Copper is taken up efficiently from albumin and α2-macroglobulin by cultured human cells by more than one mechanism

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Copper is taken up efficiently from albumin and α2-macroglobulin by cultured human cells by more than one mechanism. To examine this, the kinetics of uptake from purified human albumin and these plasma proteins. To examine this, the kinetics of uptake from purified human albumin and α2-macroglobulin, and the effects of inhibitors, were measured using human hepatic (HepG2) and mammary epithelial (PMC42) cell lines. At physiological concentrations (3–6 μM), both cell types took up copper from these proteins independently and at rates similar to each other and to those for Cu-dihistidine or Cu-nitrilotriacetate (NTA). Uptakes from α2-macroglobulin indicated a single saturable system in each cell type, but with different kinetics, and 65–80% inhibition by Ag(I) in HepG2 cells but not PMC42 cells. Uptake kinetics for Cu-albumin were more complex and also differed with cell type (as was the case for Cu-histidine and NTA), and there was little or no inhibition by Ag(I). High Fe(II) concentrations (100–500 μM) inhibited copper uptake from albumin by 20–30% in both cell types and that from α2-macroglobulin by 0–30%, and there was no inhibition of the latter by Mn(II) or Zn(II). We conclude that the proteins mainly responsible for the plasma-exchangeable copper pool deliver the metal to mammalian cells efficiently and by several different mechanisms. α2-Macroglobulin delivers it primarily to copper transporter 1 in hepatic cells but not mammary epithelial cells, and additional as-yet-unidentified copper transporters or systems for uptake from these proteins remain to be identified.

transcuprein; uptake kinetics; iron competition; silver competition; HepG2 cells; PMC42 cells

IN NORMAL HUMANS AND ANIMALS, most of the copper entering the blood in ionic form (such as after absorption from the intestine) goes directly to the liver and kidney before reappearing in blood plasma, attached to albumin and transcuprein, is the direct source of copper entering cells of the liver and kidney after copper entering blood is diverted from the liver and kidney to the mammary gland, where it rapidly incorporates into milk and milk ceruloplasmin. In preparation for studying what controls these different patterns of copper distribution among organs, we used cultured hepatic and mammary epithelial cell models to examine how copper is taken up from the blood plasma proteins to which it is firmly bound and to obtain clues that will help us identify the receptors and/or transporters involved, the expression and/or activity of which are regulated by lactational hormones.

Little is actually known about how copper is delivered to and enters mammalian cells, including those of the liver and mammary epithelium. This is despite numerous studies with various cell lines in culture. In normal (nonlactating) rats and humans, we know that ionic copper entering blood becomes part of an “exchangeable copper pool” in plasma (35–39, 58). This pool does not include ceruloplasmin, as ceruloplasmin copper is buried in the structure and not dialyzable at neutral pH. Rather, it is composed primarily or exclusively of copper bound to high-affinity sites on albumin (35, 39, 58). Indeed, as calculated by Rae et al. (56), there are virtually no “free” copper ions in biological systems. Copper on transcuprein and albumin is rapidly exchanged between them and in vitro is released only very slowly to various buffers, including those containing high concentrations of histidine (37, 38). This implies that for cell uptake, copper is being handed from one (or both) of these plasma proteins directly to transporters in the cell membrane and is not first released to amino acids (or present as the free ion) before binding to a transporter or transport system.

It has become evident that this phenomenon of “handing” copper from protein to protein is what occurs within cells as well, where a series of copper chaperones (proteins that transport incoming copper to specific intracellular sites/proteins) have been identified (23, 25, 27, 52–54). Thus, present evidence strongly implies that protein-bound copper in blood plasma, attached to albumin and transcuprein, is the direct source of copper entering cells of the liver and kidney after...
intestinal absorption or after it otherwise enters the blood in ionic form. Our previous study (16) using rats indicated that this is also the case with mammary epithelial cells during lactation. Several questions thus arise. The first is whether transcuprein/macroglobulin and/or albumin directly deliver copper to hepatic and mammary epithelial cells, and whether other plasma factors are required. If both proteins deliver copper, then the question is whether they interact with the same transport system in the cell membrane. The ultimate questions are of course the identity of the transporter or transport mechanisms involved and details about the mechanism(s) of entry.

As regards albumin, the results of several studies have suggested that it may actually impede rather than facilitate copper uptake. The groups of Ettinger and Mc Ardle showed using cultured rat and mouse hepatocytes and fibroblasts that the presence of albumin markedly lowered the initial rates of copper uptake supplied as Cu(II) with or without histidine (15, 48, 49, 64). In addition, we showed that copper uptake by the liver was not dependent on the presence of albumin (63). Thus, in analbuminemic rats, uptake of intraperitoneally injected tracer copper was (if anything) accelerated (63). Moreover, receptors for albumin have not been detected on hepatic cells. Thus, the function of copper binding to albumin in blood plasma may not be for it to deliver the metal to cells. Rather, it may have a protective role in plasma, allowing safe sequestration of excess copper ions entering the blood. [Because of its abundance in the plasma, albumin is capable of binding a great deal of copper and will do so when excessive amounts are administered (12).]

Transcuprein is a macroglobulin (44), and so an obvious possibility is that copper bound to this protein enters cells via receptor-mediated endocytosis, using the macroglobulin receptor (8, 59). Macroglobulins are well known for their ability to act as “traps” for proteases, clearing them from plasma. Hepatocytes have macroglobulin receptors, and these cells are thought to be the main removal route for macroglobulin complexes (59). Thus, for example, almost all 125I-labeled prostate-specific antigen-α2-macroglobulin injected intravenously into rats went directly to the liver (9), with small amounts also going to the kidney and spleen, the same initial distribution pattern that occurs with radioactive copper tracer (68). However, other cells also have these receptors, including fibroblasts (8), macrophages (30), retinal pigment epithelial cells (24), and those of the mammary epithelium (6).

Alternatively, or in addition, transcuprein and/or albumin may deliver copper to specific transporters on the cell membrane. The obvious possibilities are copper transporter (CTR)1 and divalent metal transporter (DMT)1 (also known as Nramp2 and DCT1). CTR1 is ubiquitous (52, 74) but expressed more highly in the liver and kidney than in most other tissues (28). However, CTR1 may not be the only transporter involved (33). DMT1 (also expressed in most tissues) does appear to play a role in the uptake of ionic copper across the intestinal brush border (3, 41). Then, there is the hepatocyte (and fibroblast) copper transporter studied by Ettinger and Mc Ardle and their collaborators (17, 35, 48) using CuCl2 or the di-histidine complex as a source, which does not appear to be identical to CTR1 or DMT1 (see below). In addition, there is CTR2, initially reported to be associated only with internal vesicles and organelles (57, 61) but now apparently also in the plasma membrane of some cells (5).

In this report, we focused on determining whether α2-macroglobulin (transcuprein) and albumin deliver copper directly to hepatic and mammary epithelial cells and initial characterization of the uptake mechanisms involved. We studied the uptake of copper from purified human proteins compared with Cu-di-histidine using cultured human hepatic and mammary epithelial cell models (HepG2 and PMC42 cells). The results reported here indicate that both plasma proteins independently deliver copper efficiently to both cell types but that they do so by mechanisms that differ not only with the protein involved but also among cell types.

MATERIALS AND METHODS

Cell culturing and uptake experiments. HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in MEM with nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), and 10% FBS at 37°C in 5% CO2. PMC42 cells, a human adenocarcinoma cell line derived from the pleural effusion of a breast carcinoma patient (69), were cultured in RPMI-1640 with nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), and 10% FBS. Measurements of rates of copper (and other metal ion) uptake were carried out in 6-well plates (HepG2 cells) or 12-well bicameral Transwell plates (Corning, Corning, NY) (PMC42 cells). In the latter case, 7.5 × 104 cells were plated on 1.5 diluted Matrigel (BD Biosciences, San Jose, CA) in each well and grown for 14 days in monolayers to reach a resistance of 300 Ω. Uptake experiments with 64Cu-labeled proteins [Cu(II)-dihistidine or Cu(II)-nitrilotriacetate (NTA) (1:5)] were carried out for 30 min in HEPES buffer [50 mM HEPES (pH 7.4) containing 5 mM glucose, 10 mM KCl, 1 mM MgSO4, and 130 mM NaCl], as previously described for Caco2 cell monolayers (41, 66) except that with the PMC42 cells, the metal ions were delivered from the basal chamber (“blood” side). (The copper content of the growth medium was in the range of 0.6 μM and that in the HEPES buffer was <0.1 μM.) Uptake rates were linear for at least 60 min. Uptake over 30 min was used to calculate initial rates and uptake kinetics. Uptake was total radioactivity in washed cells (HepG2 cells) plus (in the case of PMC42 cells) radioactivity in the apical medium. (Cells were washed with HEPES buffer containing 100 μM histidine.) Overall transfer across the monolayer was calculated from the radioactivity in the apical medium. Kinetic data were analyzed with Prism 5 software (Graphpad Software). 64Cu (specific activity: 20–300 mCi/μg) was obtained from Mallinckrodt Institute of Radiological Sciences at Washington University Medical School (St. Louis, MO). In one set of experiments, Caco2 cell monolayers were also used, as previously described (72), to follow the uptake of 59Fe(II) (1 μM) in 1 mM ascorbate and the same HEPES buffer. Other metal ions were added in the form of AgI(NO3), ZnCl2, and Mn(II)-histidine (1:10).

Purification of α2-macroglobulin from human plasma. Purification followed the protocol kindly provided by Salvatore Pizzo and his laboratory (Duke University, Durham, NC). For this, 300-ml batches of human plasma, obtained from subjects being treated for iron overload due to hemochromatosis (through the kindness of Dr. Richard Ajioka, University of Utah Medical Center, Salt Lake City, UT), and approval of our university Institutional Review Board (HSR no. 05-004), were fractionated with polyethylene glycol 8000, the 4–16% saturation fraction being subjected to Zn(II)-charged immobilized metal affinity chromatography (IMAC). For IMAC, the sample was applied to a 100 mM Na-phosphate buffer (pH 6.5) containing 800 mM NaCl. The column was washed with 20 mM Na-phosphate (pH 6.0) containing 150 mM NaCl, and α2-macroglobulin was eluted with 10 mM Na-acetate (pH 5.0) containing 150 mM NaCl. In some cases, samples were further purified by size exclusion chromatography (SEC) on Sephadryl S300 equilibrated with (low copper) 20 mM K-phosphate (pH 7.0). The resulting protein was pure, as shown in Fig. 1A, left,
which shows an overloaded native PAGE gel (4.5% acrylamide) stained with Coomassie blue. Treatment with 200 mM methylamine to convert the open form to the closed form of the protein (Fig. 1A) indicated that the open form had been purified, since it migrated more slowly in native PAGE. SDS-PAGE indicated subunits of 180 kDa (data not shown). Endogenous copper was removed by 2-day dialysis in 100 μM histidine in 20 mM Na-phosphate buffer (pH 7.0) and further dialysis into the HEPES cell incubation buffer. The resulting protein had <0.04 Cu atoms/subunit.

Purification of human albumin. Purification from human plasma was by a combination of pseudoaffinity chromatography with Cibacon blue (Affi-Gel Blue, Bio-Rad, Richmond, CA) using 20 mM Na-phosphate buffer (pH 7) to apply the plasma and wash the gel column and 1 M NaCl in the same buffer to elute the albumin, followed by SEC of concentrated extracts on Sephadex G150 or removal of residual contaminants with 50% saturation ammonium sulfate. Attached copper was removed by dialysis against histidine, as described for α2-macroglobulin. The resulting albumin was pure (Fig. 1A, right) and had <0.01 Cu atoms/molecule.

Copper loading of α2-macroglobulin and albumin. The stoichiometry of high-affinity copper binding to α2-macroglobulin was assessed by mixing 1.25 nmol of the copper-free tetrameric protein with various amounts of 64Cu-labeled Cu(II)-NTA (1:2 molar ratio) in the presence of 5 nmol albumin and in 20 mM K-phosphate (pH 7.0). After 1 h of incubation, the resulting 0.5-ml mixture was separated on 25-ml (1 × 25 cm) Sephadex G150 columns. Columns were stan-

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**Fig. 1.** Purity and copper loading of α2-macroglobulin (α2M) and its retention in conditioned medium. A: native PAGE of purified α2M (left) and SDS-PAGE of purified Alb (right). In the case of α2M, samples shown were (+) and were not (−) pretreated with methylamine to convert the open form to the closed form of the protein. SDS-PAGE gave subunits of 180 kDa (data not shown). B–D: Sephadex G150 chromatographic separation of 64Cu-labeled α2M, Alb, and Cu-nitrilotriacetate (NTA) after a preincubation with 5 nmol of the pure α2M subunit, 5 nmol of pure Alb, and 1.25 (B), 2.5 (C), and 7.5 (D) nmol of 64Cu-labeled Cu(II) (as the NTA complex) in 0.50 ml (see MATERIALS AND METHODS). Nanomoles of copper bound to α2M were calculated from the proportion of radioactivity associated with that protein peak. For A, B, and C, the proportions were 63%, 54%, and 26%, respectively. E: elution of radioactive copper on α2M after use in uptake experiments with cultured (PMC42) cells. Conditioned medium, from cultures incubated with 2 μM 64Cu-labeled copper bound to pure human α2M, was separated by Sephadex G150 chromatography, as in B–D, except that a slightly larger (1 × 28 cm) column was used. Radioactivity was still bound to α2M (also verified by immunoblot analysis of 64Cu peak fractions).
Copper is bound mainly to these proteins. For comparison, plasma (35, 36) and concentrations of associated with macroglobulin per milliliter of human and rat increasing amounts of 64Cu-labeled Cu(II)-NTA (see MATERIALS and METHODS) in the presence of 5 nmol of albumin and then radioactivity in the two peaks obtained by SEC reflected that proportions of copper bound to each protein. Figure 1, B–D, shows examples obtained with three different amounts of added copper. Immunoblot analysis and column standardizations verified that the first peak contained only Cu-α2-macroglobulin (M₇, 770 k) and the second peak only Cu-albumin (M⁷, 70 k).

The first thing evident was that, when very little copper was added (1.25 nmol), a larger proportion bound to the macroglobulin than albumin, confirming what we have already known for some time, namely, that transcuprein (α₂-macroglobulin in humans and α₁-inhibitor-3 in rodents) has an even higher affinity for copper than albumin. When larger amounts of copper were added, increasing proportions bound to albumin (Fig. 1, C and D). The amount bound to α₂-macroglobulin appeared to plateau at 0.5 Cu atoms/subunit, or 2 Cu atoms/molecule of 4 subunits. This is in the range of what might be expected from in vivo data that showed that ~100 ng Cu associated with macroglobulin per milliliter of human and rat plasma (35, 36) and concentrations of ~1 mg/ml α₂-macroglobulin (59). This ratio of Cu to protein was used in all subsequent uptake experiments.

The question was now whether Cu, attached to either albumin and α₂-macroglobulin, would be taken up by cells at rapid rates. To study this, we used copper-protein concentrations (1–2 μM) at the low end of the physiological range, where ~6 μM copper is bound mainly to these proteins. For comparison, we also examined the uptake of 1 μM Cu from the dihistidine complex. As shown in Fig. 2A, both cell types took up copper from both proteins rapidly. At 1 μM Cu, the rates of uptake from albumin (Fig. 2A, hatched bars) and α₂-macroglobulin (Fig. 2A, shaded bars) were in the same range. This was true for both cell types (Fig. 2A, left vs. right), although in both cases, rates for Cu-albumin were significantly lower (P < 0.001). In hepatic cells (Fig. 2A, left), the rates of uptake from copper histidine were in the same range as those for Cu on α₂-macroglobulin. In the mammary epithelial cell model (PMC42 cell monolayers; Fig. 2A, right), Cu-dihistidine was significantly more effective than either of the copper proteins at this concentration (P < 0.001). It should be noted that PMC42 cells were in the form of a monolayer with tight junctions, growing on filters in bicameral chambers, and 64Cu-loaded proteins (Cu-histidine) were applied on the basolateral (blood) side. These results showed clearly, for the first time, that both albumin and the macroglobulin were independently
capable of transferring copper directly to cells. Uptake of copper as the free ion was not occurring (after dissociation of the copper from the proteins) except perhaps in the process of binding to a transporter, transport system, or reductase on the cell surface. No low-molecular-weight $^{64}$Cu was detected in the conditioned medium (example in Fig. 1E) fractionated on SEC columns, as shown in Fig. 1, B–D, and dialysis of $^{64}$Cu-loaded proteins in PBS resulted in <5% release over 24 h [as previously described (35)]. Preliminary EPR analysis indicated specific copper binding (J. Goto, M. Moriya, and M. Linder, unpublished observations).

To assess whether the uptake of copper from albumin would also be robust if only a small proportion had copper bound to it (as is the case in vivo), we also examined uptake from tracer $^{64}$Cu-NTA-treated whole plasma fractions separated by SEC, as shown in Fig. 1. B–D. With whole plasma, the tracer bound to albumin versus macroglobulin fractions at a ratio of 14:1, and the ratio of apo to holo Cu-albumin was ~250:1. Nevertheless, as shown in Fig. 2B, there was good uptake of copper. More was taken up from albumin than from the macroglobulin because the peak albumin fraction had ~10 times more $^{64}$Cu than that of the macroglobulin, and there was ~150 times more albumin than macroglobulin offered to the cells on a molar basis (~46 μM albumin and ~0.3 μM macroglobulin).

Kinetics of copper uptake from albumin, α₂-macroglobulin, and histidine. The next step was to monitor the effects of different concentrations of Cu-loaded proteins on the initial rates of uptake and to compare them with the results for Cu-dihistidine for both cell types. Figure 3 shows the results for HepG2 cells. Covering the range of copper concentrations (as Cu-protein complexes) from 0.1 to 20 μM, there appeared to be one saturable uptake mechanism for Cu delivered on α₂-macroglobulin (Fig. 3A). Software analysis indicated a $K_m$ of 3 μM and a $V_{max}$ of 22 pmol·min$^{-1}$·mg cell protein$^{-1}$. Thus, the uptake mechanism for copper delivered by this protein was sensitive and efficient. For Cu-albumin, there appeared to be two separate uptake mechanisms in the physiological range from 0.1 to 6 μM (Fig. 3, B and C): one higher-affinity, lower-capacity system ($K_m$ 0.36 μM and $V_{max}$ 2.0 pmol·min$^{-1}$·mg cell protein$^{-1}$; Fig. 3B) and the other a lower-affinity, higher-capacity system ($K_m$ 2.4 μM and $V_{max}$ 18 pmol·min$^{-1}$·mg cell protein$^{-1}$; Fig. 3C), resembling that for Cu-α₂-macroglobulin. The data for Cu-dihistidine (Fig. 3D) suggested there might be three different uptake systems, with $K_m$ values of ~1, 4, and 10 μM, with the latter two having the highest uptake capacities. Kinetic analysis of the data indicated one low-capacity ($V_{max}$ 2.9 pmol·min$^{-1}$·mg cell protein$^{-1}$), high-affinity ($K_m$ 0.6 μM) system and ambiguous kinetics.

![Fig. 3. Kinetics of copper uptake by HepG2 cells from α₂M, Alb, and His. A: initial rates of copper uptake (in pmol·min$^{-1}$·mg cell protein$^{-1}$) from α₂M at different Cu-α₂M concentrations, from 0.1 to 20 μM Cu. Kinetic analysis indicated a $V_{max}$ value of 22 pmol·min$^{-1}$·mg$^{-1}$ and a $K_m$ value of 3 μM. B: initial rates of copper uptake from Alb at concentrations from 0.1 to 0.4 μM. Kinetic analysis indicated a $V_{max}$ value of 2.0 pmol·min$^{-1}$·mg$^{-1}$ and a $K_m$ value of 0.36 μM. C: Cu-Alb uptake data for 0.4–10 μM gave a $V_{max}$ value of 18 pmol·min$^{-1}$·mg$^{-1}$ and a $K_m$ value of 2.4 μM. D: initial rates of copper uptake from Cu(II)-di-His at concentrations from 0.1 to 20 μM (left) and from 0 to 3.5 μM (right). The latter gave a $V_{max}$ value of 2.9 pmol·min$^{-1}$·mg$^{-1}$ and a $K_m$ value of 0.6 μM. Data for higher concentrations did not yield standard kinetic values.](http://ajpcell.physiology.org/)
above 3 µM Cu. Except at the lowest copper concentrations, uptake of Cu from histidine was higher than that from the two proteins, being about twice as high at 4 µM and three to four times higher at 10 µM. Thus, apart from plasma proteins being able to deliver copper at about the same rate as Cu-histidine in the physiological range of copper concentrations of the exchangeable copper pool (3–6 µM or below 10 µM), the data indicated that there was more than one mechanism of uptake in the case of ionic copper or that delivered on albumin, in contrast to a single mechanism in the case of Cu-α2-macroglobulin.

Figure 4 shows the data for uptake by mammary epithelial cells (PMC42 cells). Uptake of copper from α2-macroglobulin (Fig. 4A) was as robust as for hepatic cells but had different kinetics, with a lower $K_m$ (22 vs. 3 µM) but higher capacity ($V_{max}$ 100 vs. 22 pmol·min$^{-1}$·mg cell protein$^{-1}$). In contrast to what occurred with HepG2 cells, the kinetics of Cu uptake from albumin did not show a saturable transport system in the range of 0–4 µM (Fig. 4B). Rather, uptake rates increased almost linearly with Cu-albumin concentrations up to 25 µM. This suggested that Cu-albumin might be diffusing paracellularly through the monolayer. However, we have shown when cells are grown in bicameral chambers under the conditions used, the monolayers have tight junctions and a resistance of 300 Ω (which was the case here as well) and thus contain no gaps through which ions could diffuse. In addition, the application of [14C]mannitol or phenol red to the basal chamber did not result in leakage into the apical chamber under the same conditions. The overall kinetics of the uptake systems for Cu-dihistidine (Fig. 4C) in PMC42 cells had some similarity to those for HepG2 cells (Fig. 3D), but kinetic analysis indicated a lower-affinity ($K_m$ 21 µM) saturable system with twice the capacity. In PMC42 cells, the uptake of copper from histidine was again about twice that for α2-macroglobulin and fourfold higher than from albumin. Uptake of copper from the NTA complex was also examined in PMC42 cell monolayers (Fig. 4D) and showed even higher rates of uptake ($V_{max}$ 140 pmol·min$^{-1}$·mg cell protein$^{-1}$) and a $K_m$ value of 11 µM in the physiological range (Fig. 4D, bottom) and increasing rates at higher concentrations (Fig. 4D, top). Taken together, the results of these kinetic experiments in two very different kinds of cells indicated that there were one or more uptake mechanisms with different kinetics for Cu delivered either tightly bound to albumin or α2-macroglobulin or kept soluble in the medium by chelation with histidine or NTA.

The mammary epithelial cell monolayers (PMC42 cells) allowed us not only to measure rates of copper uptake but also the overall transfer (of the entering copper radioisotope) from

Fig. 4. Kinetics of copper uptake by PMC42 cells from αM, Alb, His, and NTA. A: initial rates of copper uptake (in pmol·min$^{-1}$·mg cell protein$^{-1}$) from αM at concentrations from 0.1 to 15 µM Cu. Kinetic analysis indicated a $V_{max}$ value of 100 pmol·min$^{-1}$·mg cell protein$^{-1}$ and a $K_m$ value of 22 µM. B: initial rates of copper uptake from Alb at concentrations from 0.1 to 25 µM gave values for $V_{max}$ and $K_m$ values of 194 pmol·min$^{-1}$·mg cell protein$^{-1}$ and 131 µM, respectively. C: initial rates of copper uptake from Cu(II)-di-His concentrations of 0.1–10 µM gave $V_{max}$ and $K_m$ values of 206 pmol·min$^{-1}$·mg cell protein$^{-1}$ and 21 µM, respectively. D: initial rates of copper uptake from Cu(II)-NTA concentrations of 0.1–20 µM (left) and 0–8 µM (right). Only the latter gave kinetic values for $V_{max}$ (140 pmol·min$^{-1}$·mg cell protein$^{-1}$) and $K_m$ (11 µM).
the basal to apical medium. Transfer from cells to the apical medium would represent copper secreted across the apical membrane in these polarized monolayers, akin to what occurs when milk is secreted. The data shown in Fig. 5 show rates of appearance of copper in the apical medium with different Cu-protein (or chelate) concentrations administered to the basolateral side of the monolayer. Calculations of copper release rates (in pmol·min⁻¹·mg cell protein⁻¹) were made on the basis of ⁶⁴Cu specific activity in the basolateral medium. Rates generally increased in linear fashion in relation to basal copper concentrations, and rates were similar for Cu on both plasma proteins and Cu-histidine in the physiological range (<10 μM). For Cu-NTA, rates were higher. Cu-albumin was the least efficient, and there was a distinct lag in apical secretion until the Cu-albumin concentration exceeded 3 μM, implying potential differences in the intracellular routes taken by copper entering from the two plasma proteins.

Differentiation of copper uptake mechanisms by responses to the presence of high concentrations of other metal ions. The uptake systems or transporters that could be involved in the uptake of copper may at least in part also absorb other transition metal ions. Large concentrations of such potentially competing ions would thus be expected to inhibit uptake of copper, depending on which transporter is involved. For example, the hepatic copper uptake system characterized by Ettinger et al. (17) was markedly inhibited by Mn(II) and Cd(II) but not by Ni(II) and only marginally inhibited by Zn(II) at molar ratios of 5:1 (inhibitor:Cu) and using 10 μM Cu(II)-dihistidine. DMT1 is also used by ions of iron, copper, and manganese (3, 19, 22, 41, 60), although its importance for Fe(II) uptake has been emphasized. CTR1, on the other hand, is thought to be more specific but is inhibited by (the rare) silver ion [Ag(I)] (32, 33, 41, 60), although its importance for Fe(II) uptake has been emphasized. CTR1, on the other hand, is thought to be more specific but is inhibited by (the rare) silver ion [Ag(I)] (32, 33, 41, 60). If the transporters involved in uptake of copper from albumin and transcuprein (and histidine or NTA) are identical, one would expect that various, potentially competing metal ions would identically influence copper uptake from these proteins and chelators. We thus examined the effects of ions of iron and silver on copper uptake from these factors in both cell types. Cells were incubated with Cu-proteins or Cu-chelators at two concentrations (1–2 and 8–10 μM Cu) in the presence and absence of high concentrations (50–200 μM) of the potentially competing metal ions.

The results of our experiments with HepG2 cells are shown in Fig. 6. High (50 μM) concentrations of iron [as Fe(II)-NTA] had little or no statistically significant inhibitory effects on the uptake of copper delivered by either α₂-macroglobulin, albumin, or histidine (Fig. 6A, top), and this was the case at both higher and lower concentrations of the Cu-complexes tested (see Fig. 6A for indications). In contrast, the uptake of 10 μM copper from NTA was significantly (~40%) impaired. Much higher concentrations of Fe(II)-NTA (200 μM) had a more severe inhibitory effect on 10 μM copper uptake from NTA (60% inhibition), and the same was the case for 10 μM copper delivered on histidine (Fig. 6A, bottom). These high concentrations of Fe(II) also inhibited copper delivery from plasma proteins but to a lesser degree. Thus, the uptake mechanisms active at high concentrations of copper chelate were only affected by very large amounts of iron, and there was a much smaller or no effect on the uptake mechanisms for copper delivered on albumin and α₂-macroglobulin (perhaps because proteins might scavenge some of the iron under these very unphysiological conditions and thus mitigate harm to the uptake systems).

The effects of Ag(I) were much more significant with regard to the uptake of copper from plasma proteins by hepatic cells (Fig. 6B). Here, there was a clear distinction between the delivery of copper on albumin versus α₂-macroglobulin: uptake from the latter was markedly inhibited, but there was only a slight inhibitory effect on uptake from albumin. This was true at both concentrations of silver ions (Fig. 6B, top and bottom), and, in the case of 8 μM Cu-albumin, 50 μM Ag(I) actually stimulated copper uptake. The presence of high concentrations

Fig. 5. Transfer of copper from mammary epithelial cells (PMC42 cells) to the apical medium after delivery of ⁶⁴Cu-labeled copper on α₂M (A), Alb (B), His (C), or NTA (D) to the basolateral surface of the monolayer. Data are ⁶⁴Cu absorbed from the basal chamber that was released to the apical medium after the administration of various concentrations of Cu attached to the proteins or chelators, using the specific radioactivity of the original ⁶⁴Cu for calculations of release rates (in pmol·min⁻¹·mg cell protein⁻¹).
of ascorbate during uptake of copper from \( \alpha_2 \)-macroglobulin did not alter uptake rates (Fig. 6C), implying no (additional) need for copper reduction, but the presence of this reductant somewhat enhanced the inhibitory effects of Ag(I) (compare shaded and solid bars, Fig. 6C). These results clearly indicate that albumin and \( \alpha_2 \)-macroglobulin mainly deliver copper to different cellular uptake systems in HepG2 cells and suggest that the macroglobulin is interacting with CTR1 [inhibited by Ag(I)]. Silver ions did inhibit 10 \( \mu \)M (but not 1 \( \mu \)M) copper uptake by HepG2 cells from histidine [50% with 50 \( \mu \)M Ag(I) and 70% with 100 \( \mu \)M Ag(I)], but there was no significant inhibition of uptake from Cu-NTA (Fig. 6B), suggesting some involvement of CTR1 with copper uptake from histidine but not NTA.

The same parameters were investigated with PMC42 cell monolayers (Fig. 7). As in hepatic cells, iron had little or no effect on the uptake of copper from the two plasma proteins (Fig. 7A) except in the case of 1 \( \mu \)M Cu-albumin, where 20–30% inhibition was observed. Uptake from histidine was also relatively unaffected. In the case of silver ions (Fig. 7B), there also were no statistically significant inhibitory effects on the uptake of copper from albumin, histidine, or NTA. In contrast to HepG2 cells, Ag(I) also had no effect on the uptake of 10 \( \mu \)M Cu delivered as the dihistidine complex. More importantly, uptake of copper from \( \alpha_2 \)-macroglobulin was not inhibited. This was a highly reproducible result and confirmed kinetic indications that the two cell types take up copper from \( \alpha_2 \)-macroglobulin by different mechanisms.

**Effects of Mn(II) and Zn(II) on copper uptake from \( \alpha_2 \)-macroglobulin by HepG2 cells.** Since the hepatocyte transporter characterized some years ago by Ettinger et al. (17, 35) showed that uptake from Cu-dihistidine by hepatocytes was inhibitable by manganese and zinc ions, we examined whether this was the case for copper delivered to HepG2 cells on \( \alpha_2 \)-macroglobulin. As shown in Fig. 8, excess of neither Mn(II) nor Zn(II) inhibited uptake. The addition of Mn(II) at concentrations of 50 and 200 \( \mu \)M (Fig. 8A, shaded and solid bars), as the 1:5 histidine complex, made no difference in the uptake of 1 \( \mu \)M Cu from \( \alpha_2 \)-macroglobulin. Neither did 100 and 400 \( \mu \)M concentrations of histidine alone (hatched and striped bars). Zinc (200 \( \mu \)M; Fig. 8B) also had no effect.

**No effects of Ag(I) on Fe(II) uptake by Caco2 cell monolayers.** To eliminate the possibility that the inhibitory effects of Ag(I) in HepG2 cells were due to the inhibition of DMT1, we investigated whether uptake of Fe(II) across the brush border of Caco2 cell monolayers [an excellent model for the intestinal mucosa (3, 41, 43, 73)] would be affected by this metal ion. It is well established that in this cell model as well as the intestinal mucosa, in vivo, DMT1 is the primary (and perhaps only) transporter for the uptake of Fe(II) from the intestinal lumen. As shown in Fig. 9, no inhibition was observed at ratios of Ag to Fe of 25:1 and 50:1. This indicates that the transport...
function of DMT1 was not inhibited by silver ions. Therefore, HepG2 cell Cu uptake from α2-macroglobulin (inhibited by silver) cannot be ascribed to DMT1.

**DISCUSSION**

The study described here was initiated as a first step to understand not only how copper is taken up from blood plasma by mammalian cells but how this is regulated in the physiological condition of lactation, during which considerable copper is transferred from the mother to the newborn through milk. In this condition, a substantial proportion of copper associated with very abundant albumin and 40% with 2-macroglobulin (transcuprein) enters the mammary gland and milk and less is taken up by the liver (16). This suggests that specific transporters or transport systems for copper are upregulated in mammary epithelial cells and downregulated in hepatic cells during the suckling period. These transporters might include CTR1, DMT1, the uptake system described by Ettinger et al. (17), and even CTR2 as well as receptor-mediated endocytosis via the macroglubulin receptor.

The cell line we used to model the mammary epithelial cell (PMC42) was originally established from the pleural effusion of a breast carcinoma patient (69). Based on morphological criteria and responsiveness to lactational hormones, the cells have retained many of the characteristics of the mammary epithelium. In culture, PMC42 cells differentiate into several cell types, with some showing characteristics of secretory cells (swollen endoplasmic reticulum and secretory vesicles) and others characteristics of myoepithelial cells (contractile fibers). As with primary cultures of the breast epithelium (13, 14), PMC42 cells grown as monolayers as organoids on filters coated with extracellular matrix can be stimulated toward milk production by sequential treatment with estradiol and progesterone and then insulin, hydrocortisone, and prolactin (1, 70, 71). These treatments not only induce the secretion of lipid (characteristic of milk) but synthesis of the milk-specific protein β-casein. The cell line also expresses a range of luminal-specific markers (cytokeratin 18 and cell adhesion molecule E-cadherin) (2) and has the morphology of mammary epithelial cells, with apical microvilli and nuclei in the cell basal region. Like human breast epithelial cells, they express CTR1 and both ATP7A and ATP7B (Ref. 50; A. Michalczyk and L. Ackland, unpublished observations). The hepatic cell model used (HepG2) also retains many of the characteristics of normal hepatocytes and expresses CTR1, DMT1, and the macroglubulin receptor (Ref. 55; M. Moriya and M. Linder, unpublished observations).

Since exchangeable copper in blood plasma is tightly bound to proteins (yet exchangeable), and the same is the case within cells, we would expect that protein-protein interactions are required for transfer of the metal ion from blood carriers to the transport systems on the cell surface. Binding and cell uptake might then be driven by mass action or, alternatively, coupled with enzymatic reduction, as occurs in the case of Fe(III) seeking to enter the enterocyte from the intestinal lumen. In the latter, a cell surface reductase (duodenal cytochrome b) produces Fe(II), which can then cross the apical membrane through DMT1 (51). Analogously, a copper reductase [NADH oxidase (62)] on the hepatocyte or mammary epithelial cell surface might reduce Cu(II) attached to albumin or macroglubulin to make it available for entry via CTR1.

The results reported here clearly show that both plasma proteins that tightly bind ionic copper in blood plasma efficiently and independently deliver the trace element to the two cell types investigated. Moreover, they do so as efficiently as complexes of ionic copper with amino acids. Human blood plasma (like that of the rat) contains ~1,200 ng Cu/ml, 65–70% of which is on ceruloplasmin and/or other components of that size (12, 35, 36). About 350 ng Cu/ml (6 μM) is with the exchangeable copper pool, which is composed principally (or exclusively) of albumin and α2-macroglobulin (transcuprein). Estimates from SEC profiles has indicated that ~60% of that is associated with very abundant albumin and 40% with α2-macroglobulin (44). Thus, the concentration of plasma copper in the exchangeable pool is 5–6 μM, with albumin-Cu at 3–3.6 μM and α2-macroglobulin-Cu at ~2–2.4 μM. α2-Macroglob-
findings that copper from 20–40 pmol·min⁻¹·mg cell protein⁻¹ with Cu-dihistidine (25–60 with Cu-NTA). Where saturable, $K_m$ values for the uptake systems for copper from these proteins were in or somewhat above the physiological range. With Cu-α2-macroglobulin, the kinetics indicated a single saturable uptake system for both cell types, but with different kinetics. The system in HepG2 cells was of much higher affinity, with half the capacity of that in PMC42 cells. In addition, the former but not the latter was inhibited by Ag(I), suggesting the involvement of CTR1. Thus, different uptake systems for Cu-α2-macroglobulin are involved. For Cu-albumin, different uptake mechanisms may also be operating in the two cell types, as the kinetic analysis indicated saturable systems in the physiological range (to 10 μM) and a much lower-affinity, higher-capacity one (although with similar uptake rates) in PMC42 cells. Uptake kinetics for Cu-dihistidine and Cu-NTA were more complex and ambiguous, consistent with multiple carriers and diffusion, especially at higher (unphysiological) concentrations. [Earlier studies implicated diffusion in the uptake of ionic copper by the rat intestinal mucosa at high copper concentrations (10–90 μM) (11).] There was little or no inhibition by Ag(I) or Fe(II) except at very high concentrations of the latter.

The major points and significance of these findings are that both plasma proteins are capable of donating copper to more than one cell type independently and that uptake occurs by different mechanisms both within and between cell types. Uptake of copper from these proteins is just as efficient as when delivered as ionic copper complexes (including Cu-dihistidine). As previously reviewed (36, 39), there is no solid evidence that Cu-histidine actually exists as a complex in blood plasma, although it may form a trimeric complex with Cu-albumin (31, 35). Size exclusion fractionations of blood plasma have never indicated the presence of radioactive or actual copper in components of the size of amino acids. In our recent HPLC-inductively coupled plasma-mass spectroscopy study upon itself is present at a concentration of ~1 mg/ml, or 1.3 μM; albumin is ~70% of plasma protein, and thus ~710 μM. From these calculations, the molar ratio of Cu to α2-macroglobulin would be ~2:1 and that for albumin ~0.005:1. Our findings that ~2 Cu atoms bind 1 tetrameric α2-macroglobulin molecule fits closely with the estimates just described and indicates that the high-affinity copper-binding sites on this protein are more or less saturated in vivo. The high-affinity copper-binding sites on albumin, on the other hand, are far from saturated in vivo (which has been clear for a long time). These data add strength to the finding that α2-macroglobulin has an even higher affinity for copper than albumin, as also indicated by the preferential binding of copper to the macroglobulin in the presence of albumin seen in our loading experiments.

The experiments we carried out with pure Cu-albumin and Cu-α2-macroglobulin were done with concentrations right in the physiological range. In HepG2 cells, the maximum rates of uptake in the physiological range of 3–6 μM Cu were 12–15 pmol·min⁻¹·mg cell protein⁻¹ with Cu-α2-macroglobulin, 10–15 pmol·min⁻¹·mg cell protein⁻¹ with Cu-albumin, and 7–20 pmol·min⁻¹·mg cell protein⁻¹ with Cu-dihistidine. In the mammary epithelium-type cells (PMC42), the maximum rates of uptake in the same physiological range were 10–20 pmol·min⁻¹·mg cell protein⁻¹ with Cu-α2-macroglobulin, 5–13 pmol·min⁻¹·mg cell protein⁻¹ for Cu-albumin, and
(12), the lowest molecular weight component detected in mouse, human, and rat plasma was ~11 kDa. Binding constants of albumin for copper at the high-affinity site have ranged from $10^{12}$ to $10^{17}$ M, with $10^{12}$ M for the albumin-Cu-histidine complex, compared with a constant of $10^{17}$ M for histidine (31, 46). Moreover, albumin (at 700 μM) is present in much higher concentrations than histidine (30–100 μM), and the constant for α₂-macroglobulin is higher than that for albumin, all of which indicate that copper would preferentially bind to these two proteins in plasma rather than histidine, the amino acid with the highest copper affinity. This is of course also the case within cells, where copper is tightly bound to, and distributed to, other proteins and compartments by its protein chaperones (52, 53).

In regard to the potential involvement of CTR1 in the uptake of copper from α₂-macroglobulin by HepG2 (but not PMC42) cells, copper transport through CTR1 is known to be inhibited by Ag(I) (32, 33, 75), and this metal ion markedly inhibited copper uptake from the macroglobulin in hepatic cells, where CTR1 is known to be highly expressed. However, we now know that at least one other copper transporter (CTR2) is also inhibited by Ag(I) (5). It seems less likely that CTR2 is involved, because it is not abundantly expressed in hepatic cells (5) and is mainly confined to the membranes of internal vesicles and organelles (57, 61), although it has been detected in the plasma membrane of transfected COS-7 cells (5). Still other transporters, not yet identified, might also be inhibited by Ag(I). The study of Ettinger et al. (17) characterizing a hepatic copper transporter in primary rat and mouse hepatocytes did not examine the effects of Ag(I). However, that transport system (which used Cu-dihistidine) was markedly inhibited by Mn(II) and Zn(II), and we found no such inhibition with copper delivered on α₂-macroglobulin, tending to confirm the concept that CTR1 is involved. [Further definitive studies are in progress to answer these questions.] It should be noted that CTR1 is thought only to transport Cu(I), which could mean that the copper bound to α₂-macroglobulin is in that state. The alternative, namely, that Cu(II) on transferrin requires a cell surface reductase (47, 62) for the release and binding of its copper to CTR1, seems unlikely, as large concentrations of ascorbate capable of reducing Cu(II) had no effect on uptake rates. Our data also suggest that uptake from higher concentrations of Cu-dihistidine also partly involves CTR1 in HepG2 (but not PMC42) cells.

It also seems unlikely that in our experiments Cu-α₂-macroglobulin was delivering copper (even partly) through receptor-mediated endocytosis via the macroglobulin receptor, which is well known to be abundant on hepatocytes (18, 67) and has also been identified on mammary epithelial cells (5). The macroglobulin receptor takes up the compact form of this protein, which is produced when the “bait region” inside the subunits is cleaved [as by proteases (59)], resulting in a marked conformational change that exposes regions to interact with the receptor and remove it from plasma (18, 20, 67). We purified and used the “open” form of the protein, which is the most abundant in blood plasma. In addition, although both cell types are thought to express the macroglobulin receptor, our data indicated that macroglobulin was delivering copper to different uptake systems in the two cell types, one inhibited and the other not inhibited by Ag(I).

It also seems unlikely that DMT1 is involved, since we observed no inhibition by excess iron (at a ratio of 25:1) on copper uptake from α₂-macroglobulin and the same was true for excess manganese, which is also thought to use DMT1 for uptake (19, 22, 60). This is consistent with extensive further evidence from our laboratory using not just cell culture but also the Belgrade rat, which produces an inactive form of DMT1 (M. Moriya, Y. H. Ho, E. Sauble, and M. Linder, unpublished observations), showing that copper uptake is unaltered. In regard to the uptake system(s) to which albumin delivers copper, there was no inhibition by silver or ferrous iron at a ratio of 50:1 and only 20% inhibition at a ratio of 100:1, indicating that neither CTR1, CTR2, nor DMT1 are likely to be involved. Although the uptake of copper from albumin by hepatocytes and fibroblasts has previously been demonstrated (17, 48, 49, 65), our study is the first to examine Cu-albumin uptake kinetics. Our HepG2 cell data indicated two saturable systems in the physiological range that were not seen in PMC42 cells, where uptake rates were almost linear with Cu-albumin concentrations, indicating a difference in the response of the two cell types to this form of copper.

Previous studies had shown that the addition of given concentrations of albumin to solutions of CuCl₂ or Cu-dihistidine decreased uptake of copper by rat hepatocytes, fibroblasts, and even trophoblasts (7, 45, 64). For example, albumin added in at a 1:1 molar ratio to Cu(II) inhibited uptake by rat hepatocytes by 50% and about eightfold in the case of fibroblasts (65). Histidine added to CuCl₂ also decreased uptake rates (64). A single, more-detailed published study where the uptake of copper in the presence of a range of albumin concentrations was studied showed that the protein had an increasingly inhibitory effect (7). Uptake of 2 μM $^{67}$Cu-dihistidine by rat hepatocytes fell dramatically with the addition of increasing amounts of albumin, falling from ~90 to ~8 pmol·min⁻¹·mg cell protein⁻¹ when albumin concentrations reached 0.1 mg/ml or more (>90% lower rates). This might suggest that with actual plasma albumin concentrations (which are orders of magnitude higher, ~45 mg/ml), copper uptake rates might dwindle to nothing. However, we found that when $^{64}$Cu tracer (picogram amounts) on albumin (obtained by addition to whole human plasma) was used for uptake experiments, rapid uptake still occurred (Fig. 2B). In this case, only ~0.4% of the total was bound to the metal. The idea of albumin inhibiting copper uptake in vivo thus seems untenable.

The rates of copper uptake and the $K_m$ values we obtained with our two cell types were of the same order of magnitude but lower than those reported by others for primary cultures of primary hepatocytes. For example, Waldrop et al. (65) reported rates of 40 pmol·min⁻¹·mg⁻¹ for rat hepatocytes with 5 μM Cu(II)-albumin compared with our rates of ~12 pmol·min⁻¹·mg⁻¹ for HepG2 cells. Bingham and McArdle (7) obtained rates of 80 pmol·min⁻¹·mg⁻¹ with rat hepatocytes and 2 μM Cu-dihistidine, 10-fold higher than our rates with HepG2 cells but only 4-fold higher than what we obtained with the mammary epithelial cell line. In regard to the kinetics of uptake from Cu-dihistidine in HepG2 cells, our evidence for a high-affinity ($K_m$ 0.6 μM), low-capacity saturable system and nonsaturable kinetics at higher concentrations differs from that of Ettinger et al. (17) for primary hepatocytes, where the $K_m$ value was in the range of 11 μM. All of these differences are
most likely due to differences between primary and immortalized cells.

The strong implication of our findings that CTR1 plays little or no role in uptake of copper from the blood plasma proteins to which it is bound, especially in the mammary epithelial cell model, may at first seem counterintuitive, as this transporter is ubiquitously expressed in mammalian cells and clearly does play a role in copper uptake elsewhere. Kuo et al. (28) demonstrated strong expression in the epithelium of ducts and alveoli of the mouse mammary gland in lactation; Kelleher and Lonnerdal (26) showed expression in the lactating gland of rats. However, the data from both these groups indicated that, normally, most or all of CTR1 was confined to cytoplasmic organelles, in part colocalizing with transferrin-receptor-containing endosomes (26), and thus largely unavailable for mediating uptake at the cell surface. Treatment of the cells with prolactin, or having pups suckle the rat dams (inducing prolactin release), greatly and transiently enhanced the proportion of CTR1 on the plasma membrane (26), which correlated with a 50% increase in the rate of copper uptake (from $^{67}$CuCl$_2$). Moreover, prolactin induced CTR1 to migrate both to the basolateral and apical portions of HC11 cells, suggesting that it plays a role not just in the uptake of copper from blood but also in the reuptake from developing milk (unless CTR1 might work in both directions, which is not inconceivable). Since our experiments with PMC42 cells were done in the absence of prolactin, when little or no CTR1 might be on the basal cell surface, it is not surprising that Ag(I) did not inhibit copper uptake. (Studies on the effects of prolactin are in progress.)

In all, our findings indicate that although CTR1 does play a role in uptake of copper from blood by hepatocytes, this is only when delivered by $\alpha_2$-macroglobulin and does not happen in mammary cells. More importantly, and as detailed earlier, our study indicates that the remaining known uptake systems that could be involved in the uptake of copper from albumin and $\alpha_2$-macroglobulin (DMT1, macroglubulin receptor, CTR2, and the Ettinger system) do not play a major role. Uptake from Cu-albumin also differs between the two cell types, particularly at lower copper concentrations, as judging from the initial kinetics of copper release from mammary epithelial cell monolayers at the apical surface, in response to Cu-albumin and Cu-macroglobulin in the basal medium, additionally implicates either that copper delivered by these proteins enter by mechanisms that lead to different intracellular pools and routes across the cell and into the apical fluid and/or that the two Cu-proteins trigger different intracellular responses of copper transporters/transport mechanisms that lead to apical copper release.

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