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Stable Polarized Expression of hCAT-1 in an Epithelial Cell Line

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Abstract. Our laboratory has recently identified and cloned three cationic amino-acid transporters of human placenta. We have now examined the plasma membrane domain localization and functional expression of one of these transporters, hCAT-1, in a polarized epithelial cell line (MDCK). To facilitate identification of expressed protein we first transferred the hCAT-1 cDNA to a vector with C-terminal green fluorescent protein (GFP). The resultant hCAT-1-CT-GFP fusion protein stimulated L-[^{3}H] lysine uptake in *Xenopus* oocytes. In confluent monolayers of stably transfected cells grown on porous nitrocellulose filters, saturable uptake of L-[^{3}H] lysine from the basolateral surface was stimulated 7-fold over that of untransfected cells. Concentration-dependence studies in Na^+-free medium at pH 7.4 demonstrated a K_m of approximately 68 ± 13 μM and a V_{max} of 970 ± 170 pmol/mg protein/min. Uptake from the apical plasma membrane surface was negligible in both transfected and untransfected cells. Consistent with these results, confocal microscopy of confluent monolayers of hCAT-1-CT-GFP-expressing cells revealed localization of the transporter solely on the basolateral domain of the cell. This is apparently the first report of a cultured polarized epithelial cell model for stable expression of a cationic amino-acid transporter. It has the potential to aid in the identification of targeting signals for transport protein localization.

Key words: Membrane localization — Cationic amino-acid transport — System y^+-polytopic protein — Green fluorescent protein (GFP)

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Introduction

Mammalian tissues express four cDNAs that code for proteins mediating high-capacity system y^+ cationic amino-acid transport, the CATs, numbered 1 through 4. Our laboratory recently demonstrated the expression of mRNAs for three of these cationic transporters in trophoblast cells directly cultured from human placenta, namely hCAT-1, hCAT-2B, and hCAT-4 (Kamath et al., 1999). The presence of multiple hCAT transporters gives polarized epithelia, such as the placental syncytiotrophoblast, the ability to regulate interaction with substrates differently at the two surfaces. In the placenta these fourteen transmembrane domain proteins play an essential role in the maternal to fetal transfer of cationic amino acids.

We have shown that human placental microvillous (apical) and basal membranes have different transport mechanisms for the uptake of cationic amino acids (Furesz et al., 1995). The system y^+ transporters of the two membranes respond differently to inhibition of cationic amino-acid transport by neutral amino acids, suggesting a polarized distribution of the hCAT transporters with different functional properties.

As a first step towards addressing the goal of determining the actual distribution of these transporter isoforms within the polarized syncytiotrophoblast monolayer we have utilized the highly polarized MDCK epithelial cell line to study the localization of hCAT-1. This well characterized epithelial cell line has proved to be an excellent model system for determining the localization and sorting of a multitude of exogenous, single transmembrane (monotopic) proteins. More recently the localization, sorting and functional characteristics of multiple membrane-spanning (polytopic) membrane-transport proteins have also been investigated in this cell line

(Pascoe et al., 1996; Perego et al., 1997, Poyatos et al., 2000; Sweet, Miller & Pritchard, 2000; Sun et al., 2000). It has an advantage over placental cell lines in determining functional polarization, as it readily forms very tight monolayers.

Specific antibodies to the various CAT transporters that work well in immunofluorescence and western blotting procedures have been difficult to prepare. Therefore we chose to approach the question of localization of transfected hCAT-1 within the epithelial cell plasma membrane domain by tagging the C-terminus with GFP. The reports that mCAT-1-GFP retains its function as a murine leukemia-virus (MLV) receptor confirmed the validity of this approach. The fusion protein permitted the observation of the receptor function of CAT-1-GFP in living cells and facilitated the isolation of stably transfected cell clones (Lee, Zhao & Anderson, 1999; Masuda et al., 1999; Lu & Silver, 2000).

We report here that in stably transfected MDCK cells grown on porous polycarbonate filters, hCAT-1-CT-GFP is localized to the basolateral surface. The transporter is fully functional and stimulates lysine uptake 7-fold more from this plasma membrane domain compared to the basolateral membrane domain of untransfected cells.

Materials & Methods

Materials

Restriction enzymes were from Promega (Madison, WI); Qiaex II gel extraction kit and QIA filter™ Plasmid Maxi Kit were from Qiagen (Valencia, CA), GeneAmp RNA PCR (polymerase chain reaction) kit and ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, from Perkin Elmer (Foster City, CA). pcDNA3.1(+) and CT-GFP Fusion TOPO™ Cloning Kit containing pcDNA3.1/CT-GFP-TOPO were from Invitrogen (Carlsbad, CA). L-[^{3}H]lysine (specific activity 81Ci/mmol) was obtained from NEN-DuPont (Boston, MA). Opti-Fluor was from Packard Instruments (Downers Grove, IL). MDCK cells (strain II) were obtained from ATCC (Manassas, VA). Chemically defined fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Geneticin (G418) and Lipofectamine Plus™ were obtained from Invitrogen/Gibco, (Grand Island, NY). Penicillin, streptomycin, and L-glutamine were from the Washington University Tissue Culture Support Center. Costar Transwell filters (pore size 0.4 μM) were from Corning (Corning, NY). Protease inhibitor cocktail tablets, Complete™, were from Boehringer Mannheim (Indianapolis, IN). Trizma base, sodium chloride, ethylenediamine tetra-acetic acid (EDTA), magnesium chloride, sucrose, bovine serum albumin (BSA), glycerol, lysine, and NP-40 were reagent grade from Sigma (St. Louis, MO). Acrylamide, sodium dodecylsulphate (SDS), bromophenol blue and prestained molecular weight markers were electrophoresis grade from BioRad (Hercules, CA). Hybond C nitrocellulose was from Amersham Pharmacia Biotech (Piscataway, NJ). Polyclonal rabbit antibody raised against recombinant GFP was from Invitrogen (Carlsbad, CA); rhodamine-conjugated goat anti-rabbit antibody from Jackson ImmunoResearch Laboratories (Westgrove, PA) and alkaline phosphatase-conjugated goat anti-rabbit IgG from CALBIOCHEM® (San Diego, CA). Alkaline phosphatase colorimetric substrate, nitroblue tetrazolium/5-bromo-4-chloro3-inodolyl phosphate (NBT/BCIP) was from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

hCAT-1-CT-GFP Vector

hCAT-1-cDNA in the pGEM-T Easy vector (Kamath et al., 1999) was excised with *Not*I and purified with QIAEX II gel extraction kit as described in Kamath et al., 1999. PCR was run according to kit directions using this purified cDNA, the forward primer: nt 117–136 (TTCCCGTCATATTCCAGCTC) and reverse primer (5′T) 2045–2016 (TCTTGCACTGGTCCAAGTTG) using conditions for 3-temperature PCR described previously (Kamath et al., 1999). This hCAT-1 cDNA of nt 117–2034 (A) (nt positions based on hCAT-1 sequence reported by Yoshimoto, Yoshimoto & Meruelo 1991) starts 31 nt upstream from the initial methionine, and ends at the last translated nt, with an extra base, A, to place the cDNA in the same reading frame as the vector's GFP sequence. hCAT-1 cDNA (117–2034 (A)) was inserted into the pcDNA3.1/CT-GFP-TOPO vector and used to transform *E. coli* cells as described by the vector manufacturer. Colonies were grown and clones of plasmid DNA were isolated as described previously (Kamath et al., 1999). *Sac*I restriction enzyme digest of plasmid DNA was used to identify clones with hCAT-1 in the correct orientation. Appropriate clones were grown and purified as described by QIAfilter™ Plasmid Maxi Kit. Purified clones were sequenced using ABI Prism™ BigDye™ Terminator kit essentially as described previously (Kamath et al., 1999).

hCAT-1-CT-GFP was linearized by *Sph*I digestion and then used as described by Kamath et al., 1999 to study lysine transport in *Xenopus* oocytes injected with hCAT-1-CT-GFP cRNA.

An hCAT-1 vector without GFP was constructed by inserting the *Not*I-excised hCAT-1 into pcDNA3.1(+). Plasmids with hCAT-1 inserted in the correct orientation were isolated as described above. The correct insertion of the vector was confirmed by sequencing across both insertion sites.

Cell Culture and Transfection

MDCK cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin G in 5% CO_2. Transfection of MDCK cells was performed using lipofectamine plus reagent following the manufacturer's instructions. Stable cell lines were obtained by G418 screening (600 μg/ml), and positive clones were selected by GFP fluorescence using an Olympus IMT-2 with a reflected light fluorescent attachment. Transient transfections, carried out on 100 mm dishes, were allowed to grow for 2 days post-transfection before trypsinization and plating on filters for an additional 4 days. Mock-transfected MDCK cells, stable and transient transfectants were seeded at 1.5×10^6 cells/24-mm filter and grown for 4 days to allow the development of a tight monolayer.

Immunofluorescence

Subcellular localization of hCAT-1-CT-GFP fluorescence was observed in living cells by confocal microscopy. Transfected MDCK cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and visualized directly with an upright Zeiss microscope equipped with a 63× water objective and a BioRad MRC 1024 confocal adaptor. A krypton-argon laser was used with an epifluorescence filter set designed for fluorescein. Stacks comprising 10–12 images, at planes separated by 0.5 μM, were projected together as a single image using BioRad Confocal Assistant Software Version 4.02.

For indirect immunofluorescence visualization, cells were fixed with ice-cold, freshly depolymerized 3% paraformaldehyde in PBS, pH 7.4, for 15 min at 4°C. After quenching with 50 mM NH_4Cl in PBS containing 1% (BSA) for 15 minutes, the cells were permeabilized with 0.1% Triton X-100 in PBS-1% BSA for 15 min at room temperature (RT). The cells were then incubated with a polyclonal rabbit antibody against recombinant GFP for primary detection of hCAT-1-CT-GFP (1:500 dilution in PBS-1% BSA for 1 hr at RT), washed 3 times with PBS, and then incubated with rhodamine-conjugated goat anti-rabbit secondary antibodies (1:250 in PBS-1% BSA for 30 min at RT). After final washing the cells were mounted and visualized with the 40× oil objective in the rhodamine and fluorescein channel.

Preparation of a Total-Membrane Fraction from MDCK Cells

A composite membrane fraction was prepared by differential centrifugation of total-cell homogenates from untransfected and hCAT-1-CT-GFP-transfected MDCK cells (Chuang & Sung, 1998). Briefly, the cells were washed and scraped from 3 × 15 cm dishes in an ice-cold buffered medium (in mM: 50 Tris, pH 7.4, 150 NaCl, and 2 EDTA) and centrifuged for 5 min at 1000 × g. Cells were resuspended in isotonic buffered medium (in 10 mM: 10 Tris, pH 7.2, 0.2 $MgCl_2$, 250 sucrose, 1 EDTA, plus protease inhibitors), sheared with 5 passes through a 22-gauge needle and then homogenized with 20 strokes of a motor-driven teflon pestle. The cell homogenates were centrifuged at 3,000 × g for 10 min, to remove the nuclei and unbroken cells. The supernatant was centrifuged at 100,000 × g for 45 min, and the pellets were resuspended in choline buffer (*see* transport assay) and stored at −20°C.

Western Blotting

Membrane protein (38 μg) was solubilized in 4× concentrated electrophoresis sample buffer (to give a final 1× concentration of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue) and subjected to electrophoresis using 10% acrylamide mini gels (Laemmli, 1970). Proteins were transferred from gel to nitrocellulose and immunoblotted as described in Tollefsen et al., 1990. A polyclonal rabbit antibody raised against recombinant GFP was used at a 1:2000 dilution. Following a detergent wash, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG at a 1:2500 dilution. Color was developed for 10 min with the alkaline phosphatase colorimetric substrate, NBT/BCIP.

Transport Assays

Prior to measuring transport of [^{3}H]lysine by monolayers of filter-grown cells, the cells were washed twice and then starved for an hour in Na^+-free, choline buffer (in 10 mM: 10 Hepes, 4.2 KCl, 1.2 $MgSO_4$, 5.0 glucose, 120 choline chloride, 10 choline phosphate, 0.75 $CaCl_2$, pH 7.2 with Tris base). Uptake of [^{3}H]lysine (2 μCi/ml) in transport solution (choline buffer + 1 μM cold lysine) was measured in triplicate at 37°C by the addition of 2 ml of [^{3}H]lysine solution to the basolateral or apical surface of the filters. Saturable [^{3}H]lysine uptake was determined by subtracting the uptake in the presence of 20 mM non-radioactive lysine. In all experiments the medium in the chamber not containing radioactivity contained 2 ml of transport solution alone. After incubation for the specified period of time, the filters were rapidly washed, excised, and solubilized in 1% NP-40, 20 mM Tris pH 7.4, for 1 hr. The solubilized cells were then vortexed vigorously and aliquots were assayed for radioactivity in a liquid scintillation counter with 5 ml of Optifluor, and for protein by the modified Lowry assay (Markwell et al., 1978). The results are reported in pmoles per mg protein per time. Kinetic and statistical analyses were performed exactly as described in Kamath et al., 1999.

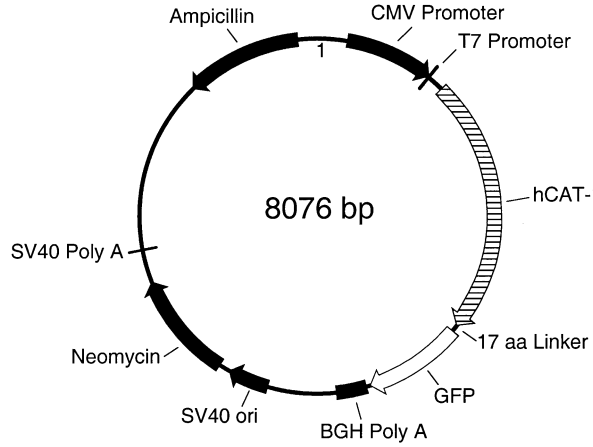

Fig 1. hCAT-l-CT-GFP vector map. Schematic map of the vector used in the stable transfection of MDCK cells, which summarizes the features used for the expression of hCAT-1-CT-GFP fusion protein.

Results

We tagged the hCAT-1 cDNA with GFP on its C terminus to observe its localization within the cell. cRNA expressed from the vector shown in Fig. 1 was injected into *Xenopus* oocytes and stimulated lysine uptake (*data not shown*). This confirmed the expression of functional transport activity by the fusion protein. MDCK cells were transfected with the hCAT-1-CT-GFP vector using the lipofectin method. A CMV promoter in the vector allowed constitutive expression of the fusion protein while a neomycin-resistance gene allowed selection of transfected MDCK colonies with G418 (Fig. 1). Three large hCAT-1-CT-GFP-expressing colonies with identical morphology were isolated after 12 days of selection in 600 μg/ml of G418 selection, pooled, and used for all further studies.

Expression of GFP-Tagged Protein of the Expected Molecular Weight

hCAT-1 has a predicted molecular weight of approximately 68 kDa with two putative N-glycosylation sites (reviewed in Devés and Boyd, 1998). To show that the hCAT-1-CT-GFP-expressing cells produced a protein of the expected molecular weight, we isolated total cellular membrane from untransfected and transfected cells. Equal amounts of protein were subjected to 10% SDS-PAGE and transferred to nitrocellulose. Immunoblotting was carried out with a polyclonal antibody against GFP. The band of

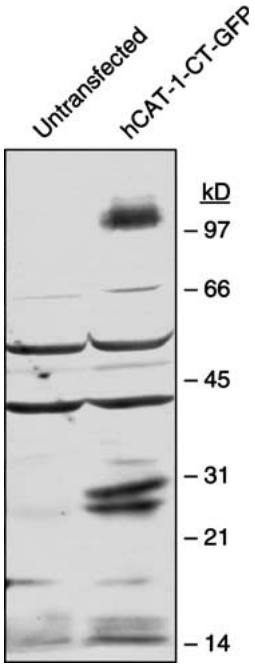

Fig 2. Immunoblot of hCAT-1-CT-GFP in transfected MDCK cells. Total cellular membranes were isolated from transfected and untransfected MDCK cells. 38 μg protein from each was loaded onto a 10% polyacrylamide gel for SDS-PAGE followed by western transfer to nitrocellulose. Immunoblotting for GFP revealed a unique antigen of ~97 kD only in transfected cells that corresponds to the predicted molecular weight of the fusion protein (hCAT-1, 68 kD, plus GFP with linker, 29 kD).

approximately 97 kD was observed only in membranes from transfected cells, and corresponded to the expected molecular weight of the full-length hCAT-1-CT-GFP fusion protein (Fig. 2). The GFP band also observed at approximately 27 kD in membranes from transfected cells is likely to represent a degradation product of the tagged protein. Upon treatment of the cells with 10 mM sodium butyrate for 20 hr (known to increase the expression of proteins under the control of CMV promoters), the 27 kD and 97 kD bands were the only two immunoreactive bands that showed increased expression (*data not shown*). The remaining bands, which are identical in the two lanes, can be substantially reduced in intensity by shortening the enzyme-substrate development time.

Basolateral-Specific Expression of hCAT-1-CT-GFP in MDCK Cells: Fluorescence Confocal Microscopy

The cellular distribution of hCAT-1-CT-GFP was determined by confocal microscopy of the transfected cell line. A confluent monolayer of cells grown on a plastic dish was washed twice with PBS and viewed directly. The cells in horizontal (XY) cross section images were obtained by confocal laser analysis. The

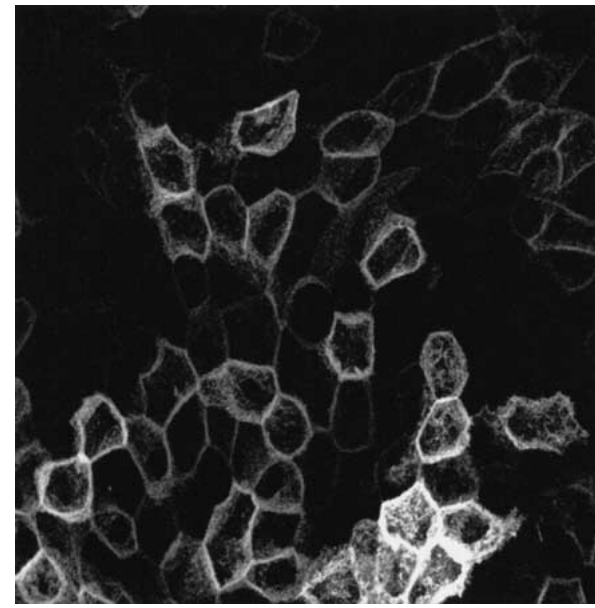

Fig 3. Cellular localization of hCAT-1-CT-GFP. Stably transfected MDCK cells were grown to confluency on a plastic dish. A series of 10 confocal images (every 0.5 μm were taken from basal to the apical surface and then projected as a cumulative image. hCAT-1-CT-GFP localizes predominantly to the basolateral domain as evidenced by the hollow cup-shaped fluorescence pattern. No significant apical fluorescence is detectable in these cells.

cumulative projected view of these series of images of hCAT-1 cells is shown in Fig. 3. A classic "chicken-wire" basolateral pattern of expression was observed with no punctate apical (microvillar) staining at any elevation.

A similar pattern was observed using indirect immunofluorescence with a polyclonal anti-GFP antibody and a rhodamine-labeled secondary antibody with paraformaldehyde-fixed, permeabilized cells (*data not shown*). No signal was observed from non-permeabilized transfected cells in the rhodamine channel, although GFP fluorescence was observed in the same cells. These results are consistent with the cytoplasmic location of C-terminal-tagged GFP.

Basolateral Specific Expression of hCAT-1-CT-GFP in MDCK Cells: Transport Measurement

To measure transport by hCAT-1-CT-GFP, transfected and untransfected cells were plated at 1.5-fold confluency and grown for 4 days on porous polycarbonate filters. A time course of lysine uptake established a one-minute incubation as appropriate to reflect the initial rate of uptake by the cell lines (Fig. 4). We then examined the uptake of lysine individually from the apical or basolateral surfaces of transfected and untransfected cells. Saturable lysine uptake was predominantly from the basolateral surface, in keeping with a previous report (Sepulveda & Pearson, 1985). Saturable apical uptake in transfected and

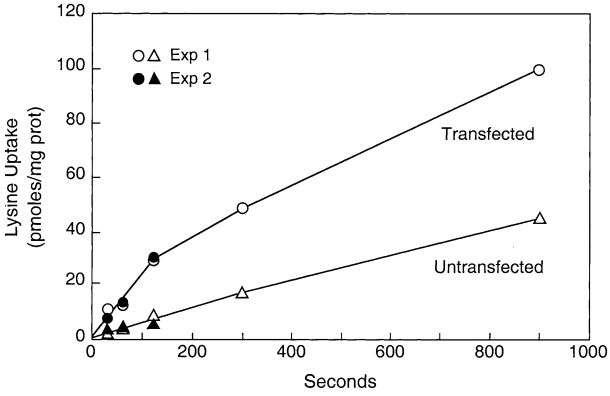

Fig 4. A time course for lysine uptake in hCAT-1-GFP-transfected cells. The time course of L-[^{3}H]lysine (1 μM) uptake at 37°C by transfected and untransfected cells was determined. The filled and clear symbols represent data from two separate experiments. Each point represents the mean saturable lysine uptake using triplicate filters. Lysine uptake is linear for 120 seconds in both untransfected and hCAT-1-CT-GFP-transfected cells.

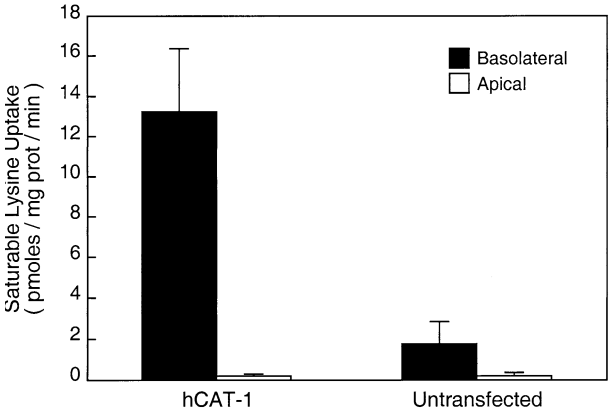

Fig 5. Stimulation of lysine uptake in hCAT-1-CT-GFP transfected cells. Uptake of 1 μM L-[^{3}H]lysine was measured at 37°C for 60 seconds from the basolateral or apical side of separate confluent filter grown cells. Solid bars show basolateral uptake and open bars show apical uptake of L-[^{3}H]lysine. L-[^{3}H]lysine uptake in the presence of 20 mM unlabeled L-lysine has been subtracted from each condition. hCAT-1-CT-GFP in transfected cells produced an approximately 7-fold stimulation of lysine uptake as compared to untransfected cells. Error bars represent standard deviation, $n = 4$ experiments.

untransfected cells was negligible. The saturable basolateral uptake of lysine by hCAT-1-CT-GFP-transfected cells was 7-fold greater than that by untransfected cells (Fig. 5).

We addressed the issue of whether the cytoplasmic C-terminal GFP tag in the hCAT-1-CT-GFP fusion protein unduly influences the observed polarized localization. To accomplish this, we performed transient transfections of MDCK cells with a plasmid (pcDNA3.1) containing untagged hCAT-1, followed by measurement of directional lysine uptake. Simultaneous, separate transient transfections with hCAT-1-CT-GFP vector and mock-transfected cells served

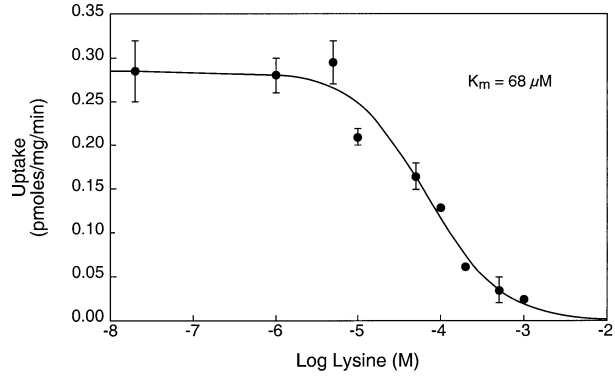

Fig 6. Concentration dependence of lysine uptake by hCAT-1-CT-GFP. The uptake of L-[^{3}H]lysine (0.02 μM) at 37°C for 1 min by transfected and untransfected cells was determined in the presence of increasing concentrations of unlabeled lysine. For each lysine concentration, saturable uptake by untransfected cells was subtracted from saturable uptake by transfected cells. The data were fit to a Henri-Michaelis-Menten equation describing the relationship of hCAT-1-CT-GFP transporter activity to lysine concentration with an added diffusion term data. Data are means ± standard error of 2 experiments determined in triplicate.

as positive and negative controls, respectively. MDCK cells transfected with hCAT-1 exhibited an increase in saturable lysine uptake activity from the basolateral side when compared to mock-transfected cells. In two experiments, uptake increased by 1.6-and 2.9-fold (4.8 ± 0.2 *vs.* 3.0 ± 0.4 (± SD) pmoles/mg prot/min, and 7.2 ± 0.5 *vs.* 3.0 ± 0.4 pmoles/mg/min), respectively. We estimate our transfection efficiency to be around 25%. All the transiently transfected and mock-transfected cell lines had negligible apical uptake (< 0.3 pmoles/mg prot/min). These experiments confirm that the observed basolateral membrane-specific localization and function of hCAT-1-CT-GFP are independent of the addition of the GFP tag, and are an inherent property of hCAT-1 in MDCK cells.

Concentration Dependence of hCAT-1-CT-GFP Transport Activity in MDCK Cell Monolayers

The concentration dependence of lysine uptake by the cloned trophoblast hCAT-1 transporter expressed in MDCK cells was examined by varying the concentration of non-radioactive lysine from 0 to 20 mM. Fitting a Michaelis-Menten equation to the data demonstrated a K_m = 68 ± 13 μM and a V_{max} = 970 ± 170 pmoles/mg/min (n = 2 experiments) (Fig. 6). This K_m value is similar to that of hCAT-1 expressed in oocytes (98 μM) (Kamath et al., 1999).

Discussion

This study established a method to study the cellular localization and function of the various placental

syncytiotrophoblast hCAT protein isoforms in a model epithelial cell. We show that the protein coded for by the cDNA of the hCAT-1 isoform is localized to the basolateral surface domain when stably transfected and expressed in polarized MDCK cells. This is the first demonstration of specific membrane localization for a member of a family of highly homologous placental cationic amino-acid transporters in MDCK cells. Previous transport studies of endogenous system y^+ have described predominantly basolateral uptake of lysine in MDCK cells (Sepulveda & Pearson, 1985), rabbit oxyntic glands (Barahona & Bravo, 1993), and human intestinal Caco-2 cells (Thwaites et al., 1996). It will be interesting to see if hCAT-1 is the major isoform expressed in these cell lines or tissues.

The correct localization of the hCAT isoforms in human placental trophoblast is almost certainly essential to maternal-fetal transport of cationic amino acids. We have previously shown that system y^+ cationic amino-acid transport in placental basal and microvillous membranes differ from each other in the degree of inhibition by certain neutral amino acids in the presence of sodium (Furesz et al., 1995). The enhanced lysine uptake stimulated by hCAT-1 mRNA expression in oocytes is strongly inhibited by neutral amino acids and sodium reminiscent of placental basal membrane y^+ activity (Kamath et al., 1999). Recently data from our laboratory using the same oocyte expression system indicated that hCAT-2B activity interacts relatively weakly with amino acids and sodium (unpublished observations). These results suggest that differences in the properties of the various isoforms of the hCAT proteins coupled with differential cellular localization may be important to the physiologic regulation of cationic amino-acid transport by placental syncytiotrophoblast. The GFP-expression system demonstrated that hCAT-1 localized to the basolateral membrane of the MDCK cells. An analogous localization in the placental syncytiotrophoblast would facilitate the inhibition of basal membrane system y^+ activity by neutral amino acids and sodium and the predominant uptake of lysine and arginine from the microvillous membrane. This would lead to 'reservoir' function for the syncytiotrophoblast, which would allow it to serve the nutritional demands of the fetus more efficiently. Such a function has been previously suggested from in vivo concentration measurements (Philipps et al., 1978).

In the absence of specific antibodies to the hCAT proteins, we tagged the cytoplasmic tail of the first of these transporters, hCAT-1 with GFP to observe its cellular localization in the highly polarized MDCK cell line. The available evidence indicates that the addition of a tag occasionally randomizes the plasma-membrane expression of a polarized wild type protein (e.g., the apically targeted aminoisobutyric transporter, GAT-3 with a c-Myc epitope tag) (Muth, Ahn & Caplan 1998). We confirmed that the transiently transfected untagged hCAT-1 transporter had the same, completely polarized (basolateral) distribution, as did hCAT-1-CT-GFP.

The steady-state distribution of exogenous membrane proteins transfected into MDCK cells seem to mimic the membrane localization seen in the epithelial tissue of origin. This cell line has been widely used for this purpose (reviewed in Hubbard Stieger & Bartles, 1989; Mellman, 1995; Weimbs et al., 1997; Ikonen & Simons, 1998, and Mostov, Verges & Altschuler, 2000). This utility of MDCK cells has been extended to show that neuronal proteins found in the axon sort to the apical surface of MDCK cells, whereas most dendritic proteins sort to the basolateral surface (reviewed in Rodriguez Boulan & Powell, 1992). While there are clearly exceptions for both epithelial and neuronal proteins (*see* reviews above), the MDCK cell has been a useful model to guide investigations into the sorting process of membrane proteins.

Similarly, many investigators have used the MDCK model to investigate the differential localization of various epithelial nutrient transporters. The renal organic cation transporter 2 (rOCT2) fused to C terminal GFP also showed a specific basolateral distribution when localized in MDCK cells (Sweet et al., 2000). The various isoforms of the glycine transporters GLYT1 and GLYT2 are asymmetrically distributed in an isoform specific manner. GLYT1a, GLYT2a and GLYT2b are localized apically while GLYT1b is localized basolaterally in response to an amino- terminal signal and two di-leucine motifs located in the carboxyl tail of this protein (Poyatos et al., 2000). The facilitative glucose transporters are also differentially localized in MDCK cells; GLUT1 and GLUT2 are found on the basolateral surface, GLUT3 and GLUT5 on the apical surface, while GLUT4 is found in an intracellular compartment (Pascoe et al., 1996). The basolateral and apical sorting signals have not been identified for the GLUT's, which share a high degree of sequence homology, but interestingly considerable sequence heterogeneity in the NH2 and COOH-terminal cytoplasmic tails. The cellular localization of the GLUT isoforms in MDCK cells exactly mimic their localization in the tissues of origin.

Following the stable transfection of heterologous proteins into MDCK cells, deletion and domain-swapping experiments have identified basolateral targeting signals in many single-pass transmembrane proteins. These protein-based basolateral sorting signals frequently contain a critical tyrosine residue, a di-hydrophobic motif, a cluster of acidic residues or a combination of these elements. A consensus from numerous studies is that basolateral sorting signals are almost always found in the cytoplasmic domain

of single transmembrane proteins (reviewed by Aroeti et al., 1998). This signaling domain may interact with a recently identified novel clathrin adaptor complex to mediate basolateral targeting in polarized epithelial cells (Fölsch et al., 1999).

Much less is known about the signals that mediate the targeting of proteins with multiple membrane-spanning domains. Early indications are that basolateral sorting signals for polytopic membrane proteins, like the ones for monotopic proteins are located in the cytoplasmic portions of the proteins. Recently Perego et al. (1997) mapped an 8-amino-acid segment, rich in basic residues within the carboxyl-terminus cytosolic tail of the polytopic dog betaine transporter, which confers basolateral targeting and residence to this protein. Chimeric constructs of a monotopic and a polytopic apical resident protein containing this sequence of amino acids in the cytoplasmic tail was sufficient to redirect them to the basolateral surface. Similar results and conclusions were drawn regarding a 21-amino-acid sequence in an intra-cytoplasmic loop of the 7-transmembrane neutral M3 muscarinic acetylcholine receptor in MDCK cells (Nadler, Kumar & Nathanson, 2001).

We have used the GFP-expression system to demonstrate that hCAT-1 is localized to the basolateral membrane of MDCK cells. We are now using this procedure to facilitate determination of the cellular distribution, vectorial targeting pathways, and targeting motifs of the other placental cationic amino-acid transporters (abstract by Cariappa et al., 2001). This system has the potential to determine the mechanisms that underlie the establishment and maintenance of polarity of placental syncytiotrophoblast transporters and facilitate maternal-fetal transport of important nutrients.

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