Human Copper Transporter hCTR1 Mediates Basolateral Uptake of Copper into Enteroctyes

IMPLICATIONS FOR COPPER HOMEOSTASIS

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Copper is essential for human growth and survival. Enteroctyes mediate the absorption of dietary copper from the intestinal lumen into blood as well as utilizing copper for their biosynthetic needs. Currently, the pathways for copper entry into enterocytes remain poorly understood. We demonstrate that the basolateral copper uptake into intestinal cells greatly exceeds the apical uptake. The basolateral but not apical transport is mediated by the high affinity copper transporter hCTR1. This unanticipated conclusion is supported by cell surface biotinylation and confocal microscopy of endogenous hCTR1 in Caco2 cells as well as copper influx measurements that show saturable high affinity uptake at the basolateral but not the apical membrane. Basolateral localization of hCTR1 and polarized copper uptake are also conserved in T84 cells, models for intestinal crypt cells. The lateral localization of hCTR1 seen in intestinal cell lines is recapitulated in immunohistochemical staining of mouse intestinal sections. Biochemical and functional assays reveal the basolateral localization of hCTR1 also in renal Madin-Darby canine kidney cells and opossum kidney cells. Overexpression of hCTR1 in Madin-Darby canine kidney cells results in both apical and basolateral delivery of the overexpressed protein and greatly enhanced copper uptake at both cell surfaces. We propose a model of intestinal copper uptake in which basolateral hCTR1 plays a key role in the physiologically important delivery of copper from blood to intracellular proteins, whereas its role in the initial apical uptake of dietary copper is indirect.

Copper is required by higher eukaryotes, including mammals, for a variety of metabolic processes, for mitochondrial respiration as an essential cofactor in cytochromes, in the synthesis of collagen and the production of connective tissue and skin, and in the synthesis of neurotransmitters and brain peptides as well as in protection against reactive oxygen species in cells and tissues by virtue of its catalytic involvement in superoxide dismutase. At the same time, elevated levels of copper are toxic, as a result of its redox chemistry and its propensity to form stable adducts with protein sulfhydryl residues. Thus, most organisms have developed a series of sophisticated mechanisms to regulate the acquisition and excretion of this metal. About 1–2 mg of copper is delivered to humans in their daily diet to be absorbed in the intestine or excreted, largely in the feces (1).

Copper uptake in eukaryotes is mediated by CTR proteins that were first characterized in Saccharomyces cerevisiae (2, 3). In humans high affinity copper entry is mediated largely by hCTR1, the human copper transporter. hCTR1 was first cloned and its sequence determined in 1997 (4). The mouse homolog, mCTR1, is 92% identical to hCtrl (5). Deletion of mCTR1 results in embryonic lethality, death occurring in utero in midgestation, around E9.5 (6, 7). hCTR1 is a small, methionine-rich protein (190 amino acid residues) that has three transmembrane segments and oligomerizes to form functional trimers that most likely cooperate to form a specific ion pathway for copper across the membrane (8–12). The complex mediates copper uptake with a $K_m$ value in the 2–5 μM range. However, it is clear that other systems may be involved in cellular copper entry, as ctrl$^{-/-}$ mouse fibroblasts still carry out copper uptake at about 20–30% that of the rate of normal cells (13). Candidates for other copper uptake pathways include DMT1, the relatively nonspecific divalent cation transporter that has been shown to transport copper (14, 15). Copper exit and delivery of cellular copper into the secretory pathway of cells is accomplished by the ATP-dependent copper transporters, ATP7A and ATP7B, which are members of the P-type ATPase family (16). These are two intrinsic membrane proteins that utilize the energy of hydrolysis of ATP to fuel the transport of copper across cell membranes (17). Disruption of ATP7A or ATP7B leads to the two human genetic diseases, Menkes and Wilson diseases, respectively.

In polarized epithelia of the intestine and kidney, absorptive transepithelial transport of ions and nutrients is accomplished by separating the entry step (from the lumen into the cells) from the exit step from the cells into the blood. This is done by the exclusive localization of entry mechanisms to the apical surface and of exit mechanisms to the basolateral surface. Such polarization is an essential property of epithelia and is maintained by the presence of tight junctions between epithelial cells. In keeping with this well established model it has been supposed that major copper entry mechanisms will be limited to the apical epithelial membrane and P-type ATPases to the basolateral side (18). The two major candidates so far identified for mediating copper uptake are DMT1, a nonspecific divalent cation transporter, shown to be at the apical surface of human...
intestinal epithelial cells (19), and hCTR1. It has also been argued that whereas hCTR1 may be responsible for copper uptake in the brain, its involvement in dietary copper uptake in intestinal tissue remains uncertain (20). It has recently been reported after immunohistochemical staining of tissue that hCTR1 is present in intracellular locations in mouse intestine (21, 22) and perhaps at the apical surface of intestinal cells (21). Interestingly, in this latter study, when hCTR1 was selectively disrupted in mouse intestine, cellular intestinal levels of copper were elevated rather than lowered. In another recent study it was reported that hCTR1 in both placental tissue and a placental cell line was found both in the cytoplasm and at the lateral membrane (23). The functional consequences of these different locations on copper uptake have not previously been reported.

In the present work we utilize Caco2 cells, a well established model for enterocytes, T84 cells (an intestinal crypt cell model), and also MDCK2 and OK renal cell lines that represent cells from the distal and proximal nephron, respectively. Measurements of copper enterocyte demonstrate a saturable high affinity uptake from the basolateral but not apical cell surface. Confocal imaging and cell surface biotinylation confirm the basolateral localization of hCTR1 in these cells. Evidently, intestinal cells acquire their essential copper for intracellular processes from the blood as do other cells and must utilize an alternative uptake mechanism to carry out the absorption of dietary copper.

EXPERIMENTAL PROCEDURES

**Cell Cultures**—Cell cultures were maintained in a humidified incubator at 37 °C under 5% CO2 atmosphere in growth media supplemented with an antibiotic-antimycotic mixture of 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone (Invitrogen #15240), 5 µg/ml Plasmocin (InvivoGen #ant-mpt), and 15 mM Hepes buffer (Invitrogen #15140). All cells were subcultured at 1:5 ratios, except for T84 cells, which were subcultured at a 1:2 ratio; growth media were exchanged twice per week. Specifically, Caco2 cells were cultured in Dulbecco’s modified Eagle's medium (DMEM) medium (Invitrogen #11995) containing 20% fetal bovine serum (Atlantic Biologicals #s11550), T84 cells were grown in a 1:1 mixture of DMEM and Ham’s F-12 medium (Invitrogen #11765) supplemented with 5% FBS, whereas, MDCK and OK cells were maintained in DMEM medium supplemented with 10% FBS.

For generation of tetracycline-regulated MDCK cell lines that stably express the C-terminal-tagged FLAG-hCTR1, we used a Flip-In Invitrogen system (catalog #K6010). Briefly, MDCK cells were transfected with pFRT/lacZeo to create a Zeocine-resistant, Flip-In host cell line. Next, pcDNA5/FRT vector containing a FLAG-tagged hctr1 construct was co-transfected with pOG44 plasmid into the MDCK Flip-In host cell line and selected for hygromycin resistance. To convey tetracycline regulation, the hygromycin-resistant cells were then transfected with a regulatory plasmid, pcDNA6/TR (Invitrogen #V1025) and selected for Blasticidin resistance. In the absence of tetracycline, a Tet repressor protein expressed from pcDNA6/TR vector represses transcription of the hctr1 gene, whose expression is induced upon the addition of tetracycline.

**Antibodies**—The affinity-purified rabbit anti-hCTR1 antibody raised against the C-terminal peptide (SWKKAVVVDITECH) was described previously (9). The anti-hCTR1 antibodies described here were raised against a 46-amino peptide corresponding to the cytoplasmic loop (KIARESSLRKSQVSIRYNSMPVGPNGTILMETHKTVQGQMLSFPH) purchased from Biopptide Co., LLC (San Diego, CA). This peptide was used to immunize rabbits (Cocalico Biologicals, Reamstown, PA). Preimmune and immune sera were tested for reactivity against the immunizing peptides, against purified hCTR1 expressed in yeast (a kind gift from Dr. V. Unger, Yale University), and against membranes from various cells expressing hCTR1.

For affinity purification of antibody, hCTR1 cytoplasmic loop peptide was coupled to Actigel ALD resin matrix using instructions provided by the manufacturer (Sterogene, Carlsbad, CA). Post-coupled resin was washed and packed into columns and used to purify anti-hCTR1 antibody from whole rabbit sera. Bound antibody was eluted with ActiSep elution medium (Sterogene), which was subsequently removed by desalting into PBS. The antibody was concentrated to the original serum volume in spin concentrators (Vivascience AG, Hanover Germany) and stored in 0.05% azide.

**Cell Surface Biotinylation**—Cells were grown on 24-mm polystyrene membrane Transwells with 0.4-µm pores (Corning, #3450) until confluent. Caco2 cells were grown on membranes that were coated with collagen (Sigma #C8919) and diluted 1:100 in 70% ethanol, and then Transwells were dried for ~2 h. When the cells were confluent, the growth medium was exchanged every 1–2 days. Formation of tight junctions was monitored by the measurement of transepithelial electrical resistance using an EVOM meter and STX2 electrodes (World Precision Instruments). Cells were judged to be ready for biotinylation when the transepithelial electrical resistance value was ~250 Ω × cm2 for Caco2, ~2500 Ω × cm2 for T84, ~4000 Ω × cm2 for MDCK, ~2000 Ω × cm2 for OK cells. Before biotinylation cells were placed on ice for ~10 min, and all the subsequent steps were done on ice with all the solutions pre-chilled to ~4 °C. Cells were washed twice with PBS supplemented with 0.1 mM CaCl2 and 1.0 mM MgCl2, and then transferred into 0.5 ml of the biotinylation buffer (PBS, pH 7.5, supplemented with 10 mM triethanolamine, 2 mM CaCl2, and 150 mM NaCl) to the working concentration of 0.8 mg/ml. An aliquot of 0.5 ml of the biotin reagent was added to the apical compartment, keeping the basolateral compartment filled with buffer, or 1.5 ml of the biotin reagent was added to the basolateral compartment, keeping the apical compartment filled with buffer only. Plates were incubated on ice with a slow rocking motion. The reaction was quenched by replacing the biotinylation solution with 1 ml of apical and 2 ml of basolateral quench buffer (PBS supplemented with 0.1 mM CaCl2, 1.0 mM MgCl2, and 100 mM glycine) and incubated at 4 °C for 20 min with a slow rocking motion. The quenching procedure was repeated once more. Then quench buffer was
removed, and the permeable membrane was excised from the Transwells with a scalpel. The membrane was placed in a 1.5-ml centrifuge tube with 1 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) and incubated at 4 °C with end-over-end rotation for 1 h. The permeable membrane was removed, and samples were vortexed briefly and spun at 10,000 × g for 10 min. Supernatant was transferred to a fresh tube. An aliquot of 50 μl was taken for Western blot analysis of the total cell lysate. 700 μl of the sample was placed into a Zeba spin column (Pierce #89868), and 100 μl of streptavidin-coupled Sepharose beads (Pierce #20349) was added. Samples were incubated overnight with end-over-end rotation in the cold room, they were then spun down at 500 × g for 5 min to remove buffer, and the beads were washed 3 times with lysis buffer, twice with salt wash buffer (0.1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5), and once with buffer (10 mM Tris, pH 7.5). The final spin to remove buffer was at 10,000 × g for 5 min. Biotinylated protein was eluted from the streptavidin-coupled beads with 50 μl of 2× sample buffer (working concentration of 80 mM DL-dithiothreitol, 5.6% SDS, 0.008% bromophenol blue, 16% glycerol, and 240 mM Tris, pH 8.9). Mini spin columns were placed into fresh 1.5-ml centrifuge tubes, and samples were incubated at room temperature overnight. Final elution step was done by spining samples for 10 min at 10,000 × g.

Confocal Microscopy—Cells were seeded onto permeable membranes in 1.12-cm² Transwells (Corning #3460) and grown until ~95% confluence. They were then washed in PBS and fixed on ice in −20 °C acetone for 30 s and then re-hydrated in PBS for 10 min. Cells were blocked with 1% bovine serum albumin, 1% gelatin in PBS overnight at 4 °C. Primary antibodies, rabbit anti-loop hCTR1 (1:100), and mouse anti-albumin, 1% gelatin in PBS overnight at 4 °C. Primary antibodies were used in confocal microscopy (see above) followed by biotinylated secondary antibodies and then an avidin-conjugated peroxidase enzyme. Immunohistochemical staining was visualized by adding a chromogenic peroxidase substrate, 3,3′-diaminobenzidine tetrahydrochloride (Vector Laboratories #SK-4100), which produces a brown precipitate. As a negative control, instead of the primary antibody, a rabbit immunoglobulin G (Santa Cruz Biotechnology #sc-2027) was used.

Cell Fractionation—Fractionation of cells was carried out using a five-step sucrose gradient with a total volume of 36 ml consisting of the following sucrose concentrations layered from the bottom to top: 3.0 ml of 2.0 M sucrose, 6 ml of 1.6 M sucrose, 12 ml of 1.4 M sucrose plus homogenate, 12 ml of 1.2 M sucrose, and 3.0 ml of 0.8 M sucrose. Cells were washed twice with PBS and scraped from the plates and collected by centrifugation for 10 min at 1000 × g at 4 °C. They were then homogenized on ice with a Dounce homogenizer using HB buffer, ~3 ml per 500 μl of cell pellet (HB buffer: 250 mM sucrose, 10 mM Tris, pH 7.4, and 2 mM EDTA supplemented with protease inhibitor mixture table Roche #11836153001). Cell homogenate was spun at low speed to remove nuclei and unbroken cells, and supernatant was adjusted with 2.55 M sucrose to a final concentration of 1.4 M sucrose and loaded onto the gradient. Tubes were spun in a Beckman SW28 rotor at 25,000 rpm for 2.5 h at 4 °C. Three fractions enriched in plasma membrane, Golgi, and endoplasmic reticulum containing visible bands of protein were collected from the top to bottom. To remove sucrose each fraction was diluted to 25 ml with HB buffer and spun at 30,000 rpm for 30 min in Beckman 50.2Ti rotor. The pellets were re-suspended in an appropriate volume of TE buffer (10 mM Tris-HCl, 2 mM EDTA, pH 7.4).

64Cu Uptake Assays—For copper uptake experiments, cells were plated on 4.67-cm² permeable membrane supports in Transwells (Corning) and grown as described above for biotinylation. The cells were washed twice in PBS, and fresh DMEM medium with 10% FBS was added to the apical (1.35 ml) and to the basal (1.8 ml) compartments. Cells were incubated for 30 min at 37 °C. Copper uptake was initiated by adding 150 μl apically or 200 μl basolaterally of 10× copper solution containing trace levels of 64Cu (MIR radiological sciences, Washington University Medical School) and the desired concentration of CuCl₂. Cells were incubated for the desired times at 37 °C, and
Copper transport was terminated by the addition of ice-cold stop buffer (150 mM NaCl, 5 mM KCl, 2.5 mM MgCl$_2$, 25 mM Hepes, pH 7.4, and 10 mM Na$_2$-EDTA), after which cells were washed 3 additional times with ice-cold stop buffer. The media were then aspirated, and membrane supports were excised from the Transwells with a scalpel and placed in a container with added Eco-Lume scintillation liquid (ICN Biomedicals #882470) for scintillation counting (Beckman-Coulter LS6500). All transport determinations were carried out in triplicate. $^{64}$Cu content of the initial tracer-containing buffer was determined for the calculation of specific activity. Copper uptake was then expressed as pmol of copper taken up by the cells per mg of protein after determination of the protein content of the cell monolayer by the Bio-Rad protein assay (Bio-Rad #500-0006). Inhibitory effects of silver and cadmium ions on $^{64}$Cu uptake were determined by including a 50-fold molar excess of AgNO$_3$ or CdCl$_2$ in the transport medium in each compartment of the Transwell and then running the copper uptake assay as described above. Our transport experiments were carried out under conditions designed to optimize the determination of the true initial rate of copper uptake into the cells (that is, only a very small fraction, less than 2%, of the applied apical copper enters the cells). Under these conditions we found that less than 0.15% of the applied apical copper appeared in the basolateral solution, indicating that the cell monolayer was not leaky.

**Western Blots**—Sample preparation for Western blot analysis included addition of 2× sample loading buffer (5% SDS, 1.6 M urea, and 240 mM Tris-HCl, pH 6.8) and 1.5% mercaptoethanol followed by heating for 10 min at 50 °C. About 30–50 μg of samples were loaded in each lane of 12% Laemmli gels. Gels were run at constant 24 mA for about 2 h and then transferred to polyvinylidene difluoride membranes (Millipore #IPVH00010) at 180 mA for 3 h. Membranes were blocked with 5% milk and then treated with antibodies diluted in 1% milk. For detection of hCTR1 protein, the following primary antibodies were used: rabbit anti-C terminus hCTR1 antibody at 1:10,000 dilution or rabbit anti-loop hCTR1 antibody at 1:500 dilution followed by anti-rabbit IgG horseradish peroxidase conjugate (Amersham Biosciences, NA934V). Blots were developed using SuperSignal West Pico peroxide and enhancer solutions (Pierce, #1856135 and 1856136). The intensity of the Western signals was measured using chemiluminescent imaging with the ChemiDoc XRS (Bio-Rad) and quantitated using QUANTITY ONE Version 4.6.2 software (Bio-Rad).

**RESULTS**

**Copper Uptake Measurements in Intestinal Cells**—Caco2 cell line, derived from human colon adenocarcinoma, can be cultured to a high confluence as a continuous monolayer, forming tight junctions and spontaneously differentiating into polarized epithelial cells with characteristics of mature enterocytes, including formation of an apical brush border. Caco2 monolayers serve as a well established model for the evaluation of metal ion absorption (19). T84 cells, derived from a human colorectal carcinoma, are also capable of developing tight junctions and desmosomes, and they differentiate into polarized columnar cells resembling intestinal crypt epithelia (24).

We initially characterized the relative ability of intestinal cells to carry out copper uptake across either their apical or basolateral plasma membranes by measuring radioisotopic copper uptake from each of these sides independently. The results of these experiments revealed a striking asymmetry in the rates of copper uptake (Fig. 1). Using 1 μM copper there was a 12-fold higher rate of copper uptake from the basolateral than the apical surface in Caco2 cells and about 20-fold higher basolateral uptake in T-84 cells. At 3 μM copper this difference decreased somewhat to about 7- and 10-fold in the Caco-2 and T-84 cells, respectively, but remained high, indicative of the basolateral membrane being a predominant route of copper entry.

The observation that with the increasing copper concentrations the ratio between basolateral and apical transported changing suggested that the copper dependence of uptake differed at two membranes. Consequently, we determined the concentration dependence for copper transport at each membrane by measuring the initial rates of uptake using a wide range of copper concentrations. The data for Caco2 cells are shown in Fig. 2. Uptake across the apical membrane increases monotonically as copper concentration is increased (Fig. 2A). Uptake across the basolateral surface is composed of two components. In the range of physiological copper concentrations (1–10 μM) the basolateral uptake is largely composed of a sat-
urating component (see the inset in Fig. 2) and a small contribution from a non-saturating component. Correction for the linear component of this data yielded a maximal transport rate for the saturable component of 891 pmol of copper/mg × h, with a $K_m$ of 5.4 μM.

There are two unexpected aspects to these observations. First, it was anticipated that in intestinal cells copper uptake would be high from the apical surface, because such uptake would have to satisfy both intracellular needs as well as provide copper for further distribution to the body. Clearly this is not the case. Uptake at the basolateral surface is not only higher, but it is also mediated via a high affinity system, whereas the lower apical transport is of low affinity. The $K_m$ value for basolateral transport obtained is close to the values of 1–5 μM previously reported for hCTR1 (8–10, 25). This observation was also unexpected as it has been assumed that CTR1 plays a role in apical copper uptake. To clarify these issues, we investigated the involvement of hCTR1 in basolateral and apical transport in more detail.

It has been previously demonstrated that Ag(I) ions are effective inhibitors of hCTR1-mediated copper entry into cells (8) and that metal ion transport via DMT1, a divalent cation transporter, is inhibited by the addition of Cd(II) ions (26). In Fig. 3 we show that the presence of a 50-fold excess of silver caused a 90% inhibition of copper uptake across the basolateral membrane of Caco-2 cells with 3 μM copper and 70% with 7 μM copper, whereas the 50-fold excess of cadmium was without effect. In contrast, at the apical surface, silver (or cadmium) had almost no effect on copper uptake. Together with the $K_m$ values, these inhibition studies strongly suggested the involvement of hCTR1 in the basolateral uptake of copper and not the apical uptake while providing no evidence of a significant role for DMT1 under these conditions.

Localization of hCTR1 in Intestinal Cells—Measurements of copper uptake pointed to the presence of functional hCTR1 at the basolateral membrane surface. To test this prediction and establish the cellular localization of CTR1, we employed two approaches, cell surface-specific biotinylation and immunocytochemistry using confocal fluorescent microscopy.

Caco2 cells were grown under conditions identical to those used for copper transport. Proteins at the cell surface were then biotinylated from either the basolateral or apical side of Caco2 monolayers using a membrane-impermeable reagent; the biotinylated proteins were collected on avidin beads, and the presence of hCTR1 in each fraction was determined using an anti-CTR1 antibody. An antibody against basolateral marker Na,K-ATPase was utilized as a control to establish the appropriate polarization and formation of tight junctions in cell culture. The results of these studies are shown in Fig. 4. The hCTR1 is strongly biotinylated from the basolateral but not the apical surface. Quantitation of these
data showed that less than 1% of the total hCTR1 is present at the apical surface. The β-subunit of the Na,K-ATPase, which is known to be localized exclusively at the basolateral side, shows the same distribution. A final confirmation of the polarized distribution of hCTR1 at the basolateral membranes was obtained using confocal microscopy. The images displayed in Fig. 5 clearly show the typical basolateral “chickenwire” staining pattern of the Na,K-ATPase (Fig. 5, center panel) and the same membrane localization for hCTR1 (left panel). Co-localization of these transporters is confirmed in the merged images (right panel). The basolateral presence of hCTR1 is also confirmed in the xz and yz stacks shown in Fig. 5. Thus, the biochemical labeling and immunocytochemical localization of hCTR1 confirm its appropriate localization and make it most likely that the high affinity transport at the basolateral membrane is mediated via CTR1.

Copper Uptake and Localization of hCTR1 in Renal Cells—
The unexpected localization of hCTR1 in intestinal epithelia led us to examine renal epithelial cells to determine whether this orientation was conserved, as is often the case for the polarization of membrane transporters. We utilized MDCK cells, a model for distal renal cell epithelia, and OK cells as a model for proximal renal epithelia. The results of measurements of copper uptake into monolayers of MDCK and OK cells, carried out in the same way as for intestinal cells, are shown in Fig. 6. The rates are about an order of magnitude lower than seen in intestinal cells. This correlates well with the 10-fold lower expression of hCTR1 seen in Western blots of the plasma membrane fractions of the renal cells compared with the intestinal cells (data not shown). Furthermore, the influx rates were about 7-fold higher across the basolateral surface of MDCK and OK cells compared with uptake across their apical surfaces. In keeping with our observations in the intestinal cells, cell surface biotinylation reveals that hCTR1 is located at the basolateral surface of the renal cell lines (see Fig. 4). Confocal fluorescence microscopy carried out on MDCK cells in the same way as described above for Caco2 cells confirmed colocalization with the Na,K-ATPase β-subunit in a pattern consistent with its basolateral location (Fig. 7).

Because endogenous levels of hCTR1 in the renal cells are quite low compared with intestinal cells and our hCTR1 antibodies are of relatively low affinity, we decided to enhance the expression level of hCTR1 in the renal cells. We utilized an MDCK cell line we had engineered in which an epitope-tagged form of hCTR1 is overexpressed under the control of a tetracycline-regulated promoter (see “Experimental Procedures”). We have shown that Western blot analysis of the plasma membrane fraction of these cells indicates a substantial increase in the expression level of hCTR1 when grown in the presence of tetracycline (27). However, when copper uptake assays were performed, a striking observation was made. The cells that were induced to express hCTR1 at elevated levels showed influx rates of 3 μM copper that were higher (about 2-fold) from the apical than from the basolateral surface (see Fig. 8a). Cell surface biotinylation of these cells revealed that the overexpressed hCTR1 was delivered predominantly to the apical plasma membrane (Fig. 8b) and that the level of hCTR1 in the apical membrane was higher than in the basolateral membrane. It is also evident in these cells that whereas the exclusive basolateral targeting of endogenous hCTR1 is disrupted by overexpression, exclu-
sive basolateral delivery of endogenous Na,K-ATPase is maintained (Fig. 8b). We have also previously shown that overexpression of the β1 subunit of the Na,K-ATPase in these same MDCK cells does not alter its exclusive basolateral targeting (28). The mechanistic basis of the loss of selective basolateral localization of overexpressed hCTR1 in MDCK cells is the subject of ongoing studies.

**Localization of hCTR1 in Intestinal Tissue**—Because our results in intestinal cell lines suggested that present models for dietary copper uptake are in need of modification, it was important to establish that the functional and biochemical data obtained in intestinal cell lines applied to the in vivo situation. We carried out immunohistochemical staining of sections of mouse intestine. In Fig. 9 we show the results of such studies on duodenum. Clearly, we see Na,K-ATPase is localized to the lateral membranes, as expected. In addition hCTR1 is also seen predominantly at the lateral membrane with some intracellular staining just below the apical membrane surface. Thus, the basolateral localization in Caco-2 and T-84 cell lines derived from intestine and reported here are recapitulated in histochemical staining of intestinal tissue.

**DISCUSSION**

We have found that in human intestinal cells hCTR1, the major human high affinity copper transporter, is localized at the basolateral surface where it mediates copper uptake from the blood. This finding was not expected, as the first step in the absorption of copper from the diet is uptake from the lumen across the apical membrane of intestinal cells and from the intestinal cells to blood and then on to other tissues. The functional polarization of hCTR1 is substantiated by the exclusive basolateral (and not apical) cell surface biotinylation of the transporter. Confocal imaging of intestinal cell monolayers confirms the basolateral location. Copper uptake is also inhibited by silver ions from the basolateral but not the apical surface. In keeping with this basolateral localization in intestinal cells, a basolateral orientation of endogenous hCTR1 is also found in MDCK and OK cells and mod-

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**FIGURE 8. Tetracycline induced Flip-In-MDCK cells.** Flip-IN MDCK cells were grown in Transwells until confluent. The hCTR1 expression was then induced with 1 μg/ml tetracycline for 48 h, and the copper uptake was assayed (a), or biotinylation was performed (b) as described above. A, apical side; B, basolateral side.

**FIGURE 9. Immunohistochemistry of hCtr1 in the mouse small intestine.** Tissue slices obtained from the duodenum of an adult male mouse were immunoprobed with either hCTR1 antibody (panel A and B), β1-subunit of Na,K-ATPase (a basolateral marker, panels C and D), or rabbit IgG (a negative control, panel E and F). Binding of each antibody was visualized with 3, 3′-diaminobenzidine tetrahydrochloride. The figures display 100× magnifications of sections of villi in the duodenum, showing either a longitudinal sectional view (panels A, C, and E) or a chickenwire pattern resulting from a more oblique section (panel B, D, and E).
hCTR1 Polarity in Epithelial Cells

uptake via hCTR1 and delivery to a series of protein-specific chaperones, such as Atox1, or CCS, that then load the apoenzymes with copper. The basolateral location of hCTR1 we have observed fulfills this function. However, apical entry of dietary copper uses a different pathway. It is clear that hCTR1 is an essential component of copper metabolism, and genetic deletion has an embryonic lethal phenotype (6, 7). It is also clear that hCTR1 is responsible for the majority (see below) of copper uptake by mammalian cells. However, there have been few studies before this that have provided evidence for the location of hCTR1 in epithelial cells. In two recent studies it was reported that hCTR1 was intracellular in mouse intestinal cells (21, 22); one of these reported some apical localization (21). Earlier studies of hCTR1 location in Caco-2 cells reported that the protein was present in both the plasma membrane and intracellular locations (29). In this latter study lateral membrane staining of Caco-2 cells is clearly evident. In another study of placental tissue and cells derived from placenta, it was reported that hCTR1 was intracellular and localized to the basolateral plasma membrane (23). In none of these studies was a functional effect on copper transport of the reported localization tested.

Our results also demonstrate a basolateral location for endogenous hCTR1 in kidney cells. Although the kidney is not a major pathway for copper excretion, renal cells have the same requirements for copper as all other cells, and it seems likely then that they obtain this copper from the blood and not from the luminal filtrate. In the case of several other transporters that are expressed in both renal and intestinal cells, such as the Na,K-ATPase, their basolateral localization is conserved in both epithelia. The expression levels of hCTR1 are much higher in intestinal cells than in renal cells. It seems likely that the higher expression of hCTR1 in intestine is a reflection of the intracellular need for copper in cells that are actively engaged in the significantly higher dietary uptake and recycling of iron.

An interesting observation that may be of less direct significance to physiology but may be of considerable experimental interest is the effect of overexpression of hCTR1 in renal cells. In MDCK cells that are engineered to overexpress hCTR1 under the control of a tetracycline-regulated promoter, high levels of overexpression result in the appearance of hCTR1 at both the apical and basolateral membranes. In these cells the exclusively basolateral expression of the Na,K-ATPase is unaffected, which implies that the trafficking pathways for the basolateral transport proteins, hCTR1 and Na,K-ATPase, are different. This conclusion is supported by our observation that overexpression of the basolateral β1-subunit of the Na,K-ATPase using this same promoter system does not cause any change in its localization (28).

If hCTR1 is in fact at the basolateral cell surface, how do we account for the uptake of dietary copper from the intestinal lumen? In intestinal cells the essential delivery of extracellular copper (in the blood) to intracellular target proteins that require copper is mediated by the uptake through hCTR1 at the basolateral plasma membrane. Existing models for copper absorption in the intestine by enterocytes follow conventional models of nutrient absorption. There are transporters responsible for uptake from the lumen at the apical cell surface and distinct transporters responsible for exit from the cell into the blood at the basolateral surface. It is apparent that the uptake of dietary copper, which takes place at the apical surface of intestinal cells, is mediated by a system other than hCTR1. The identity of that system is not clear, but some likely candidates can be considered.

There are at least three likely candidates. First, the apical uptake could be mediated by DMT1, the divalent metal ion transporter that has been shown to be at the apical surface of Caco2 cells (19). However, the lack of inhibition by Cd(II) ions, another high affinity substrate of DMT1 (26), as well as the lack of saturation of copper uptake at the apical surface argue against this possibility. DMT1 mediates a co-transport of protons (14, 19, 26), so that a thorough analysis of the apical extracellular pH dependence of copper uptake may be required before this possibility can be eliminated. On the other hand, it should also be pointed out that one previous study has shown that partial knock-down of DMT1 expression using an antisense oligonucleotide strategy results in a decrease in apical uptake of copper by Caco2 cells (15). Second, when copper uptake has been measured in ctr1−/− cells derived from mouse embryos, the rate of copper uptake is still about 30% that observed in wild-type animals (13). The hCTR1-independent uptake of copper in these cells was not inhibited by iron, the preferred substrate for DMT1. The identities of the protein(s) mediating this process are unknown, but they represent candidates for apical transport in the intestine. Third, it is possible that the uptake of copper at the apical intestinal membrane is not mediated by a copper-specific transporter but occurs by endocytosis. The lack of saturation of copper uptake at the apical membrane certainly supports this explanation but might also merely result from the action of an unidentified transporter with a very low affinity for metal ions. We are currently attempting to identify the components of the transepithelial uptake of copper in the intestine. These studies will be carried out under conditions that are optimized to dissect the contributions of the paracellular and transcellular pathways of transepithelial transport.

That intestinal hCTR1 is not essential for dietary copper uptake into enterocytes is implied by the results of some recent interesting experiments in mice. These studies employed animals in which only the intestinal hCTR1 gene was ablated. The mice had (counter-intuitively) higher levels of copper, but not other metals, in their intestinal cells than did wild-type animals (21). This implies that a non-CTR1-mediated pathway is involved in the intestinal cell accumulation. Copper-dependent enzymes in cells that lacked hCTR1 did not have their necessary copper, suggesting that the elevated copper was probably in a biologically unavailable pool. Thus, although hCTR1 is not involved in the apical entry of copper into intestinal epithelial cells, it is involved in making the copper that does enter biologically available. When hCTR1 is absent from the intestinal cells, the copper that (still) enters across the apical membrane does not enter the pool that renders bio-available the copper that is normally delivered from the blood. Also, because other tissues
in the mice lacking intestinal hCTR1 were copper-deficient, it implies that hCTR1 is needed for intestinal cells to be able to pass copper onto other tissues via the blood. hCTR1 is, thus, needed in the uptake from the blood and also in a less well-defined way to allow copper that enters the cell from the lumen to be biologically incorporated and passed onto other tissues.

In Fig. 10 we present a model that suggests how hCTR1 plays an indirect role in copper acquisition into the biologically available pool in intestinal epithelia after hCTR1-independent uptake from the lumen. The precise role of hCTR1 and the identity of the apical uptake system await characterization. Our model combines the basolateral location of hCTR1 demonstrated in the current work with a low affinity entry process we observe at the apical surface. The model also provides an explanation for why intestinal cells that selectively lack hCTR1 might be expected to still accumulate copper and yet not be able to pass it on to other tissues in the organism. The model also provides a function for the intracellular presence of hCTR1 recently reported in intestinal cells (21, 22, 28) and observed by us as sub-apical staining in mouse duodenal tissue.

A recent interesting examination of copper homeostasis in Drosophila showed that the Ctr1B, one of several copper import proteins in the fly, was apically located in fly intestinal cells (30). It is interesting that this Ctr1B protein is similar to the yeast Ctr3 and lacks the methionine motifs that are present in the human transporter, hCTR1. The closest homologs in flies to yeast Ctr3 and lacks the methionine motifs that are present in the human transporter, hCTR1 are Ctr1A and Ctr1C, whose roles in copper homeostasis remain to be elucidated.

Interestingly, in the case of the zinc transporters ZIP4 and ZIP5, it was known that both proteins were important in zinc homeostasis and that ZIP4 was important for the uptake of dietary zinc. It has been shown that ZIP5 functions to remove zinc from the blood via its location on the basolateral surface of the intestine, whereas ZIP4 can be recruited to the apical surface of enterocytes (31). It was also shown that ZIP5 locates specifically to the basolateral surface of MDCK cells (32). Thus, the uptake and homeostasis of zinc in the intestine occurs via the actions of two complimentary zinc uptake proteins, one at each membrane surface of the enterocytes. This situation is reminiscent of what we are suggesting occurs in copper homeostasis.

In summary, we have shown that endogenous hCTR1, the major human copper uptake protein, is exclusively localized at the basolateral membrane of intestinal epithelial cells. This localization is also maintained in renal cells. Our findings require a re-examination of the pathways involved in the homeostasis of dietary copper in humans. hCTR1 localization satisfies the synthetic needs of intestinal cells for copper, where it can mediate the uptake from blood, but its involvement in the absorption and bioavailability of dietary copper is less direct.

Acknowledgments—We thank Dr. Svetlana Lutsenko (Oregon Health Sciences University) for thoughtful comments on the manuscript and Dr. Angela Tyner (University of Illinois at Chicago) and Dr. Ann Hubbard (Johns Hopkins University) for kindly providing mouse intestinal sections.

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