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Transcuprein is a macroglobulin regulated by copper and iron availability

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Abstract

Transcuprein is a high-affinity copper carrier in the plasma that is 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-inhibitor III, the main macroglobulin of rodent blood plasma. Albumin (65 kDa) copurified in variable amounts and was concluded to be a contaminant (although it can transiently bind the macroglobulin). The main macroglobulin in human blood plasma (α_2-macroglobulin), which is homologous to α_1-inhibitor III, also bound copper tightly. Expression of α_1I3 (transcuprein) mRNA by the liver was examined in rats with and without copper deficiency, using quantitative polymerase chain reaction methodology and Northern blot 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_1I3 mRNA, but circulating levels of transcuprein did not differ. Iron deficiency, which increased liver copper concentrations by threefold, 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 carry transport copper in the blood, and that their expression can be modulated by copper and iron availability.

Keywords: Transcuprein; α_1-Inhibitor III; α_2-Macroglobulin; Copper; Regulation; mRNA; Liver; Iron

1. Introduction

Transcuprein was first identified as a copper transport protein in the blood plasma of rats after the injection or intragastric administration of trace quantities of high-specific-activity ^{67}Cu(II) [1]. Immediately after treatment or upon direct addition of a 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 was first deposited into the liver and the 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 the liver and the kidney, with the kinetics of a precursor/product relationship [1,5]. From the liver (and perhaps also from the kidney) [3], a major portion of the copper that had 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 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

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ionic Cu(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 dialyzable and is incorporated by the liver during ceruloplasmin synthesis and secretion [2,4,11].

Both albumin and transcuprein bind copper very tightly. (The same binding characteristics were observed 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 reported to vary 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 Cu/ml plasma. Yet, the total copper in plasma is only about 1 μg/ml; 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 Cu(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 nonradioactive albumin, the label instantly redistributes 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 mechanism by which 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 the liver and to the kidney in the initial distribution phase, as shown in analbuminemic rats [5]. This suggests that transcuprein, rather than albumin, is the actual donor in hepatocytes and kidney cells.

Transcuprein has an apparent molecular weight of 270k [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 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 reverse transcription–polymerase chain reaction (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 the expression of transcuprein, as does iron status.

2. Materials and methods

2.1. 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 the 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, which was designed by E.A. Ulman in accordance with American Institute of Nutrition 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), 70% carbohydrates (45% cornstarch, 10% maltodextrin, 10% sucrose and 5.0% cellulose), 5.0% fat (corn oil), 3.5% salt mix S18101 without added copper and 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-normal or iron-normal, the former by providing cupric sulfate (7.86 mg/L) in the drinking water and the latter by injecting 25 mg of iron as iron dextran (iron dextran injection; Phoenix, Inc., St. Joseph, MO) intraperitoneally after 2–3 weeks on the diet. Rats with excess iron were given an extra 25 mg of 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 Institutional Animal Care and Use Committee (IACUC).

2.2. 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 (Salt Lake City, UT), courtesy of Dr. Richard Ajioka. Samples were stored frozen at −20°C.

2.3. Purification of transcuprein

Purification was performed according to procedures previously described [24], using blood plasma labeled in vitro with ^{67}Cu(II) or ^{64}Cu(II) (10–50 ng/ml actual Cu) as the 1:1 molar complex of nitrilotriacetate. ^{67}Cu(II) and ^{64}Cu(II) were obtained as chloride salts in 0.1 M HCl from the reactor at the University of Missouri (Columbia, MO) 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 performed on Sephadex G150 and Sephacryl S300 (Sigma, St. Louis, MO), using 20 mM K phosphate buffer (pH 7.0) with 20 mM -amino caproic acid (to inhibit nicking by plasmin) and 0.02% NaN_3. Copper–chelate affinity chromatography was performed 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 0.5 M NaCl added. The 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-inhibitor III [29]. Here, ^{67}Cu-labeled plasma was incubated with Cibacron blue Sepharose CL-4B gel (Sigma) for 30 min with shaking, and effluent was collected by filtration. The latter was applied to DEAE Sepharose CL-6B chromatography (Sigma) after dialysis into the equilibration buffer (50 mM Tris–HCl, pH 7.4, containing 50 mM NaCl). After washing, elution was performed with a linear gradient of 0.10–0.40 M NaCl in the same buffer. Purification using the procedures of Lonberg-Holm et al. [30], involving polyethylene glycol, was also carried out.

2.4. 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, and 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].

2.5. Ferritin iron determinations

Liver homogenates (10% tissue) were heated to 70°C 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 with bipyridyl after upon heating with Na bisulfite [33].

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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 R-250 (BioRad) and transfer to Immobilon PSQ membranes (Millipore, Bedford, MA). The primary antibody to 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 used was alkaline-phosphatase-conjugated donkey antisheep IgG (Sigma). Other primary antibodies against human albumin that were employed for immunoblotting were raised in goats (Sigma), with binding detected with alkaline-phosphatase-conjugated rabbit–anti-goat IgG (Biomeda, Foster City, CA). In some cases, purified transcuprein samples were incubated overnight at 37°C in 0.25 M Tris–HCl buffer (pH 8.0) containing 0.05 M phenanthroline and 5% NP40, with and without 200–250 mU/ml peptide-*N*-glycosidase F (Sigma), after denaturation in 0.25% SDS/0.05 M mercaptoethanol.

2.7. Immunoprecipitation and extraction

Rocket immunoelectrophoresis was carried out as previously described [34], using antibodies against human α_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 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 (P2545; Sigma) to remove endogenous immunoglobulins, then by incubating with primary antibody and by precipitating the complexes with protein A macrobeads (P1925; Sigma) in 20 mM phosphate-buffered saline (PBS; pH 7.0). Washed precipitates (washed thrice with cold PBS) were boiled with SDS-PAGE sample buffer to extract antigenic proteins for SDS-PAGE separation. Primary antibodies included those raised in rabbits against human ceruloplasmin (Sigma) and horse spleen ferritin (Dako, Carpinteria, CA).

2.8. Amino acid sequencing

Protein bands of transcuprein were separated on preaged 7.5% acrylamide resolving gels in SDS-PAGE. For N-terminal sequencing, which was performed at the UCLA Protein Microsequencing Facility, bands were transferred to Immobilon PSQ membranes (Millipore) and lightly stained with Coomassie brilliant blue R-250 (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°C, 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.

2.9. RNA extraction and Northern analysis

Total RNA was extracted from liver tissues 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 RapidHyb reagents and the protocol of Amersham Pharmacia Biotech. After high-stringency washing (once in 2× SSC, 0.1% SDS, 20 min at room temp, then twice in 0.1 SSC, 0.1% SDS, 42°C for 15 min), membranes were exposed to X-ray film (Kodak X-Omat, Rochester NY) for autoradiography. Loading was assessed by comparing the ethidium bromide staining of 28*S* and 18*S* rRNA bands on 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).

2.10. Quantitative PCR of α_1-inhibitor III (transcuprein) mRNA

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

(+)5′-ATGAGCAGGTCCTCATCAAAGC (22 nucleotides; T_m=64°C)
(−)5′-ACAATCTGTTGCAGCACTC (19 nucleotides; T_m=58°C).

Mimic primers were as follows:

(+)5′-*ATGAGCAGGTCCTCATCAAAGC*CGCAAGTGAAATCTCCTCCG (42 nucleotides)
(−)5′-*ACAATCTGTTGCAGCACTC*ATTTGATTGTGGACCATGGC (39 nucleotides).

Italicized portions of the sequences are identical to those of α_1-inhibitor-III-specific primers.

3. Results

3.1. Purification and sequencing of transcuprein

Transcuprein was purified from 1- to 10-ml batches of ^{67}Cu(II)-labeled rat plasma by various combinations of procedures, as described in Materials and Methods. The most rapid and useful method consisted of taking the void volume fraction, which is obtained by applying the plasma to Sephadex G150 size exclusion chromatography, and fractionating it on a gel with a larger pore size (Sephacryl S300), followed by copper–chelate affinity chromatography using an imidazole gradient for the 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 associated with the second 280-nm absorbing peak, which coincides with albumin elution [1]. Also shown is the analysis of actual copper (by furnace atomic

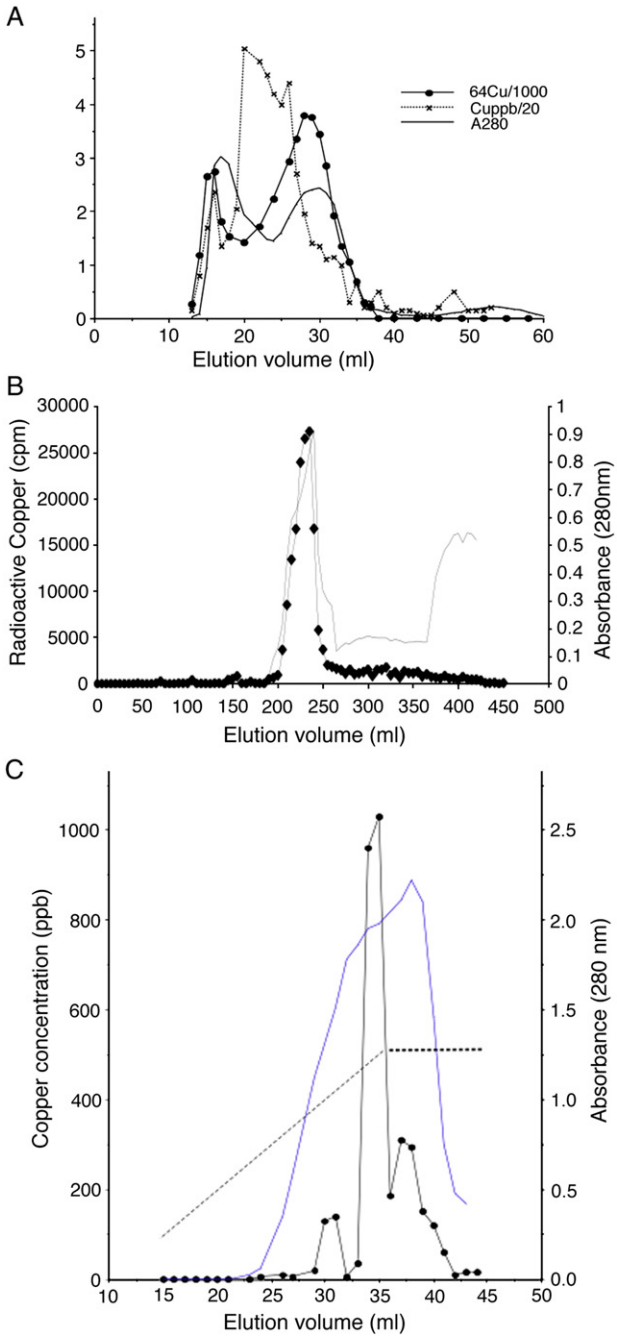

Fig. 1. Chromatography of ^{67}Cu-labeled rat plasma extracts during the purification of transcuprein by Sephadex G150 (A), Sephacryl S300 (B) and copper–chelate affinity chromatography (C) showing absorbance at 280 nm (solid line) and ^{67}Cu radioactivity (black diamonds) or actual Cu concentrations determined by atomic absorption spectrometry (X–X, or black dots in C). (C) Elution of proteins bound to copper–chelate affinity gel upon application of 20 ml of a 10- to 40-mM imidazole gradient (upangled line), followed by an additional 20 ml of 40 mM imidazole.

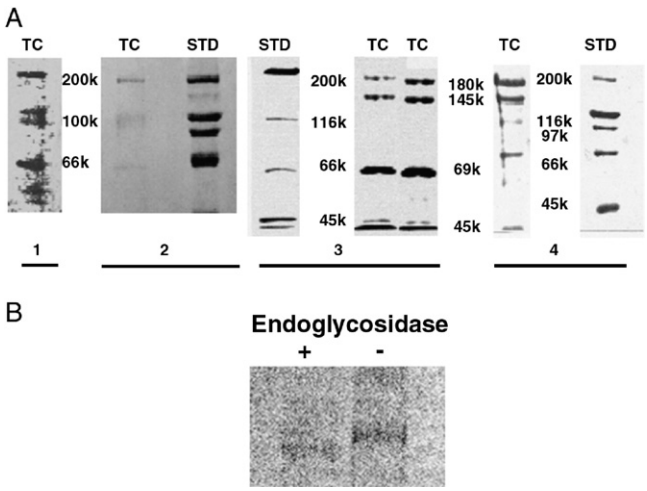

Fig. 2. SDS-PAGE of purified transcuprein. Results of representative purifications (A), separately underlined and labeled 1–4, showing protein components of transcuprein preparations (TC) and their apparent sizes (k) in kilodaltons, based on protein standards (STD). In the third example, the two TC lanes comprised samples collected from earlier and later parts of the copper–chelate affinity peak, respectively, eluting with imidazole. In the last example, purification was performed 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.

absorption spectroscopy; X–X). A significant portion of copper eluted in the void volume. The largest portion of the copper was observed with ceruloplasmin (eluting between the main A_{280} peaks), which is not labeled by in vitro ^{64}Cu or ^{67}Cu(II) addition, and a small amount was observed with albumin (seen as a shoulder to the right of the ceruloplasmin peak). (Previous studies have shown that, whether added in vitro or in vivo, the radiotracer on rat plasma transcuprein behaved in the same way with regard to its binding affinity and the release of radioactive tracer in response to nonradioactive Cu(II) and other agents [4].)

Following the application of void volume peak to Sephacryl S300 (Fig. 1B), radioactivity eluted in the middle of the column volume (M_r of about 270 kDa), in conjunction with a major peak of protein (A_{280}). When 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 prelabeled, the radioactive copper associated with transcuprein survived copper–chelate chromatography and was not displaced (data not shown).

The protein eluting from copper–chelate columns was analyzed by SDS-PAGE. Examples from several purifications and different portions of the A_{280} peak are shown (Fig. 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- to 69-kDa component was present in varying proportions, suggesting that it might be a contaminant. In addition, there often were one to two 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, third and fourth examples). The 190- and

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   1 mkkdreaqlc lfsallaflp faslLngnsk ymvlvpsqly tetpekiclh lyhlnetvtv
  61 taslisqrgt rklfdelvvd kdlfhcvsft iprlpsseee esldiniega khkfserrvv
 121 lvknkesvvf vqtdkpmykp gqsvkfrvvs mdknlhplne lfplayiedp kmnrimqwqd
 181 vktenglkql sfslsaepiq gpykivilkq sgvkeehsft vmefvlprfg vdvkvpnais
 241 vydeiinvta catytygkpv pghvkislch gnptfssetk sgckeedsrl dnngcstqev
 301 nitefqlken ylkmhqafhv natvteegtg sefsgsgrie vertrnkflf lkadshfrhg
 361 ipffvkvrlv dikgdpipne qvlikardag ytnatttdqh glakfsidtn gisdyslnik
 421 vyhkeessci hsstaerha eahhtayavy slsksyiyld teaqvlpcnq ihtvqahfil
 481 kgqvlgvlqq ivfhylvmaq gsilqtgnht hqvepgesqv qgnfaleipv efsmvpvakm
 541 liytilpdge viadsvkfqv ekclrnkvhl sfspsqslpa sqthmrvtas pqslcglrav
 601 dqsvllqkpe aelspsliyd lpqmqdsnfi assndpfede dyclmyqpia rekdvyryvr
 661 etglmaftnl kiklptycnt dydmvplavp avaldsstdr gmyeslpvva vksplpqepp
 721 rkdpppkdpv ietirnyfpe twiwdlvtvn ssgvtelemt vpdtitewka galclsndtg
 781 lglssvasfq afqpffvelt mpysvirgea ftlkatvlny lptslpmavl leaspdftav
 841 pvennqdsyc lgangrhtss wlvtpkslgn vnfsvsaear qspgpcgsev atvpetgrkd
 901 tvvkvlivep egikkehtfs sllcasdael setlslllpp tvvkdsarah fsvmgdilss
 961 aikntqnliq mpygcgeqnm vlfapniyvl kylnetqqlt ekikskalgy lragyqreln
1021 ykhkdgsysa fgdhngqgqg ntwltafvlk sfaqarafif ideshitdaf twlskqqkds
1081 gcfrssgsll nnamkggvdd eitlsayitm allesslpdt dpvvskalsc lesswenieq
1141 ggngsfvytk almayafala gnqekrneil ksldkeaike dnsihwerpq kptksegyly
1201 tpqassaeve msayvvlarl taqpapsped lalsmgtikw ltkqqnsygg fsstqdtvva
1261 ldalskygaa tfsksqktps vtvqssgsfs qkfqvdksnr lllqqvslpy ipgnytvsvs
1321 gegcvyaqtt lrynvplekq qpafalkvqt vpltcnnpkg qnsfqislei symgsrpasn
1381 mviadvkmls gfiplkptvk klerlghvsr tevttnnvll yldqvtnqtl sfsfiiqqdi
1441 pvknlqpaiv kvydyyetde vafaeysspc ssddqnv
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Fig. 3. Full amino acid sequence of the α_1-inhibitor III 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).

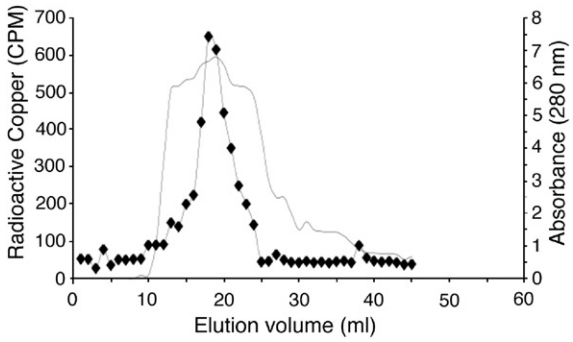

Fig. 4. Elution of transcuprein during purification, in accordance with published methods used for α_1-inhibitor III [29]. The flow-through from Cibacron blue chromatography of radiolabeled rat serum was applied to ion exchange chromatography on DEAE Sepharose and washed with 50 mM NaCl–Tris buffer. 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 NaCl gradient (100–400 mM). Pooled eluate (15–24 ml) was then applied to a Sephacryl S300 column (not shown). SDS-PAGE analysis of the resulting preparation is presented in Fig. 2A (fourth example).

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-inhibitor III [35]. The full sequence of this protein (in precursor form), as well as the sequences we obtained (underlined), is 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 endoplasmic-reticulum-bound polyribosomes. The internal sequence obtained corresponded exactly to Residues 154–171 of α_1-inhibitor III, 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-kDa component of transcuprein was within the range reported for α_1-inhibitor III (180–210 k) [37], with variability arising due to 10–23% carbohydrates. Moreover, the incubation of this with peptide-*N*-glycosidase F at 37°C for 20 h reduced the apparent molecular weight by 19 k (10%; Fig. 2B).

The 145-kDa contaminant of purified rat transcuprein was identified by sequencing as rat α_1-macroglobulin, with 16 N-terminal 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- to 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 the isolation of α_1-inhibitor III [29,30]. Using a combination of Cibacron blue pseudoaffinity chromatography [30] (to remove albumin), fractionation of flow-through on DEAE Sepharose [30] (Fig. 4) and Sephacryl S300 chromatography of the DEAE ^{67}Cu peak, the sample in Fig. 2A (fourth example) was obtained. This showed the same components as with the previous methodology: 190- and 66-kDa contaminants, as well as the 145-kDa contaminant.

All of this indicated that the major (large) component of transcuprein is α_1-inhibitor III, a member of the macroglobulin family. However, this is a major macroglobulin only in rodents, while in humans and most other mammals, the major macroglobulin 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 into half, resulting in two fragments in the range of 90 kDa [39], such as those encountered by us in some preparations (Fig. 2A, first and second examples).

α_2-Macroglobulin has a high homology to α_1-inhibitor III, particularly in its histidine- and cysteine-rich regions (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 immunoelectrophoresis (with rabbit antihuman 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 purified transcuprein preparations, and attempts to completely remove it from purified transcuprein with Cibacron blue gel beads were not

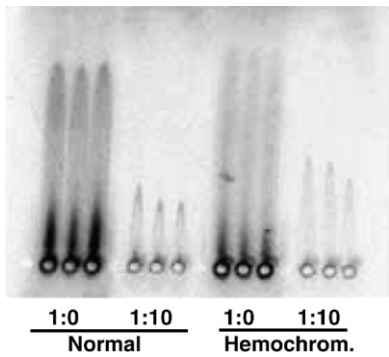

Fig. 5. Copper binding to human α_2-macroglobulin. To demonstrate that α_2-macroglobulin is the human transcuprein, traces of ^{64}Cu(II) were added to samples of pooled human plasma. Samples on the left half of the gel were from normal subjects; those on the right were from 2 patients with iron overload (hemochromatosis). Portions (6 μl) of undiluted (larger rockets) or 10-fold-diluted plasma (smaller rockets) were applied to adjacent wells, in triplicate. The resulting gels were developed by autoradiography in a phosphorimager, detecting ^{64}Cu.

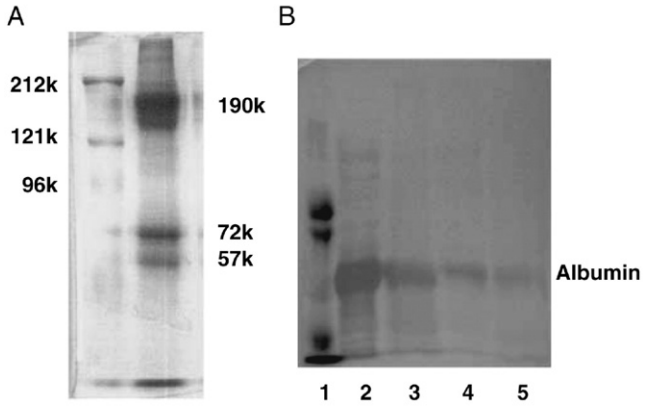

Fig. 6. Nonspecific coprecipitation of albumin with transcuprein and other proteins. (A) SDS-PAGE of immunoprecipitates extracted from rockets obtained by the immunoelectrophoresis of human plasma with antibody against human α_2-macroglobulin (Lane 2) in comparison with prestained High Range Protein Standards (212, 121 and 96 kDa) (Lane 1). (B) SDS-PAGE immunoblot with antihuman 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, whole human plasma; Lane 1, prestained protein standards (188, 100 and 45 kDa). In the case of ferritin rockets, pure horse spleen ferritin had been mixed with 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 predepletion (of the transcuprein and ceruloplasmin-containing fractions) of rat immunoglobulins with protein A (see Materials and Methods). Transcuprein/α_1-inhibitor III and ceruloplasmin in the resulting supernatants were immunoprecipitated with primary antibodies and protein A beads.

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. The SDS-PAGE of proteins extracted from rockets obtained by the 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 of about 57 kDa and near the bottom (probably the heavy and light chains of IgG, respectively; unpublished data). However, rockets from the immunoelectrophoresis of human plasma (+horse spleen ferritin), with antibodies against not only α_2-macroglobulin but also human ceruloplasmin or horse spleen ferritin, all 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 the liver and other tissues proceeded normally in the absence of albumin (Nagase analbuminemic rats) [5]. This proves that albumin did not have to be part of transcuprein. Taken together with other data, we thus conclude that it consists of only one kind of protein, namely, α_1-inhibitor III 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 four purified preparations ranged from 1 to 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 the measured copper contents do not reflect the actual situation for transcuprein in vivo and that it picked up additional copper during its purification, since 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/ml macroglobulin and 120 ng/ml Cu in the transcuprein fraction. The ability to bind additional copper atoms, but with complex stoichiometry, was confirmed by in vitro equilibrium dialysis studies (Jeremy Goforth, unpublished data).

3.2. Effects of dietary copper and iron availability on the 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 synthesis rate and blood plasma concentrations are increased by iron deficiency [26,27,40,41]. To begin the testing of this concept, we examined the expression of transcuprein mRNA in 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, and the other half were given deionized water 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 1A). Expression of transcuprein mRNA was assessed in three ways: by competitive PCR, noncompetitive PCR and Northern analysis, based on the known

Table 1
Effects of dietary copper and iron deprivation on hematocrit, plasma ceruloplasmin, liver copper and ferritin iron (data are presented as mean±S.D., with *n* inside parentheses)

Parameter	Nutritional copper or iron status		
	Deficient	Supplemented	Excess
(A) Rats on low-copper diets			
Hematocrit (%)	40±6 (5)	45±6 (5)	–
Plasma ceruloplasmin (*p*-phenylene diamine oxidase activity) (nmol/min/ml)	0.072±0.008 (3)**	0.125±0.010 (3)	–
Liver copper (μg/g)	2.1±0.4 (4)**	3.5±0.9 (5)	–
(B) Rats on low-iron diets			
Hematocrit (%)	14±5 (6)**	45±6 (8)	45±5 (5)
Liver ferritin Fe (μg/g)	4±4 (6)**	31±7 (8)	100±37 (5)**
Liver copper (μg/g)	11±5 (4)*	6±2 (4)	4±1 (4)

* $P<.05$, difference from excess-Fe-treated rats.
** $P<.025$–$.001$, difference from supplemented rats (Student's *t* test).

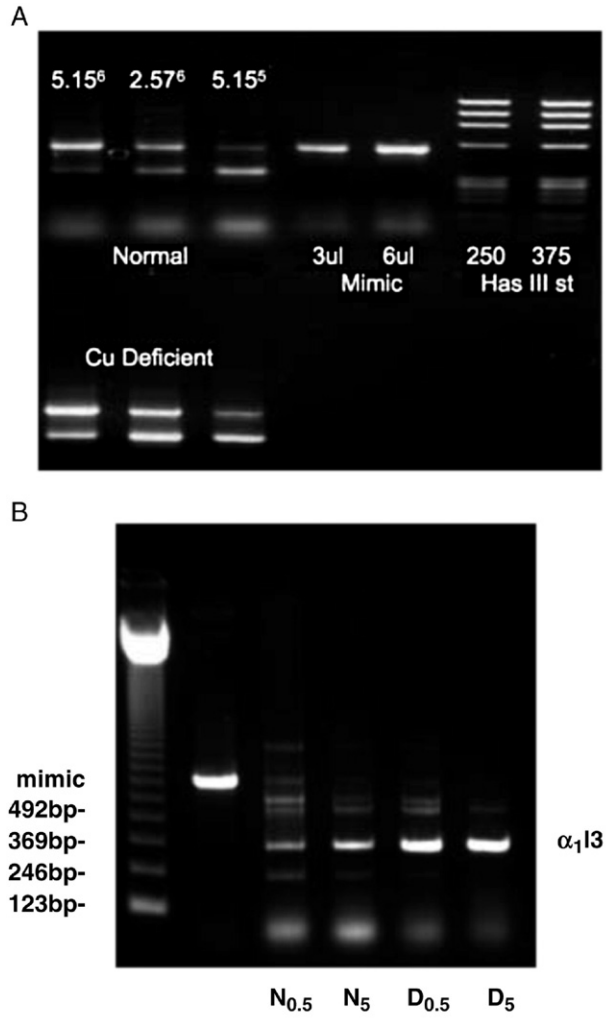

Fig. 7. Effect of copper deficiency on the expression of liver α_1-inhibitor III (α_1I3) mRNA (transcuprein), as determined by competitive and noncompetitive PCR. RNA was extracted from the livers of rats on a low-copper diet, with and without copper supplementation in the drinking water. (A) Results of competitive PCR, based on the 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 the amplification of the liver transcuprein/α_1-inhibitor III 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, 2.57×10^6 and 5.15×10^5 molecules, respectively, as indicated. The products obtained with a given amount of Mimic cDNA alone are shown to the right, along with bands for densitometric standards [375 ng of Hae-digested DNA (Mimic kit)]. (B) Noncompetitive RT-PCR of RNA from normal (supplemented; N) and deficient (D) rat livers (four lanes on the right), using two amounts of cDNA template (0.5 and 5 μl, shown in subscripts), showing the amplification of α_1I3 cDNA. The left lane contains the DNA ladder, and the next lane contains the 540-bp Mimic cDNA.

nucleotide sequence of rat α_1-inhibitor III mRNA. Total RNA was extracted from the livers of copper-normal and copper-deficient rats. For PCR, this was reverse transcribed into cDNA and analyzed for transcuprein mRNA content.

Fig. 7A shows an example of the results of applying the quantitative PCR Mimic system of Clontech (see Materials and 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 the mRNA of 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 that with cDNA from copper-sufficient (normal) livers (upper panels). Calculations of the actual change in two trials indicated that copper deficiency increased transcuprein mRNA concentrations by 65% and 83%. Direct noncompetitive PCR of 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-inhibitor III cDNA as probe (Materials and 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 (G3PDH); below]. Clearly, more expression was observed in the case of RNA from copper-deficient livers.

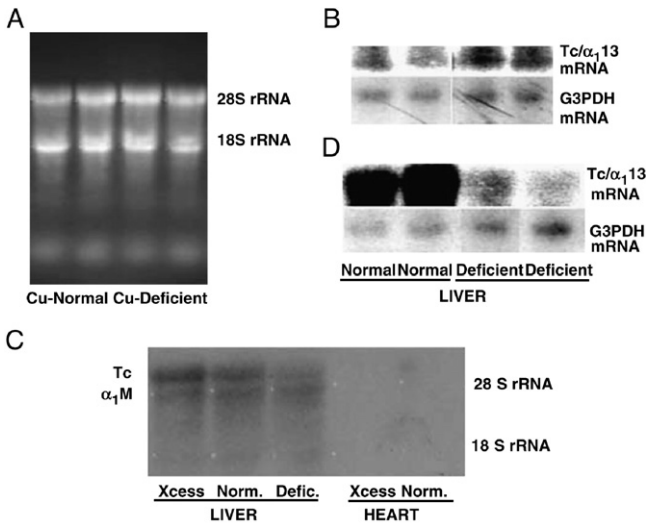

Fig. 8. Effects of copper deprivation and iron status on the expression of α_1-inhibitor III (α_1I3) mRNA (transcuprein) (Tc/α_1I3), as determined by Northern analysis. (A) Representative nylon membrane of total RNA, after capillary transfer from agarose-gel-containing ethidium bromide, showing the loading of RNA from copper-normal and copper-deficient rat livers. (B) Portions of the resulting Northern autoradiograph showing hybridization with Tc/α_1I3 cDNA and that of G3PDH control, for copper-normal and copper-deficient rats, as indicated in A. (C) Northern analysis of liver and heart total RNA from rats with normal (Norm.), deficient (Defic.) and excess (Xcess) iron status, showing the expression of Tc/α_1I3 mRNA above the 28*S* rRNA migration mark. (D) Northern analysis of total liver RNA from normal and iron-deficient rats, showing hybridization with Tc/α_1I3 and G3PDH cDNA, respectively.

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 Fig. 8C and D, iron availability correlated positively with transcuprein mRNA expression. There was little detectable expression in RNA from iron-deficient rat livers compared with RNA from normal livers (Fig. 8C and 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 1B), 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-inhibitor III protein (Materials and Methods). As shown in Fig. 9A (and other immunoblots that are not shown), there was a weak positive relationship between transcuprein levels and iron status when comparing plasma samples from deficient and nondeficient animals, with apparent densities of the bands increasing by about 35% (from deficiency to normal) from 38±5 to 51±2, respectively (mean±S.D.; $n=4$). In contrast, we could not detect a clear difference in the samples from copper-deficient and copper-supplemented rats (Fig. 9B). However, using standards, we found that the method was insufficient to detect 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 (Fig. 5).

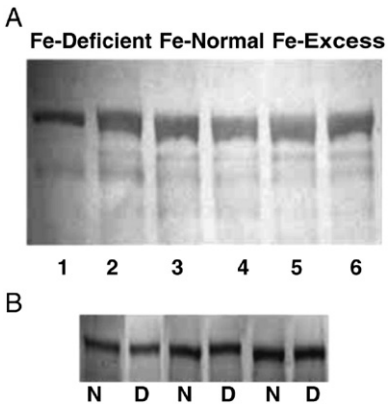

Fig. 9. Expression of transcuprein protein in the serum of rats with varying iron and copper status, as determined by Western blotting. (A) Expression of the α_1-inhibitor III (190 kDa) component in iron-deficient (Lanes 1 and 2), normal (Lanes 3 and 4) and excess-iron-treated (Lanes 5 and 6) rats. Equal (5 μl) portions of plasma were analyzed. (B) Expression in copper-deficient (D) and copper-supplemented (N) rats.

4. 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 the injection of nanogram 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, as 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-inhibitor III [35,37], the main macroglobulin found in rodents, and that the main macroglobulin found in human plasma (α_2-macroglobulin) also strongly binds ionic ^{64}Cu tracer; we concluded that the albumin isolated with transcuprein is not essential for its function but is due to contamination and it's ability to interact with the macroglobulin. We also demonstrated that expression of transcuprein/α_1-inhibitor III in rat livers at both mRNA and proteins levels is influenced by nutritional copper status and 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) were consistent with the findings for the rats.

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; and ceruloplasmin, which serves as a ferroxidase and a radical scavenger and also is the preferred source of copper for the 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 circulation [36] via macroglobulin receptors on hepatocytes [46,47], with a half-life of about 7 min in rats [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 nonproteolytic proteins, including basic polypeptides [48] and some hormones (platelet-derived growth factor [49] and cytokines [50,51]). [Interestingly, there are other connections with hormones: The expression of α_1-inhibitor III (and α_2-macroglobulin) is controlled by cytokines IL-6 and TNFα [52] and 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 two to five 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 evidence that the transport of Ag(I) in the blood of rats occurs in another macroglobulin, α_1-macroglobulin [56,57].

This 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°C). [Copper binding was not due to albumin contamination of 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 data).] These findings and the fact 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-inhibitor III (not present in humans) have a great deal of sequence homology, particularly in histidine-rich and cysteine-rich regions, allow the designation of the former as the transcuprein of human plasma. [There is much less homology between α_1-macroglobulin, which is present in rodents and nonrodents [36,46,59], and α_1-inhibitor III (or α_2-macroglobulin).] Both rat α_1-inhibitor III [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, seven 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 five 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 the 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-inhibitor III was enhanced by copper deficiency. The effect was not large, although it was reproducible. The level of liver mRNA about doubled with copper deficiency that decreased plasma ceruloplasmin oxidase activity and liver copper concentrations by 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, which is also produced by the liver. In iron deficiency with a 25–30% reduction in blood hemoglobin, human transferrin blood levels rise by 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 control but also translational control [26,61]. Transcriptional regulation appears to involve the 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]. Hypoxia-inducible factor 1 elements are also present in the promoter, which 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 way opposite to that seen in copper (i.e., there was a positive relationship). mRNA expression was very low in iron deficiency and increased upon the 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 detected changes in circulating rat transcuprein/α_1-inhibitor III were not as large as those in mRNA, particularly with excess iron, suggesting translational and transcriptional control. However, our preliminary data for human plasma from subjects with iron overload, as compared to normal subjects, also showed a substantial difference in α_2-macroglobulin levels, consistent with positive regulation by iron status. Thus, iron, rather than copper, could be responsible for actual regulation. However, there is a reciprocal relationship between copper and iron in terms of their accumulation within tissues, in general, and within the liver, in particular [42,43]. (Indeed, in iron overload, plasma ceruloplasmin levels are also lower [65].) Because iron deficiency increased liver copper concentrations by twofold, 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 its functioning as a supplier of copper to the liver, as there was still plenty of transcuprein present. The reason for the increased copper in the liver in iron deficiency is thus likely to be due to a reduction in copper excretion.

Acknowledgments

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