Published in final edited form as:

J Nutr Biochem. 2007 September ; 18(9): 597–608. doi:10.1016/j.jnutbio.2006.11.005.

Transcuprein is a Macroglobulin Regulated by Copper and Iron Availability

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SUMMARY

Transcuprein is a high affinity copper carrier in the plasma involved in the initial distribution of copper entering the blood from the digestive tract. To identify and obtain cDNA for this protein, it was purified from rat plasma by size exclusion and copper chelate affinity chromatography, and amino acid sequences were obtained. These revealed a 190 kDa glycosylated protein identified as the macroglobulin, α_1 inhibitorIII, the main macroglobulin of rodent blood plasma. Albumin (65 kDa) co-purified in variable amounts and was concluded to be a contaminant (although it transiently can bind the macroglobulin). The main macroglobulin in human blood plasma (α_2 -macroglobulin), homologous to α_1 inhibitorIII, also bound copper tightly. Expression of α_1 I3 (transcuprein) mRNA by the liver was examined in rats with and without copper deficiency, using quantitative PCR and Northern analysis. Protein expression was examined by Western blotting. Deficient rats with 40% less ceruloplasmin oxidase activity and liver copper concentrations expressed about twice as much α_1 I3 mRNA, but circulating levels of transcuprein did not differ. Iron deficiency, which increased liver copper concentrations 3-fold, reduced transcuprein mRNA expression and circulating levels of transcuprein relative to what occurred in rats with normal or excess iron. We conclude that transcupreins are specific macroglobulins that not only carry zinc but also transport copper in the blood; and that their expression can be modulated by copper and iron availability.

Keywords

Transcuprein; α_1 inhibitorIII; α_2 macroglobulin; copper; regulation; mRNA; liver; iron

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INTRODUCTION

Transcuprein was first identified as a copper transport protein in the blood plasma of rats after injection or intragastric administration of trace quantities of high specific activity $^{67}\text{Cu}(\text{II})$ [1]. Immediately after treatment, or upon direct addition of the radioisotope to plasma samples, ^{67}Cu associated with two plasma proteins: albumin, and a 270 kDa component that did not react with antibodies against albumin or ceruloplasmin. The latter was named transcuprein. By following the time course of their ^{67}Cu -labeling in vivo, transcuprein and albumin were shown to participate in the initial distribution of copper to tissues [1–3]. In this initial distribution, most of the copper first deposited in the liver and kidney [1,4]. Transcuprein and albumin appeared to be the main sources of copper for this deposition: Not only were they the first plasma components binding the radioisotope, but radioactive copper bound to them was rapidly lost as it was gained by liver and kidney, with the kinetics of a precursor/product relationship [1,5]. From the liver (and perhaps also the kidney) [3], a major portion of the copper that just entered reemerged in the plasma on ceruloplasmin [1,3] which, in turn, was found to be a major source of copper for other tissues [6–8]. Although there was some copper associated with the amino acid fraction [4,9], repeated studies in rats indicated little or no initial ^{67}Cu labeling of this fraction [1,3]. (The copper with albumin may however be in the form of a histidine/Cu/albumin complex [10].) Thus, transcuprein and albumin appear to be the primary components of the exchangeable copper pool of plasma and interstitial fluid. At physiological pH, radiolabeled copper bound to either protein can exchange with excess ionic $\text{Cu}(\text{II})$ in the medium or be removed by high concentrations of chelating agents, including histidine [3,4]. Ceruloplasmin, on the other hand, is not a participant in the exchangeable pool. Although it accounts for two thirds or more of the copper in rat and human blood plasma, its copper is not dialysable and is incorporated during ceruloplasmin synthesis and secretion by the liver [2,4,11].

Both albumin and transcuprein bind copper very tightly. (The same binding characteristics occurred when radioactive copper was added in vivo or in vitro to whole plasma [1,3,4,15].) Most albumins have an N-terminal copper binding site of very high affinity, involving a histidine residue in the third position [6,12–14], with dissociation constants variously reported as from 10^{-11} [14] to 10^{-22}M [12], similar to values for copper metallothionein (10^{-17} – 10^{-19} [4]). The abundance of albumin provides the blood plasma with a huge potential for binding excess copper: enough to bind up to 40 μg of Cu per ml of plasma. Yet, the total copper in plasma is only about 1 $\mu\text{g}/\text{mL}$, and of that only a small fraction is actually bound to albumin. Although present in much lower amounts than albumin, transcuprein is able to successfully compete for $\text{Cu}(\text{II})$ in the blood plasma [1,4,5,9].

Transcuprein and albumin rapidly exchange copper with each other [1,15]: When ^{67}Cu -transcuprein is mixed with non-radioactive albumin, the label instantly re-distributes to both proteins, and vice versa, depending upon their relative concentrations. Since the off-rate for Cu from these proteins is very slow [3,4], the rapidity of exchange indicates that transcuprein and albumin do this by direct interaction. Protein to protein exchange also appears to be the way copper is distributed within cells via so called copper chaperone proteins [16–20]; in fact, it has been calculated that there is less than one free copper atom

per cell [21].) This mode of transport may protect against potentially destructive effects of free copper ions and chelates [22,23].

Although albumin is normally involved, it appears that transcuprein alone can target incoming copper to liver and kidney in the initial distribution phase, as shown in analbuminemic rats [5]. This suggests that transcuprein rather than albumin is the actual donor to hepatocytes and kidney cells.

Transcuprein has an apparent molecular weight of 270 k [1]. Upon purification, during which the presence of the protein was followed with ^{67}Cu , it was initially found to be composed of proteins of about 200 and 65 kDa, although traces of material in the range of 100 kDa were sometimes also detected [15,24]. The studies reported here were designed to obtain cDNA for these transcuprein in order to characterize and further study the protein and its copper transport function. The approach taken was to obtain N-terminal and internal amino acid sequence to be used in RT-PCR cloning and screening. As will be shown, the large “subunit” of transcuprein was identified as a member of the macroglobulin family, one form of which is already recognized as a carrier of the metal ion, zinc [25]. The small “subunit” was due to albumin contamination. In analogy with the iron carrier (transferrin) and iron availability [26,27], copper availability appears to influence expression of transcuprein, as does iron status.

METHODS AND MATERIALS

Animals and treatments

Untreated adult Sprague Dawley rats obtained from Simonson Laboratories (Gilroy, CA) were the source of the blood plasma used to purify transcuprein. To examine effects of copper and iron deficiency, batches of rats (usually 10) were placed on distilled water and a pelleted low copper or low iron diet, designed by E.A. Ulman in accordance with AIN guidelines [28] and produced by Research Diets, Inc. (New Brunswick, NJ). The low copper diet (0.4–0.6 ppm Cu) contained 20% protein (casein, + 0.3% DL-methionine), 66% carbohydrate (45% cornstarch, 10% maltodextrin, 10% sucrose, 5.0% cellulose), 5.0% fat (corn oil), 3.5% salt mix S18101 without added copper, 1.0% vitamin mix V10001 (+ 0.2% choline bitartrate). The low iron diet (about 5 ppm) was identical except that the “salt mix” contained normal amounts of cupric carbonate and no iron salts. Half of each batch of rats was made copper or iron-normal, the former by providing cupric sulfate (7.86 mg/L) in the drinking water, the latter by injecting 25 mg of iron as iron dextran (Iron Dextran injection, Phoenix, Inc., St. Joseph, MO) i.p., after 2–3 weeks on the diet. Rats with excess iron were given an extra 25 mg Fe injection 5 days before sacrifice. Rat tissues and plasma were analyzed after 6–7 weeks on the diets. All protocols were approved by the university IACUC.

Human plasma (IRB HSR# 05-004)

Blood plasma from normal individuals was obtained from volunteers through the Student Health Center. That from subjects being treated for iron overload due to hemochromatosis

was obtained from the University of Utah Medical Center in Salt Lake city, courtesy of Dr. Richard Ajioka. Samples were stored frozen at -20°C .

Purification of transcuprein

Purification was by procedures previously described [24], using blood plasma labeled *in vitro* with ^{67}Cu (II) or ^{64}Cu (II) (10–50 ng actual Cu/ml) added as the 1:1 molar complex of nitrilotriacetate. ^{67}Cu (II) and ^{64}Cu (II) were obtained as chloride salts in 0.1M HCl from the reactor at the University of Missouri (Columbia) and from MIR Radiological Sciences at George Washington University (St. Louis, MO), respectively. In some cases, purification was followed by copper analysis. Size exclusion chromatography was on Sephadex G150 and Sephacryl S300 (Sigma, St. Louis, MO), using 20 mM K phosphate buffer, pH 7.0, with 20 mM ϵ -amino caproic acid (to inhibit nicking by plasmin) and 0.02% NaN_3 . Copper chelate affinity chromatography was on Sepharose 6B-bound iminodiacetic acid (Pharmacia Biotech, Piscataway, NJ) precharged with Cu(II), for which the equilibration buffer was the same phosphate buffer (size exclusion chromatography) but with added 0.5 M NaCl. Elution of copper binding proteins was induced by a linear gradient of 10–40 mM imidazole, followed by 40 mM imidazole, in the same buffer. In some cases, a combination of pseudoaffinity, ion exchange, and size exclusion chromatography was applied, as described for the isolation of α_1 inhibitorIII [29]. Here, ^{67}Cu -labeled plasma was incubated with Cibacron blue Sepharose CL-4B gel (Sigma) for 30 min, with shaking, and the effluent collected by filtration. The latter was applied to DEAE-Sepharose CL-6B chromatography (Sigma) after dialysis into equilibration buffer (50 mM Tris.HCl, pH 7.4, containing 50 mM NaCl). After washing, elution was with a linear gradient of from 0.10–0.40 M NaCl in the same buffer. Purification by the procedures of Lonberg-Holm et al. [30], involving polyethylene glycol, was also carried out.

Copper and ceruloplasmin determinations

Copper was determined by furnace atomic absorption spectrometry, using a Varian Zeeman 800 instrument (Sugarland TX), as previously described [31,32]. Plasma/serum samples were assayed directly; liver samples were wet ashed in trace element grade nitric acid plus H_2O_2 prior to analysis. Ceruloplasmin oxidase activity was determined with p-phenylene diamine, as previously described [31].

Ferritin iron determinations

Liver homogenates (10% tissue) were heated to 70° for 10 min to obtain a heat supernatant, which was titrated with antiserum against horse spleen ferritin to maximally precipitate the ferritin, as previously described [33]. Washed immunoprecipitates were assayed for ferritin iron by the bipyridyl method, upon heating with Na bisulfite [33].

SDS-PAGE and immunoblotting

These procedures were performed as previously described [31,32], using 1.5 mm slab gels in the Hoefer Mighty Small electrophoresis system, High Range Protein Standards (BioRad, Hercules, CA), gel staining with Coomassie Brilliant Blue G250 (BioRad), and transfer to Immobilon PSQ membranes (Millipore, Bedford, MA). The primary antibody against rat

transcuprein was raised in sheep against two synthetic polypeptides from different portions of the transcuprein large subunit that were linked as haptens to keyhole limpet hemocyanin antigen (prepared for us by Chiron, Clayton, Victoria, Australia). For immunoblotting, the secondary antibody was alkaline phosphatase-conjugated donkey anti-sheep IgG (Sigma). Other primary antibodies employed for immunoblotting were raised in goats against human albumin (Sigma), binding being detected with alkaline phosphatase-conjugated rabbit-anti-goat IgG (Biomed, Foster City, CA). In some cases, purified transcuprein samples were incubated overnight at 37° in 0.25M Tris-HCl buffer, pH 8.0, containing 0.05M phenanthroline and 5% NP40, with and without 200–250 mU/mL peptide-N-glycosidase F (Sigma) after denaturation in 0.25% SDS/0.05M mercaptoethanol.

Immunoprecipitation and extraction

Rocket immunoelectrophoresis was carried out as previously described [34], using antibodies against human alpha-2-macroglobulin raised in goats (BioRad) or rabbits (Sigma). Samples containing radioactive copper were developed by autoradiography, using a phosphorimager, or stained for protein with Coomassie Brilliant Blue. For analysis of the proteins in the immunoprecipitates, unstained rockets were cut from the gel and extracted by homogenizing with SDS-PAGE sample buffer and heating, prior to SDS-PAGE and immunoblotting. Alternatively, immunoprecipitation was carried out by treating partially purified plasma extracts with protein A immobilized on cross-linked 4% beaded agarose (Sigma, P2545) to remove endogenous immunoglobulins, then incubating with primary antibody, and precipitating the complexes with protein A macrobeads (Sigma, P1925) in 20 mM phosphate buffered saline (pH 7.0; PBS). Washed precipitates (3x with cold PBS) were boiled with SDS-PAGE sample buffer to extract the antigenic proteins for SDS-PAGE separation. Primary antibodies included those raised in rabbits against human ceruloplasmin (Sigma) and horse spleen ferritin (Dako, Carpinteria, CA).

Amino acid sequencing

Protein bands of transcuprein were separated on pre-aged 7.5% acrylamide resolving gels in SDS-PAGE. For N-terminal sequencing, performed at the UCLA Protein Microsequencing Facility, bands were transferred to Immobilon PSQ membranes (Millipore) and lightly stained with Coomassie Brilliant Blue G250 (BioRad). For internal sequencing, subunits in stained SDS-PAGE gels were cut out, minced, and subjected to in-gel digestion with modified trypsin (Promega, Madison, WI) in 200 mM NH_4HCO_3 , overnight at 37°, with shaking, followed by repeated extraction with 1% trifluoroacetic acid in 60% acetonitrile. Concentrated extracts were sent to the California Institute of Technology Protein/Peptide Micro Analytical Laboratory, for separation of peptides and sequencing.

RNA extraction and Northern analysis

Total RNA was extracted from liver tissue with RNAzol B (Tel-Test, Inc., Friendswood, TX). Total RNA was separated in formaldehyde gels containing 1.0 % agarose [32] and transferred to nylon membranes (Zeta-Probe; BioRad) by capillary action. Membranes were hybridized with random-primer labeled cDNA plasmid inserts, using ^{32}P -dCTP and the RapidHyb reagents and protocol of Amersham Pharmacia Biotech (Piscataway, NJ). After high stringency washing (once in 2X SSC, 0.1% SDS, 20 min at room temp, then twice in

0.1 SSC, 0.1% SDS, at 42° for 15 min), membranes were exposed to x-ray film (Kodak X-Omat, Rochester NY) for autoradiography. Loading was assessed by comparing ethidium bromide staining of 28 S and 18 S rRNA bands on the gels and membranes (evaluated by densitometry) and directly by hybridizing the same membranes and/or parallel membranes with cDNA for glyceraldehyde phosphate dehydrogenase. Autoradiographs were recorded by the SpeedLight Photoimaging System (San Diego, CA).

Quantitative PCR of α_1 inhibitorIII (transcuprein) mRNA

This was performed using the strategy and reagents provided by the Mimic system of Clontech (Palo Alto, CA). A 340 base pair portion of the nucleotide sequence of α_1 inhibitorIII mRNA was identified (Fig. 1) that had low homology to the other macroglobulin expressed by rat liver (α_1 macroglobulin). To amplify this sequence by RT-PCR of rat liver total RNA, oligonucleotide primers were designed and obtained from Ana-Gen Technologies (Palo Alto, CA). The sequences of the α_1 inhibitorIII-specific primers (distinguishing between it and α_1 -macroglobulin) were:

(+) 5'-ATGAGCAGGTCCTCATCAAAGC (22 nucleotide; Tm 64° C)

(-) 5'-ACAATCTGTTGCAGCACTC (19 nucleotides, Tm 58° C)

Mimic primers were:

(+) 5'-ATGAGCAGGTCCTCATCAAAGCCGCAAGTGAAATCTCCTCCG (42 nucleotides)

(-) 5'-ACAATCTGTTGCAGCACTCATTTGATTGTGGACCATGGC (39 nucleotides)

Underlined portions of sequence are identical to those of the α_1 inhibitorIII-specific primers.

RESULTS

Purification and sequencing of transcuprein

Transcuprein was purified from 1–10 ml batches of ^{67}Cu (II)-labeled rat plasma, by various combinations of procedures, as described in Methods. The most rapid and useful method consisted of taking the void volume fraction obtained by applying the plasma to Sephadex G150 size exclusion chromatography, and fractionating it on the larger pore size exclusion gel, Sephacryl S300, followed by copper-chelate affinity chromatography using an imidazole gradient for elution of bound protein. Examples are given in Fig. 1. As seen previously [1,5], some of the added radioactive Cu(II) associated with a peak in the void volume of Sephadex G150 (Fig. 1A, black dots) and the rest with the second 280 nm-absorbing peak which coincides with albumin elution [1]. Also shown is the analysis of actual copper (by furnace atomic absorption spectroscopy) (X---X). A significant portion of copper eluted in the void volume. The largest portion of the copper was with ceruloplasmin (eluting between the main A280 peaks), which is not labeled by in vitro ^{64}Cu or ^{67}Cu (II) addition; and a small amount was with albumin (seen as a shoulder to the right of the ceruloplasmin peak). (Previous studies had shown that whether added in vitro or in vivo, the

radiotracer on rat plasma transcuprein behaved the same with regard to its binding affinity and release of radioactive tracer in response to non-radioactive Cu(II) and other agents [4].)

Following application of the void volume peak to Sephacryl S300 (Fig. 1B), radioactivity eluted in the middle of the column volume (M_r about 270 k), in conjunction with a major peak of protein (A_{280}). When the radiocopper-labeled transcuprein fractions were applied to the copper-chelate gel column, the bound protein eluted with high concentrations of imidazole (35–40 mM; Fig. 1C), upon application of the 10–40 mM gradient (up-angled line). Varying amounts of actual copper (black dots; determined by atomic absorption) eluted roughly in parallel with the protein. It is noteworthy that when pre-labeled, the radioactive copper associated with transcuprein survived the copper-chelate chromatography and was not displaced (data not shown).

The protein eluting from the copper chelate columns was analyzed by SDS-PAGE. Examples from several purifications and different portions of the A_{280} peak are shown (Figs. 2A). Protein bands of 180–200 and 65–69 kDa were always present, and their combined apparent molecular weights added up to the M_r value of 270 kDa obtained for transcuprein [1]. However, the 65–69 kDa component was present in varying proportions, suggesting it might be a contaminant. In addition, there often were 1–2 diffuse bands in the region of 100 kDa. These accumulated with time as the density of the 190 kDa component diminished (data not shown), indicating that these were degradation products of the larger protein. Occasionally, contaminants of 145 and 45 kDa were also detected (Fig. 2A, 3d and 4th examples). The 190 and 66 kDa components, as well as the 145 kDa contaminant, were processed and sent for N-terminal and internal amino acid sequencing.

The 190 kDa protein of transcuprein was identified as the major rat macroglobulin, α_1 inhibitorIII [35]. The full sequence of this protein (in precursor form), as well as the sequences we obtained (underlined) are given in Fig. 3. The N-terminal sequence of the 190 kDa SDS-PAGE band corresponded exactly to residues 25–39 of the precursor protein, suggesting that the first 24 residues constitute its signal sequence for synthesis on the ER-bound polyribosomes. The internal sequence obtained corresponded exactly to residues 154–171 of α_1 inhibitorIII, the main macroglobulin in rodent plasma [36]. Both sequences also had homology to human α_2 macroglobulin residues 29–43 (46%) and 157–174 (72%), respectively, the main macroglobulin in human plasma [36]. The apparent molecular weight of the 190 k component of transcuprein was in the range of that reported for α_1 inhibitorIII (180–210 k) [37], variability being due to 10–23% carbohydrate. Moreover, incubation of this with peptide-N-glycosidase F at 37° for 20 h reduced the apparent molecular weight by 19 k, or 10% (Fig. 2B).

The 145 kDa contaminant of the purified rat transcuprein was identified by sequencing as rat α_1 macroglobulin: The N-terminal 16 amino acids being identical. α_1 Macroglobulin has another subunit fragment of 45 kDa [30], and a subunit of this size accompanied the 145 kDa contaminant (Fig. 2A, example 3). The 65–69 kDa component was rat albumin, and this was confirmed several times.

To confirm the macroglobulin identity of transcuprein, it was purified from ^{67}Cu -labeled rat serum by methods used for isolation of α_1 inhibitorIII [29,30]. Using a combination of Cibacron blue pseudoaffinity chromatography [30] (to remove albumin), fractionation of the flow-through on DEAE Sepharose [30] (Fig. 4), followed by Sephacryl S300 chromatography of the DEAE ^{67}Cu peak, the sample in Fig. 2A (4th example) was obtained. This showed the same components as with the previous methodology: 190 and 66 kDa, as well as the 145 kDa contaminant.

All of this indicated that the major (large) component of transcuprein is α_1 inhibitorIII, a member of the macroglobulin family. However, this is the major macroglobulin only in rodents, while in humans and most other mammals it is α_2 macroglobulin. α_2 Macroglobulin is known to be a major carrier of zinc in human blood [25] and has been shown also to bind copper ions in vitro [38]. All macroglobulins can bind specific proteins and are particularly known for their ability to trap proteases to render them harmless [36]. Proteases can cut the 180 kDa subunit in half, resulting in two fragments in the range of 90 kDa [39], such as encountered by us in some preparations (Fig. 2A, 1st and 2nd examples).

α_2 Macroglobulin has a high homology to α_1 inhibitorIII, particularly in its histidine and cysteine-rich region (see later). To see whether α_2 macroglobulin might be the transcuprein of human plasma, we added trace amounts of radiolabeled copper and subjected samples to rocket immuno-electrophoresis (with rabbit anti-human α_2 macroglobulin), using autoradiography to develop the gel. Consistent with this concept, highly radioactive rockets were obtained (Fig. 5), indicating that this macroglobulin also binds ionic copper tightly.

The question was then whether albumin was truly a component of transcuprein as well, or just a contaminant. The fact that albumin and transcuprein rapidly exchange copper with each other [1,15] indicates they can bind to each other. More consistent with contamination, variable amounts of albumin ended up in the purified transcuprein preparations, and attempts to completely remove it from purified transcuprein with Cibacron blue gel beads were not successful. It was also present in washed transcuprein immunoprecipitates. However, albumin also showed up in immunoprecipitates of other plasma proteins. Results from some of these experiments are presented in Fig. 6. SDS-PAGE of proteins extracted from the rockets obtained by immunoelectrophoresis of human plasma with antibody against human α_2 macroglobulin (Fig. 6A) showed a major band in the region of 190 kDa, a band of 72 kDa (consistent with albumin), and one about 57 kDa and near the bottom (probably the heavy and light chains of IgG, respectively; unpublished data). However, rockets from immunoelectrophoresis of human plasma ($\pm$ horse spleen ferritin) with antibodies against not only α_2 macroglobulin but also human ceruloplasmin or horse spleen ferritin, all also contained albumin, as shown in the sample Western blot in Fig. 6B. Similar results were obtained for immunoprecipitates of rat transcuprein and ceruloplasmin (data not shown; see legend to Fig. 6B). Thus, washed immunoprecipitates of various plasma proteins all showed albumin contamination.

We had shown previously that copper bound to transcuprein, and copper uptake by liver and other tissues proceeded normally in the absence of albumin (Nagase albuminemic rats) [5]. This proves that albumin did not have to be part of transcuprein. Taken together with the

other data, we thus conclude that it consists of only one kind of protein, namely α_1 inhibitorIII (in rats) and α_2 macroglobulin (in humans).

The copper content of some of the purified transcuprein preparations obtained was also examined. Calculated values for 4 purified preparations ranged from 1–13 Cu atoms per molecule of 190 kDa, and there was no correlation between copper content and the degree of albumin contamination. However, we think that measured copper contents do not reflect the actual situation for transcuprein in vivo, and that it picked up additional copper during its purification, since the binding in whole plasma is more like 0.5 copper atoms per 190 kDa. This calculation is based on a ballpark concentration of 1 mg macroglobulin/mL and 120 ng Cu/mL in the transcuprein fraction. The ability to bind additional copper atom, but with complex stoichiometry, was confirmed by in vitro equilibrium dialysis studies (Jeremy Goforth, unpublished data).

Effects of dietary copper and iron availability on expression of transcuprein

Since transcuprein is involved in plasma copper transport, it seemed possible that expression of this protein would vary in relation to the availability of copper to the liver, which produces this protein for the circulation. This is the case for transferrin, the plasma protein involved in iron transport: Its rate of synthesis and blood plasma concentrations are increased by iron deficiency [26,27,40,41]. To begin to test this concept, we examined the expression of transcuprein mRNA by livers of rats on copper deficient and copper sufficient diets. In two different studies, weanling rats were placed on starch-based low copper diets for 6–7 weeks. Half were given deionized water to drink, the other half with added copper. In both studies, copper deficiency was induced, as evidenced by a 40% lower plasma ceruloplasmin oxidase activity and liver copper concentration (Table 1, A). Expression of transcuprein mRNA was assessed in three ways: by competitive and non-competitive PCR and by Northern analysis, based on the known nucleotide sequence of rat α_1 inhibitorIII mRNA. Total RNA was extracted from the livers of copper normal and copper deficient rats. For PCR, this was reverse transcribed into cDNA and analysed for transcuprein mRNA content.

Fig. 7A shows an example of the results of applying the quantitative PCR Mimic system of Clontech (see Methods). As the concentration of competitor (Mimic) cDNA decreased (from left to right), the amount of transcuprein cDNA-amplified product (target) versus Mimic cDNA product increased. In the case of the cDNA transcribed from mRNA of the deficient rats (lower panels), this was clearly more competitive with the Mimic cDNA: At all Mimic cDNA concentrations, the proportion of target (transcuprein) amplification product was greater than with the cDNA from copper-sufficient (normal) livers (upper panels). Calculations of the actual change in two trials indicated that copper deficiency increased transcuprein mRNA concentrations 65 and 83%. Direct, non-competitive PCR of the cDNA from normal and deficient rats (Fig. 7B) gave similar results, with increases of 60 and 106% by densitometry.

The same result was obtained by Northern analysis, using a 340 bp cDNA fragment of α_1 inhibitorIII cDNA as the probe (Methods). Fig. 8A shows an example of the RNA loading of a membrane after transfer from an ethidium bromide-containing agarose gel. Fig. 8B

shows the corresponding autoradiographic bands obtained with the transcuprein probe (above) and with the control probe (glyceraldehyde-3-phosphate dehydrogenase; below). Clearly, more expression was observed in the case of RNA from the copper deficient livers.

Since iron status can influence copper metabolism, and vice versa [4,42,43], potential effects of iron deficiency were also examined by Northern analyses. As shown in Figures 8C and D, iron availability correlated positively with transcuprein mRNA expression. There was little detectable expression in the RNA from iron deficient rat livers as compared with RNA from normal livers (Fig. 8C, D), and expression was even higher with iron treatment (Fig. 8C). Indeed, a comparison of the data suggested that the availability of iron may be more important in determining the amount of transcuprein mRNA expressed than the availability of copper, in that the magnitude of changes was larger. However, it is noteworthy that the level of copper in the liver was elevated by the induction of iron deficiency (Table 1, B), and this could be a factor.

The expression of transcuprein protein was also examined by immunoblotting, using a polyclonal antibody raised against two synthetic polypeptides of portions of the α_1 inhibitorIII protein (Methods). As shown in Figure 9A (and other immunoblots, not shown), there was a weak positive relationship between transcuprein levels and iron status when comparing plasma samples from deficient and non-deficient animals, apparent densities of the bands increasing about 35% (from deficiency to normal), from 38 ± 5 to 51 ± 2 , respectively (Mean $\pm$ SD; n=4). In contrast, we could not detect a clear difference in the samples from copper deficient and supplemented rats (Fig. 9B). However, using standards, we found that the method was insufficient to detect less than a 50% difference (implying that the small difference detected with iron deficiency might be an underestimate). A positive correlation between iron status and human transcuprein appeared to be present in humans as well. Thus, several samples of plasma from subjects with hemochromatosis had more α_2 macroglobulin than those of normal subjects (Figure 5).

DISCUSSION

As already described, transcuprein was the name given to a large, copper binding protein in the blood plasma of rats that was labeled with tracer ^{67}Cu (II) immediately after injection of ng portions of the radioisotope or upon direct addition of the tracer to aliquots of blood plasma or serum [1,5]. Profiles of the distribution of actual copper among rat [1,4] and human [6,44,45] plasma fractions, separated by size exclusion chromatography, have shown repeatedly that a significant portion of plasma copper is associated with the void volume (transcuprein) fraction. In the case of the rat, and using both tracer ^{67}Cu and actual copper contents as markers, we isolated the protein with which this copper is associated. We found that in rats, transcuprein is the macroglobulin, α_1 inhibitorIII [35,37], the main macroglobulin found in rodents; that the main macroglobulin found in human plasma (α_2 macroglobulin) also strongly binds ionic ^{64}Cu tracer; and concluded that the albumin isolated with transcuprein is not essential for its function but due to contamination and the ability to interact with the macroglobulin. We also demonstrated that expression of transcuprein/ α_1 inhibitorIII in rat liver at both the mRNA and proteins levels is influenced by nutritional copper status, as well as by iron status (which can influence copper status).

Moreover, preliminary results comparing α_2 macroglobulin levels in plasma from normal human subjects and those with iron overload (hemochromatosis) was consistent with the findings for the rat.

At first glance, the finding that a copper transport protein belongs to the family of macroglobulins might be surprising. However, the multifunctionality of proteins is becoming more and more apparent. Some examples include the iron regulatory protein, IRP1, which functions both as an aconitase (converting citrate to isocitrate) and as a regulator of the translation and stability of mRNAs for proteins of iron metabolism; albumin itself, which carries many unrelated nutrients and is also an amino acid store; ceruloplasmin, which serves as a ferroxidase, a radical scavenger, and also is the preferred source of copper for placenta, heart and brain [3,7,8]. Macroglobulins are best known for their ability to bind and inactivate proteases [36,38], which are then cleared from the circulation [36] via macroglobulin receptors on hepatocytes [46,47], with a half-life in rats of about 7 minutes [47]. [This might explain the rapid uptake of copper from transcuprein by the liver.] However, macroglobulins also have other, seemingly unrelated properties and functions. Thus, we know that they bind and may clear or modulate the function of several small non proteolytic proteins, including basic polypeptides [48] and some hormones (platelet-derived growth factor [49] and cytokines [50,51]). [Interestingly, there are other connections with hormones: Expression of α_1 inhibitorIII (and α_2 macroglobulin) is controlled by the cytokines, IL-6 and TNF α [52], as well as by glucocorticosteroids [53]; and insulin receptor phosphorylates the macroglobulin [48].] α_1 Microglobulin [46], a lipokalin that transports small hydrophobic substances and may also have a role in the extracellular matrix, also binds to these macroglobulins. This binding prevents α_2 macroglobulin from acting as an antiprotease. More important with regard to our studies, α_2 macroglobulin is a major carrier of Zn(II) in the blood plasma of humans [25,38, 54, 55], and perhaps also in milk [56], with 2–5 Zn ions binding per subunit, and a dissociation constant in the range of 10^{-7} M [55]. Other previous studies showed that human α_2 macroglobulin also binds copper ions, in vitro [38]; and we have some evidence that transport of Ag(I) in the blood of rats occurs on another macroglobulin, α_1 macroglobulin [56].

The current work confirms that human α_2 macroglobulin binds copper tightly, doing so in the presence of the much more abundant, high affinity copper binding protein, albumin, and retaining it during rocket immunoelectrophoresis, which is carried out over many hours (typically overnight, at 4°). [The copper binding was not due to albumin contamination of the rocket immunoprecipitates, since we have confirmed that completely purified α_2 macroglobulin also binds copper tightly (A. Grana, M. Moriya, and M.C. Linder, unpublished).] These findings and the facts that (a) there is copper associated with proteins in the void volume fraction of Sephadex G150 (where α_2 macroglobulin elutes) when human plasma or serum is fractionated [4,9,44,45]; and (b) α_2 macroglobulin (hardly present in rodents) [36,58] and α_1 inhibitorIII (not present in humans) have a great deal of sequence homology, particularly in histidine and cysteine-rich regions, allows designation of the former as the transcuprein of human plasma. [There is much less homology between α_1 macroglobulin (present in rodents and non-rodents [36,46,59]) and α_1 inhibitorIII (or α_2 macroglobulin).] Both rat α_1 inhibitorIII [35] and human α_2 macroglobulin [60] contain a similar, histidine-rich region (between residues 395 and 600) that has 12 and 13 histidines,

respectively, 7 of which are conserved. Three of the conserved histidines are part of a conserved motif HXEAHHTAY. This motif also contains the EAH sequence which constitutes the high affinity copper binding site of human albumin (albeit without the N-terminal amino group). The same region extended to about residue 700 also contains 5 conserved cysteines that could be involved in copper binding, one of which is also part of the thiolester group of the “bait” region of these proteins (where proteases bind and get trapped. [Pratt and Pizzo [38] have reported that binding of copper to human α_2 macroglobulin in vitro eliminates its antiprotease activity (protease trapping).]

In further support of the involvement of transcuprein/macroglobulin in copper transport, we found that liver expression of the mRNA for α_1 inhibitorIII was enhanced by copper deficiency. The effect was not large, although it was reproducible. The level of liver mRNA about doubled with a copper deficiency that decreased plasma ceruloplasmin oxidase activity and liver copper concentrations about 40%. A similar (and similarly modest) inverse relationship to the availability of the metal it carries has been well documented for transferrin [26,27,40,41], the blood carrier of iron, also produced by the liver. In iron deficiency with a reduction in blood hemoglobin of 25–30%, human transferrin blood levels rise about 50% [40], and at least in some species there is a similar increase in liver mRNA levels for the carrier [41]. Although we were unable to detect a significant change in circulating levels of transcuprein, the immunoblotting method employed would not have detected such a small change in magnitude. In the case of transferrin and iron status, the mechanism involved in liver hepatocytes is still poorly understood (especially for the human) and may involve not just transcriptional but also translational control [26,61]. The transcriptional regulation appears to involve binding of unknown transcription factors to two sites within the first 125 bp of the transferrin gene promoter [26,62] to stimulate expression, and/or other factors that bring a region further upstream into play (–819 to –1000 bp), repressing expression [26,63]. HIF1 (hypoxia inducible factor) elements are also present in the promoter that might mediate alterations of expression related to iron status [26,64].

Surprisingly, we found that iron status also influenced the expression of the transcuprein/macroglobulin, but in the opposite way to copper, i.e. there was a positive relationship. mRNA expression was very low in iron deficiency and increased upon treatment of normal rats with iron (opposite to what occurs with transferrin). The magnitude of the mRNA changes observed with alterations in iron status were greater than those observed with alterations in copper status. Although more tentative, the changes in circulating rat transcuprein/ α_1 inhibitorIII detected were not as large as those in mRNA, particularly with excess iron, suggesting translational as well as transcriptional control. However, our preliminary data for human plasma, from subjects with iron overload as compared to the normal, also showed a substantial difference in levels of α_2 macroglobulin, consistent with positive regulation by iron status. Thus, iron rather than copper could be responsible for the actual regulation. However, there is a reciprocal relationship between copper and iron in terms of their accumulation within tissues in general and liver in particular [42,43]. (Indeed, in iron overload, plasma ceruloplasmin levels are also lower [65].) Because iron deficiency increased liver copper concentrations 2-fold, it is possible that the iron effect is indirect and mediated by alterations in hepatocyte copper contents. This remains to be further examined. The inverse relationship between liver copper levels and plasma transcuprein levels in iron

deficiency versus normal iron status does not in our view contradict it functioning as a supplier of copper to the liver, as there was still plenty of transcuprein present. The reason for the increased copper in liver in iron deficiency is thus likely to be due a reduction in copper excretion.

Acknowledgments

This work was supported in part by two Faculty Minigrants to M.C. Linder from California State University, Fullerton, two instrumentation grants from the National Science Foundation (BIR 9220666 and DUE 9352396), U.S. Public Health Service Grants RO1 HD46949 (M.C. Linder, PI), and IR24CA86307 (MIR, Washington University, St. Louis).

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Fig. 1.

Chromatography of ^{67}Cu -labeled rat plasma extracts during purification of transcuprein by Sephadex G150 (A), Sephacryl S300 (B), and copper chelate affinity chromatography (C), showing absorbance at 280 nm (solid line), and ^{67}Cu radioactivity (black diamonds) or actual Cu concentrations determined by atomic absorption spectrometry (X---X, or black dots in C). (C) Elution of proteins bound to the copper chelate affinity gel, upon application of 20 ml of a 10–40 mM imidazole gradient (up-angled line), followed by an additional 20 ml of 40 mM imidazole.

Fig. 2. SDS-PAGE of purified transcuprein: Results of representative purifications (A), separately underlined and labeled 1–4, showing protein components of the transcuprein preparations (TC), and their apparent sizes (k) in kDa, based on protein standards (STD). In the third example, the two TC lanes were of samples collected from earlier and later parts of the copper chelate affinity peak, respectively, eluting with imidazole. In the last example, purification was by Cibacron blue, DEAE-Sepharose 4B, and Sephacryl S300 chromatography. (B) The effect of treatment with (+) and without (–) peptide-N-glycosidase F on the 190 kDa transcuprein band.

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1 mkkdreaqlc lfsallaflp fasllngnsk ymvlvpsqgly tetpekiclh lyhinetvtv
61 taslisqrqt rklfdelvvd kdlfhcvstf iprlpsseee esldiniega khkfserrrv
121 lvknkesvfv vqtdkpmypk ggsvkfrvvs mdknlhpine lfplayiedp kmnrimqwgd
181 vktenglkql sfslsaepiq gpykivilkq sgvkcehsft vmefvlprfg vdvkvpnaia
241 vydeiinvta catytygkpv pghvkislch gnptfssetk sgckeedarl dnngcstgev
301 nitefqiken ylkmhqafhv natvteegt sfsqsgrie vertrnkflf lkadshfrhg
361 ipffvkvrly dikgdpipne qvlikardag ytnattdqgh glakfsidtn gisdyslnik
421 vyhkeessci hssctaerha eahhtayavy slksyiyld teagvlpcnq ihtvqahfil
481 kgqvlqvlqg ivfhylvmaq gsilqtgnht hqvepgesqv qgnfaleipv efsmvpvakm
541 liytilpdge viadsvkfqv ekclrnkvhl sfspsqslpa sqthmrvtas pqslcqlrav
601 dgsvllqkpe aelspsliyd lpgmqdsnfi assndpfede dyclmyqpia rekdvyryrv
661 etqlmaftnl kikiptycnt dydmvplavp avaldsstdr gmyeslpvva vksplpdepp
721 rkdpkppkdpv ietirnyfpe twiwdlvtvn ssgvtelemt vpditewka galclsndtg
781 lglssvasfq afqpfvvelt mpysvirgea ftlkatviny lptslpmavl leaspdftav
841 pvennqdsyc lgangrhtss wlvtpkslgn vnfsvsaeae qspgpcgsev atvpetgrkd
901 tvvkvlipev egikkehtfs slcasdael setlsallpp tvvkdssarah fsvmgdilss
961 aikntqnliq mpygcgeqnm vlfapniyvl kylnetqqlt ekikskalgy lragyqreln
1021 ykhkdgsysa fgdhngqggg ntwltafvk sfagarafif ideshitdaf twlskqkds
1081 gcfrssgsl1 nnamkggvdd eitlsayitm allesslpdt dpvvskaalc lesswenieq
1141 ggngsfvytk almayafala gnqekrneil ksldeakeke dnsihwerpq kptksegyly
1201 tpgassaeve msayvviarl tagpapsped lalsmgtikw ltkqnsygg fssdqdtvva
1261 ldalskygaa tfsksqktps vtvqssgsfs qkfqvdkenr lllqqvslpy ipgnytvsvs
1321 gegcvyaqtt lrynvplekq qpafalkvqt vpltcnnpkg qnsfqislei symgserpasn
1381 mviadvkmls gfipkptvk klerlghvtr tevttnnvll yldqvtntql sfsfliqqdi
1441 pvknlqpaiv kvydyyetde vafaeysspc ssddqnv

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Fig. 3.

Full amino acid sequence of the α_1 inhibitorIII transcuprein component. The first two amino acid sequences underlined are those obtained for the 190 kDa band of transcuprein. Also underlined is the region corresponding to that of the cDNA used for mRNA quantitation (residues 379–492) and for the “bait region” of the macroglobulin (approximately residues 601–750).

Fig. 4.

Elution of transcuprein during purification by published methods used for α_1 inhibitorIII [29]. The flow-through from Cibacron blue chromatography of radiolabeled rat serum was applied to ion exchange chromatography on DEAE Sepharose and washed with 50 mM NaCl-Tris buffer. What is shown is the elution of proteins (absorbance at 280 nm; solid line) and copper radioactivity (black diamonds) from the ion exchange column, upon application of the NaCl gradient (100–400 mM). Pooled eluate (from 15–24 ml) was then applied to a Sephacryl S300 column (not shown). SDS-PAGE analysis of the resulting preparation is in Fig. 2A, 4th example.

Fig. 5.

Copper binding to human α_2 macroglobulin. To demonstrate that α_2 macroglobulin is the human transcuprein, traces of $^{64}\text{Cu}(\text{II})$ were added to samples of pooled human plasma. Samples on the left half of the gel were from normal subjects; those on the right from two patients with iron overload (hemochromatosis). Portions (6 μL) of undiluted (larger rockets) or ten-fold diluted plasma (smaller rockets) were applied to adjacent wells in triplicate. The resulting gels were developed by autoradiography in a phosphorimager, detecting the ^{64}Cu .

Fig. 6. Non-specific co-precipitation of albumin with transcuprein and other proteins. (A) SDS-PAGE of immunoprecipitates extracted from rockets obtained by immunoelectrophoresis of human plasma with antibody against human α_2 macroglobulin (lane 2) in comparison with prestained high range protein standards (212, 121, and 96 kDa) (lane 1). (B) SDS-PAGE immunoblot with anti human albumin of proteins extracted from immunoelectrophoresis rockets obtained with human plasma and antibodies against horse spleen ferritin (lane 3), α_2 macroglobulin (lane 4) and ceruloplasmin (lane 5). Lane 2 was whole human plasma, lane 1 prestained protein standards (188, 100 and 45 kDa). In the case of the ferritin rockets, pure horse spleen ferritin had been mixed with the human plasma. The same kinds of results were obtained with immunoprecipitates of rat transcuprein and ceruloplasmin (not shown), using rat plasma prefractionated by size exclusion chromatography on Sephadex G150, and pre-

depletion of the transcuprein and ceruloplasmin-containing fractions of rat immunoglobulins with protein A (see Methods). Transcuprein/ α_1 inhibitorIII and ceruloplasmin in the resulting supernatants were immuno-precipitated with primary antibodies and protein A beads.

Fig. 7. Effect of copper deficiency on expression of liver α_1 inhibitorIII (α_1 I3) mRNA (transcuprein), determined by competitive and non-competitive PCR. RNA was extracted from livers of rats on a low copper diet, with and without copper supplementation in the

drinking water. (A) Results of competitive PCR, based on development and application of the Mimic system of Clontech. The upper and lower pairs of gel bands are examples of results for copper supplemented (normal) and copper deficient (Cu deficient) rats, respectively, in which products were separated in ethidium bromide containing agarose gel electrophoresis. In each set, the 340 and 540 bp “target” and “mimic” PCR products (lower and upper bands, respectively) reflect amplification of the liver transcuprein/ α_1 inhibitorIII cDNA and its Mimic, respectively. Three different (decreasing) amounts of competing (Mimic) cDNA were present from left to right, in this case 5.15×10^6 and 2.57×10^6 and 5.15×10^5 molecules, respectively, as indicated. The products obtained with a given amount of Mimic cDNA alone is shown to the right, along with bands for densitometric standards (375 ng of Hae digested DNA (Mimic kit)]. (B) Non-competitive RT-PCR of RNA from normal (supplemented; N) and deficient (D) rat livers (4 lanes at right), using two different amounts of cDNA template (0.5 and 5 ul, shown in subscripts), showing amplification of α_1 I3 cDNA. The left lane contains the DNA ladder, the next lane the 540 bp Mimic cDNA.

A **B**

Fig. 8.

Effects of copper deprivation and iron status on expression of α_1 inhibitorIII (α_1 I3) mRNA (transcuprein) (Tc/ α_1 I3), determined by Northern analysis. (A) Representative nylon membrane of total RNA, after capillary transfer from agarose gel containing ethidium bromide, showing the loading of RNA from copper-normal and copper deficient rat livers. (B) Portions of the resulting Northern autoradiograph, showing hybridization with Tc/ α_1 I3 cDNA and that of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control, for copper-normal and deficient rats, as indicated. (C) Northern of liver and heart total RNA from rats with normal (Norm.), deficient (Defic.) and excess (Xcess) iron status, showing expression of Tc/ α_1 I3 mRNA above 28 S rRNA migration mark. (D) Northern of total liver RNA from normal and iron deficient rats, showing hybridization with Tc/ α_1 I3 and G3PDH, respectively.

Fig. 9.

Expression of transcuprein protein in serum of rats with varying iron and copper status, determined by Western blotting. (A) Expression of the α_1 inhibitorIII (190 kDa) component in iron deficient (lanes 1,2), normal (lanes 3,4), and excess iron (lanes 5,6) treated rats. Equal (5 μ L) portions of plasma were analyzed. (B) Expression in copper deficient (D) and supplemented (N) rats.

Table 1

Effects of dietary copper and iron deprivation on hematocrit, plasma ceruloplasmin, liver copper and ferritin iron. Data are Means $\pm$ SD for the N in parentheses.

Parameter	Nutritional Copper or Iron Status		
	Deficient	Supplemented	Excess
A. Rats on low copper diets			
Hematocrit (%)	40 $\pm$ 6 (5)	45 $\pm$ 6 (5)	--
Plasma ceruloplasmin (p-phenylene diamine oxidase activity) (nmol/min/mL)	0.072 $\pm$ 0.008 (3) *	0.125 $\pm$ 0.010 (3)	--
Liver copper (μ g/g)	2.1 $\pm$ 0.4 (4) *	3.5 $\pm$ 0.9 (5)	--
B. Rats on low iron diets			
Hematocrit (%)	14 $\pm$ 5 (6) *	45 $\pm$ 6 (8)	45 $\pm$ 5 (5)
Liver ferritin Fe (μ g/g)	4 $\pm$ 4 (6) *	31 $\pm$ 7 (8)	100 $\pm$ 37 (5) *
Liver copper (μ g/g)	11 $\pm$ 5 (4) °	6 $\pm$ 2 (4)	4 $\pm$ 1 (4)

* p < 0.025- <0.001 for difference from supplemented rats by Student's t-test

° p < 0.05 for difference from excess Fe treated rats