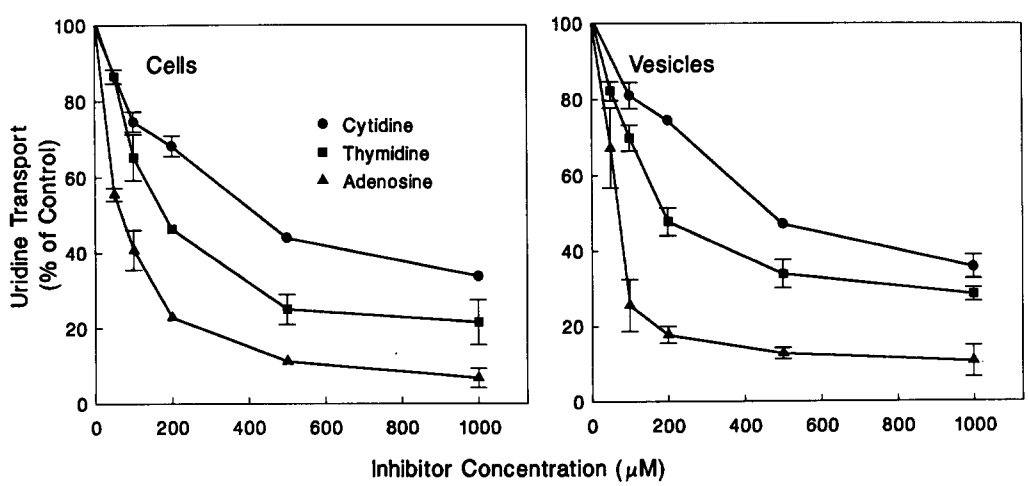

Fig. 7. Nucleoside inhibition of uridine transport in cells and reconstituted vesicles. Rates of 40 μM [^{3}H]uridine transport by cells and vesicles were determined in the absence (control) and presence of cytidine, thymidine and adenosine as described in Materials and Methods. The data plotted are means $\pm$ S.D. of triplicate determinations and are representative of experiments repeated two or more times.

experiments with vesicles the IC_{50} values for adenosine, thymidine and cytidine were 80, 190 and 470 μM, respectively. In two experiments with CEM cells the corresponding IC_{50} values were 70, 180 and 430 μM, respectively.

Discussion

[^{3}H]NBMPR has been utilized as a photoaffinity label to identify the NBMPR-sensitive nucleoside transporter of CEM cells as a 75 kDa glycoprotein. The apparent molecular weight of the transporter in CEM cells is significantly higher than that for the corresponding protein in human erythrocytes [17] and most other cell types and tissues [5]. It is, however, similar to that of the NBMPR-binding protein in the Novikoff UA rat hepatoma [32]. The NBMPR-binding protein in Novikoff UA cells is also unusual because no NBMPR-sensitive nucleoside transport can be detected in the cells. It has been speculated that the high molecular weight of the NBMPR-binding protein in Novikoff UA cells may be related to the failure of NBMPR to inhibit nucleoside transport in these cells. The number of NBMPR-binding sites in Novikoff UA cells is low ($0.4 \cdot 10^5$ sites/cell) and others [33] have suggested that there is a low level of NBMPR-sensitive transport in the cells not detected in the previous studies [32]. However, a clone of Novikoff UA has been isolated that has $1.3 \cdot 10^5$ NBMPR-binding sites per cell, but still no detectable NBMPR-sensitive nucleoside transport [34]. Thus, the NBMPR-binding protein in Novikoff UA cells must still be considered unusual. In contrast, the high-molecular-weight NBMPR-binding protein in CEM cells appears to have normal function, since nucleoside transport in these cells is almost completely inhibited by NBMPR (Belt, J.A. and Ullman, B., unpublished data) and nucleoside transport deficient mutants of CEM are also devoid of NBMPR-binding sites [20]. The high molecular weight of the NBMPR-binding protein in CEM cells appears to be due to a high degree of glycosylation, since the apparent molecular weight of the endoglycosidase F treated CEM protein is identical to that of the deglycosylated erythrocyte protein [35]. It is not known whether the high molecular weight of the Novikoff UA protein is due to glycosylation (Gati, W.P. and Paterson, A.R.P., personal communication).

Unlike most cultured cell lines which have both NBMPR-sensitive and NBMPR-insensitive nucleoside transport [1,36], the CEM cell line has very little of the latter activity (Belt, J.A. and Ullman, B., unpublished data). This has allowed the reconstitution of the NBMPR-sensitive transporter without significant interference from NBMPR-insensitive transport activity. An additional advantage of using the CEM cell line has been the availability of the nucleoside transport deficient cell line Tub4C. For the reconstitution studies proteins were solubilized from membrane preparations of each cell line with the nonionic detergent octyl glucoside. On the basis of NBMPR-binding activity, a 3–6-fold enrichment of the transporter was typically achieved in the soluble fraction from CEM cells. Tse et al. [18] have previously reported a similar selective solubilization of the NBMPR-sensitive transporter of human erythrocytes utilizing Triton X-100. Initially, a detergent dialysis method [37] was utilized in attempts to reconstitute the solubilized CEM protein, but without success (data not shown). However, the freeze-thaw sonication method of Kasahara and Hinkle [29], used previously to reconstitute the erythrocyte nucleoside transporter [18], proved to be a rapid and reproducible means to reconstitute the uridine transport activity of CEM cells. Vesicles prepared by this method possessed a significant NBMPR-sensitive uridine uptake component which can be attributed to carrier-mediated transport activity. Reconstitution of transport activity into vesicles required the addition of both exogenous phospholipid and a functional transport protein, since vesicles prepared without protein or with the soluble membrane fraction from Tub4C cells had no uridine transport activity.

A number of experiments have been performed to compare the NBMPR-sensitive uridine transport of reconstituted vesicles to that of intact cells. The uridine transport activity of the vesicles was saturable with an apparent K_m similar to that of intact cells. Similarly, the IC_{50} values for adenosine, thymidine and cytidine inhibition of uridine transport were approximately the same for reconstituted vesicles and intact cells.

In summary, although the nucleoside transporter from CEM cells has an unusually high molecular weight, it exhibits typical NBMPR-sensitive nucleoside transport activity in intact cells and when reconstituted into lipid vesicles. Reconstitution of the transporter into phospholipid vesicles provides a potentially useful approach to its further purification and characterization. This approach may also be of value in examining the proteins responsible for NBMPR-insensitive and Na^+-dependent nucleoside transport.

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