Role of Copper in the Proteosome-Mediated Degradation of the Multicopper Oxidase Hephaestin

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Running Title: Hephaestin Biosynthesis

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Summary

To elucidate the mechanisms of cuproprotein biosynthesis in the secretory pathway, a polyclonal antiserum was generated against hephaestin, a multicopper oxidase essential for enteric iron absorption. Immunoblot analysis and pulse-chase metabolic labeling revealed that hephaestin is synthesized as a single-chain polypeptide, modified by N-linked glycosylation to a mature 161-kDa species. Cell surface biotinylation and immunofluorescent studies of polarized, differentiated colon carcinoma cells detected hephaestin on the basolateral surface under steady state conditions. However, a decrease in the intracellular copper concentration resulted in a marked diminution in the abundance of this protein. Metabolic studies revealed no effect of decreased intracellular copper on the rate of hephaestin synthesis, but a dramatic, specific and reproducible increase in the turnover of the mature 161-kDa protein. Surprisingly, inhibitor studies revealed that this turnover occurs exclusively in the proteosome and consistent with this, in vitro studies identified polyubiquitinated hephaestin under conditions abrogating copper incorporation into this protein. Taken together, these studies demonstrate the presence of a quality control system for post-translational protein modification occurring beyond the endoplasmic reticulum that, in the case of hephaestin, directly links the rate of enteric iron uptake to nutritional copper status.
Introduction

Copper is an essential transition metal in all aerobic organisms, where it functions in specific cuproenzymes to facilitate electron transfer reactions necessary for respiration, antioxidant defense, connective tissue formation, neurotransmitter biosynthesis, peptide amidation, pigment metabolism and iron homeostasis (1). The useful chemical reactivity of copper also accounts for the considerable toxicity of this metal and thus specific mechanisms exist to chelate and compartmentalize this metal upon entry into the cell. For this reason, intracellular copper availability is limited under physiological conditions and the delivery of this metal to sites of cuproprotein synthesis within the cell requires delivery and transfer by specific metallochaperones (2,3). Most cuproproteins are synthesized in the secretory pathway and copper enters this compartment via P-type ATPases resident in the trans-Golgi network (1). Although copper incorporation is a critical post-translational protein modification, the precise mechanisms of this process within the secretory compartment remain poorly understood.

Recent studies on the mechanisms of copper incorporation into the multicopper oxidase ceruloplasmin have revealed a precise and sensitive mechanism for the synthesis of this cuproprotein, where copper serves as a positive allosteric factor, increasing the affinity for subsequent copper binding (4,5). The allosteric effects giving rise to this cooperativity are mediated by conformational changes within apoceruloplasmin upon copper binding, thereby permitting rapid synthesis of the holoprotein under circumstances of limited copper availability. While such a mechanism would abrogate the need for a specific metallochaperone and could accommodate the broad variation in cell-
specific expression, structure and function of the known secretory pathway cuproenzymes, further studies are needed to assess the general validity of this concept.

Hephaestin is a multicopper oxidase essential for normal iron homeostasis. Inherited loss of function of hephaestin in mice results in a microcytic, hypochromic anemia due to impaired intestinal iron transport (6). Consistent with this concept, hephaestin expression is detected in the intestine where the protein is hypothesized to play a role in iron movement across the basolateral membrane (7,8). Heterologous expression in *Saccharomyces cerevisiae* demonstrates that hephaestin oxidase activity is required for iron transport and dependent upon copper incorporation into the protein, supporting the proposed role for hephaestin as a multicopper ferroxidase (9). The deduced amino acid sequence of hephaestin includes a potential secretory peptide and a carboxyl terminal transmembrane region predicted to result in a type I membrane protein, suggesting that biosynthesis occurs in the secretory pathway (10). In the current study, the availability of a well studied colon carcinoma cell line expressing hephaestin and a polyclonal specific antiserum provided the opportunity to directly examine the mechanisms of biosynthesis of this cuproprotein.
Experimental Procedures

Cell Culture and Antibodies. A full length hephaestin cDNA was amplified from a human small intestinal cDNA library (Clontech) using nested oligonucleotide primers corresponding to the predicted open reading frame (10), inserted into pcDNA3.1 (Invitrogen) with a Kozak consensus sequence at the 5’ end and the entire nucleotide sequence verified by automated fluorescent sequencing (Perkin Elmer Life Sciences). Transient transfection was accomplished with LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Madin Darby canine kidney (MDCK), Chinese hamster ovary (CHO) and T84 cell lines were obtained from the American Type Culture Collection (ATCC) and cultured as described previously (5). Polarized T84 cells were grown on Transwell filters (Costar, Corning, NY) and maintained in a 1:1 mixture of DMEM and Ham’s F12 with 10% fetal bovine serum until confluent and polarized and tight junction permeability confirmed by paracellular flux of [14C]inulin (11). CuCl2 and bathocuproine disulfonic acid1 (BCS) were made fresh for each experiment and used at the indicated concentrations in appropriate media containing 1% bovine serum.

Rabbit polyclonal antibody to calnexin (Stressgen Biotechnologies, Victoria, Canada) and toll-like receptor 4 (TLR4) (eBioscience, San Diego, CA) and murine monoclonal antibody to cathepsin D (Santa Cruz Biotechnology, Santa Cruz, CA) were used according to manufacturer’s specifications. To generate antisera to hephaestin, a peptide corresponding to the carboxyl terminal 26 amino acids of human hephaestin was synthesized and used to produce polyclonal antisera in rabbits (Alpha Diagnostics International, San Antonio, TX). All experiments were performed with antisera purified against the hephaestin peptide coupled to AminoLink Plus Coupling Gel (Pierce).
Immunoblotting and Metabolic Labeling. Cell lysates were prepared in PBS containing 1% Triton X-100 supplemented with protease inhibitor mixture (Calbiochem) on ice for 30 min, followed by centrifugation for 10 min at 10,000 x g at 4°C. Protein concentration for all samples was determined by Bradford’s method (BioRad). Lysates were heated at 100°C for 10 min in the presence of SDS sample buffer, separated by 7.5% SDS-PAGE, transferred to nitrocellulose and detected with a donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) (12). For metabolic labeling, cells were pulse-labeled for 20 min with 60 µCi/ml of $[^{35}\text{S}]$methionine and $[^{35}\text{S}]$cysteine, chased with regular medium for the indicated time points, followed by collection of lysate for immunoprecipitation and SDS-PAGE as described previously (4). In some experiments, the immunoprecipitate was eluted by boiling in the presence of 5 mM Tris-HCl, pH 8.0 containing 0.2% SDS, split into two aliquots and incubated at 37°C overnight in the presence or absence of 0.1 mU/µl endoglycosidase H or PGNase (13). Some cells were incubated for 3 hr in media containing 10 µg/ml tunicamycin prior to pulse-chase experiments.

For lysosome and proteosome inhibition studies, T84 cells were grown in the presence or absence of BCS for 2 days, pulse-labeled with $[^{35}\text{S}]$methionine and $[^{35}\text{S}]$cysteine and chased in the presence or absence of BCS and either 5 µM MG132, 10 µM lactacystin, 5 µM epoxomicin, or lysosome inhibitor mixture consisting of 20 µM EST, 100 µM leupeptin and 100 µM pepstatin. Brefeldin A (1.6 µg/ml) and bafilomycin A1 (0.3 µM) were added 2 hr after initiation of chase. Limited trypsin proteolysis was performed on hephaestin immunoprecipitated from pulse-labeled T84 cells and incubated in 25 µl of
PBS containing 2.5-5 ng/µl bovine pancreatic trypsin for 1 hour at 37 °C. Proteolysis was quenched by addition of Laemmli sample buffer and samples analyzed as described (5).

**Cell Surface Biotinylation and Immunofluorescence Microscopy.** For biotinylation, polarized T84 monolayers were washed with PBS on ice, 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce) added for 30 min at 4°C, the reaction quenched with PBS containing 100 mM glycine, filters excised and cells lysed with PBS containing 1% Triton X-100 and protease inhibitors (14). Biotin labeled proteins were precipitated with streptavidin-agarose beads, separated by 7.5% SDS-PAGE and visualized by immunoblotting. For immunofluorescence, T84 monolayers were fixed in fresh 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, rinsed with PBS, and following blocking with PBS containing 3% non-fat dry milk and 2% BSA for 1 hour, incubated with anti-hephaestin (1:250) or anti–TLR4 (1:250) primary antibodies and donkey anti-rabbit secondary antibody conjugated to Alexa 488 (Molecular Probes) fluorophore (13,15). Inserts were placed on glass slides, mounted with ProLong Antifade (Molecular Probes) and analyzed on a laser-scanning microscope (BX61WI FV500, Olympus) as described (12).

**In vitro Ubiquitination Assay.** Hephaestin cDNA was translated in the presence of [35S]methionine using the TnT reticulocyte lysate system (Promega). Ubiquitination reaction mixtures contained 40 mM Tris-HCl pH 7.6, 5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol, 1 µM ubiquitin aldehyde (Calbiochem), 5 µM MG132, protease inhibitor mixture, 1 µg/µl ubiquitin, [35S]-labeled hephaestin, rabbit reticulocyte lysate (Promega) and either an ATP-regenerating system (0.5 mM ATP-γ-S, 10 mM phosphocreatine, 100 µg/ml creatine phosphokinase) or an ATP-depleting system (0.25 µg hexokinase, 10 mM 2-deoxy-D-glucose) as described (16). Reactions were stopped by addition of Laemmli...
sample buffer, heated at 72 °C for 15 min, subjected to 7.5% SDS-PAGE and proteins visualized by PhosphorImager (Typhoon 9410, Amersham Biosciences).
Results

Immunoblot analysis of lysates from MDCK cells transfected with a cDNA encoding human hephaestin identified two specific bands of 144 and 161-kDa and analysis of multiple human cell lines revealed endogenous hephaestin expression in the colorectal adenocarcinoma-derived cell line T84 (Fig. 1A). Pulse-chase analysis in T84 cells demonstrated that hephaestin is synthesized as a single-chain polypeptide with a precursor-product relationship between these two specific bands (Fig. 1B). Consistent with these data, endoglycosidase studies revealed that newly synthesized hephaestin is modified by N-linked glycosylation to a mature, endoglycosidase H-resistant 161-kDa species (Fig. 1C, lanes 2,3 vs. 6,7). Hephaestin synthesized in the presence of tunicamycin was smaller than the products that resulted from Endo H or PGNase digestion (Fig. 1C, lanes 4,8) suggesting that additional post-translational modifications occur during biosynthesis. Hephaestin was not detected in the media either at steady state or during the pulse-chase studies (data not shown).

To determine the cellular localization of hephaestin, T84 cells were polarized and differentiated on permeable membrane supports followed by biotinylation and streptavidin precipitation of cell-surface proteins. This analysis revealed that mature 161-kDa hephaestin is present on the plasma membrane, exclusively localized to the basolateral surface (Fig. 2). To determine if this localization is altered by intracellular copper concentration, experiments were performed following treatment of polarized T84 cells with either CuCl₂ or the copper specific chelator BCS. The results indicate that while hephaestin localizes to the basolateral surface under all conditions, the abundance
of hephaestin on the plasma membrane is greatly reduced as the intracellular copper content is decreased (Fig. 2).

To examine the cellular localization of hephaestin, filter-grown T84 cells were processed for immunofluorescence. In agreement with the data obtained from the biotinylation experiments, hephaestin was found to localize to the basolateral membrane (Fig. 3, panel A). When this experiment was repeated with cells grown in 100 μM CuCl₂ or 200 μM BCS the abundance of hephaestin was dramatically affected with the amount of plasma membrane signal detected being directly proportional to the intracellular copper content (Fig. 3, panels B,C). These findings were specific for hephaestin as neither the abundance nor the apical membrane localization of TLR4, a glycosylated protein localized to the apical membrane of T84 cells (17), was altered by changes in the intracellular copper content (Fig. 3, panels D-F). No specific staining was observed when these experiments were performed without cell permeabilization (data not shown). This membrane localization is specific to differentiated enterocytes as no plasma membrane hephaestin was detected in CHO cells transfected with the human hephaestin cDNA despite abundant intracellular expression (data not shown).

These data indicate that the plasma membrane content of hephaestin is decreased with decreasing intracellular copper content. This effect of copper is on the intracellular pool of hephaestin as can be seen in Fig. 4A, where immunoblot analysis of total cell lysates was carried out in polarized T84 cells incubated in the presence or absence of 100 μM CuCl₂ or 200 μM BCS for 4 days. A striking difference in the steady state level of the 161-kDa mature hephaestin species is observed in these experiments, with significantly greater hephaestin in the presence of increased copper and significantly decreased
hephaestin with decreased copper (Fig. 4A). Of note, the immature 144-kDa species was not affected by these changes in copper content (Fig. 4A). These effects were specific for copper and not observed with alterations in the intracellular content of zinc, magnesium, cadmium or iron (data not shown) and were specific for hephaestin with no decrease in the abundance of calnexin (Fig. 4A, lower panel) or TLR4 (data not shown) following treatment with BCS. Time course experiments revealed that these effects were maximum by 24 hr for BCS and within 48 hours for added CuCl₂ (Figure 4B). These findings were not specific to T84 cells as identical copper-dependent differences in steady state levels of hephaestin were observed in experiments with lysates from CHO and MDCK cells transfected with hephaestin (data not shown). Variation in the abundance of hephaestin in cells grown in media alone is due to the initial intracellular copper content under these conditions (data not shown).

Previous studies of the homologous multicopper oxidase ceruloplasmin revealed that failure to incorporate copper during biosynthesis results in a marked increase in trypsin sensitivity reflecting conformational differences between the apo and holoprotein (5). Pulse-chase studies in T84 cells revealed that newly synthesized, immature 144-kDa hephaestin is equally sensitive to trypsin regardless of intracellular copper content (Fig. 5, lanes 1-3). However, while the 161-kDa hephaestin present after a 4 hr chase was much more resistant to proteolysis than the immature species (Fig. 5, upper panel lanes 3 vs. lane 6), the 161-kDa hephaestin synthesized under copper-limiting conditions remained sensitive to proteolysis (Fig. 5, lanes 5,6, upper vs. lower panel).

The above data suggest that a decrease in the intracellular copper content may result in conformational changes in hephaestin that target the apohephaestin moiety for increased
turnover. Consistent with this concept, pulse-chase experiments in T84 cells cultured in standard media or media supplemented with copper or BCS revealed that while equivalent amounts of hephaestin are synthesized under each of these conditions (Fig. 6, lanes 1, 2) and there is no apparent difference in the turnover rate of the 144-kDa immature protein (Fig. 6, lanes 3-5), a striking difference is observed in the t$_{1/2}$ of the fully glycosylated 161-kDa protein (Fig. 6, lanes 6-10). This difference in half-life was readily apparent when the rate of disappearance of the mature 161-kDa protein was quantitated under each of the three conditions (Fig. 7). This effect of BCS was entirely due to abrogation of copper incorporation into newly synthesized protein within the secretory pathway, as analysis of steady state levels of cell surface hephaestin under these same conditions revealed identical turnover rates of the mature protein under normal and copper limiting conditions (Fig. 8A). Consistent with this concept, treatment of T84 cells with BCS for 4 hr was without effect on the abundance of cell-surface hephaestin (Fig. 8B) despite the fact that nearly 50% of the newly synthesized protein is turned over in this same time period under these conditions (Fig. 7).

To determine the intracellular site of hephaestin turnover under copper-depleted conditions, T84 cells grown in the presence or absence of BCS for 2 days were pulse-labeled and chased for 8 hr in the presence or absence of proteosome or lysosome specific inhibitors. Lysosomal inhibition had no effect on the increased turnover of hephaestin under copper limiting conditions, as equivalent amounts of this protein remained at the end of the chase period (Fig. 9A, lanes 3,4 vs. 5,6), despite a distinct effect on the turnover of cathepsin D (18), a known lysosomal protein (Fig. 9A). In contrast, the increase in hephaestin turnover observed in the presence of BCS was
completely abrogated by proteosome inhibitors (Fig. 9B). This effect was specific to proteosomal inhibition as no change in the abundance of cathepsin D was observed under these conditions (data not shown). Moreover, treatment of cells with either brefeldin A or bafilomycin A1, two agents known to disturb the Golgi apparatus and inhibit retrograde Golgi to ER transport (19), also abrogated the increase in hephaestin turnover observed in the presence of BCS (Fig. 9C). This effect of copper was direct, as analysis of hephaestin under conditions of in vitro translation that do not permit copper incorporation during synthesis (20) readily revealed covalent conjugates with ubiquitin that could be specifically immunoprecipitated from the reaction mixture (Fig. 10, lanes 4, 8).
Discussion

The data in this study demonstrate a dramatic and specific effect of copper on the turnover rate of the multicopper oxidase hephaestin. In polarized, differentiated T84 cells hephaestin is synthesized in the secretory pathway, modified by N-linked glycosylation and trafficked to the basolateral plasma membrane, consistent with the hypothesized role of this protein in basolateral iron efflux. The biosynthetic data indicate that the rate of hephaestin synthesis, folding and trafficking through the Golgi is unaffected by copper availability and that it is the abundance of the mature, glycosylated form of the protein that is altered by the intracellular copper content (Fig. 6, 7). As chelation with BCS completely abrogates copper incorporation into cuproproteins in both the cytoplasm and the secretory pathway (5,21), the data indicate that it is the mature, apohephaestin moiety that is unstable and rapidly degraded. The marked increase in proteolytic sensitivity of the mature, glycosylated apoprotein (Fig. 5) suggests that this difference in turnover is the result of conformational changes occurring upon copper incorporation late in the secretory pathway, analogous to what has been observed with the homologous multicopper oxidase ceruloplasmin (5,20).

While previous studies have revealed that the homologous multicopper oxidase Fet3p in Saccharomyces cerevisiae is demetallated by BCS at the plasma membrane (22), the data here clearly indicate that mature cell surface holoprotein is stable to BCS treatment (Fig. 8A,B), supporting the concept that the effect of BCS on hephaestin was entirely due to abrogation of copper incorporation into newly synthesized protein within the secretory pathway. Importantly, while these conclusions regarding BCS and copper incorporation are entirely consistent with previous findings and the known function of BCS (4,20), the
experiments shown here are indirect in terms of demonstrating the loss of copper and catalytic activity in hephaestin under these conditions.

Although the intracellular copper content does affect the steady state abundance of membrane localized hephaestin (Fig. 4), this is most likely secondary to the turnover rate and not a direct effect of copper on hephaestin trafficking to or from the plasma membrane. In support of this concept, an identical effect of copper on hephaestin turnover is observed in transfected CHO cells where no expression is detected on the plasma membrane (data not shown). Furthermore, proteosomal inhibition prevents the copper-dependent turnover of hephaestin and it is the mature, glycosylated apoprotein that accumulates under such conditions (Fig. 9A, B). These data, as well as the finding that the effect of BCS on the turnover of hephaestin is abrogated by brefeldin A and bafilomycin A1 (Fig. 9C), support the concept that apohephaestin is retrotranslocated from the Golgi to the endoplasmic reticulum prior to turnover.

In the case of proteins that are misfolded in the endoplasmic reticulum, polyubiquitination is critical for translocation and destruction (23). Although the paucity of hephaestin in BCS treated cells did not allow for direct examination of ubiquitination of the apoprotein in vivo, translation of this protein under conditions permitting synthesis of only apohephaestin demonstrates polyubiquitination (Fig. 10), a finding consistent with the proteosomal inhibition data. These findings were specific for hephaestin, as alteration of the intracellular copper content in T84 cells did not induce the unfolded protein response or alter the turnover rate of misfolded proteins in the endoplasmic reticulum (data not shown). Although analysis of ubiquitination of hephaestin in T84 cells in vivo under conditions of BCS treatment and proteosomal inhibition did not reveal
the anticipated presence of higher molecular weight ubiquitin conjugates (data not shown), the failure to observe such products may reflect the physiologic balance of ubiquitin-conjugating enzymes and deubiquitinases present under the experimental conditions (24). Nevertheless, this is an important issue and future studies will be needed to directly address this experimental finding.

Most importantly, these studies of hephaestin biosynthesis reveal a system of quality control in the late secretory pathway that involves retrotranslocation to the endoplasmic reticulum, ubiquitination and proteosomal degradation. While it is well established that during protein biosynthesis quality control systems exist in the endoplasmic reticulum to ensure that only correctly folded proteins reach their final destination (25,26), the system of quality control described here for hephaestin is likely to be widely utilized as many posttranslational modifications in addition to metal incorporation occur late in the secretory pathway. In this regard, recent studies in yeast have identified a subset of membrane-associated proteins that are ubiquitinated in response to mutations in the endoplasmic reticulum degradation machinery (27) some of which may be marked for turnover after exiting the endoplasmic reticulum and may therefore utilize a similar pathway to that of hephaestin for degradation. Interestingly, an analogous pathway of retrograde trafficking is utilized by shiga and other bacterial toxins to access the cytosol following binding at the plasma membrane (28,29), raising the possibility that microorganisms may co-op an existing quality control mechanism for retrieval of misfolded proteins from the secretory pathway to the endoplasmic reticulum.

The biotinylation and immunofluorescent data (Fig. 2, 3) reveal that hephaestin is a type I plasma membrane protein and that intracellular copper is a critical factor, albeit
indirectly, in determining this localization. The lack of plasma membrane hephaestin in transfected CHO cells regardless of intracellular copper content, as well as previous studies that have localized transfected hephaestin to the endocytic pathway in yeast and mammalian cells (9) indicate that copper is necessary but not sufficient for newly synthesized, mature holohephaestin to reach the plasma membrane and that additional factors must be required for basolateral membrane localization in enterocytes. In *Saccharomyces cerevisiae*, iron transport across the cell surface requires both the homologous multicopper oxidase Fet3 and an associated iron permease Ftr1 and proper plasma membrane targeting of these proteins requires simultaneous synthesis of both (30). Iron export in mammalian cells requires the permease ferroportin (31-33) and recent studies have revealed a direct association between this protein and the multicopper oxidase ceruloplasmin during iron movement from astrocytes (34), suggesting a potential role for ferroportin in the basolateral membrane localization of hephaestin in enterocytes. Several studies indicate that hephaestin is expressed in multiple organs (6,7,10) and it also remains possible that in specific cell types in these tissues hephaestin localization and function is intracellular as suggested in previous transfection studies in yeast (9).

Taken together, these data demonstrate a novel mechanism for the quality control of protein synthesis in mammalian cells that may have broad significance for cell biology. In terms of iron homeostasis, previous work has revealed that the abundance of hephaestin is increased as systemic iron levels decrease (7,8,35). While such regulation serves to increase the uptake of enteric iron, this would not be desirable when systemic iron levels are decreased secondary to copper deficiency as absorbed iron would only be further sequestered within the reticuloendothelial system (36). The data in this study
reveal a cell biological mechanism whereby the systemic effects of copper deficiency promote the retrograde transport and proteosome-mediated degradation of hephaestin in enterocytes, abrogating any increase in enteric iron uptake. Consistent with this physiological model, nutritional studies demonstrate a marked increase in iron accumulation within the gastrointestinal tract of copper-deficient pigs (37). Future experiments will focus on dissecting the mechanisms of recognition and retrograde transport of apohephaestin in enterocytes as well as the relationship of this cellular process to quality control mechanisms for other protein modifications occurring late in the secretory pathway.
Acknowledgments

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Footnotes

BCS, bathocuproine disulfonic acid; TLR4, toll-like receptor 4
References


Figure Legends

Fig 1. Characterization of human hephaestin. (A) Immunoblot analysis of T84 and transfected MDCK cells. Cell lysate (100 µg) was separated by 7.5% SDS-PAGE and analyzed with affinity-purified anti-hephaestin antibody followed by chemiluminescent detection. Arrows indicate two specific hephaestin bands. (B) Pulse-chase analysis of hephaestin in T84 cells. Cells were pulse-labeled for 20 min and then chased for indicated times. Lysates were prepared, and hephaestin immunoprecipitated and analyzed as described in Experimental Procedures. (C) Glycosylation studies. T84 cells were pulse-labeled for 20 min and either harvested immediately (lanes 1-4) or after 2 hr chase (lanes 5-8). Lysates were prepared, and hephaestin immunoprecipitated and digested with Endo H or PGNase prior to analysis. Some cells were treated with tunicamycin 3 hr prior to the pulse and during the chase (lanes 4 and 8).

Fig. 2. Localization of hephaestin in polarized, differentiated T84 cells. T84 cells grown on Transwell filter supports in control media, 100 µM CuCl₂ or 200 µM BCS for 4 days were treated without (-) or with biotin added to the apical (A), basolateral (B), or both (A/B) membranes. Following solubilization and streptavidin-agarose precipitation (from 800 µg total protein), biotinylated proteins were separated on 7.5% gels and hephaestin identified by immunoblotting. Arrow indicates 161-kDa mature hephaestin.

Fig. 3. Immunofluorescent localization of hephaestin in polarized, differentiated T84 cells. T84 cells grown on Transwell filter supports in control media (A,D), 100 µM CuCl₂ (B,E) or 200 µM BCS (C,F) were fixed, permeabilized, and examined following treatment with antibodies against hephaestin (A-C) or TLR4 (D-F) as described in
Experimental Procedures. Shown are en face views focusing on the mid-axes (XY), and x-z axis reconstructions (XZ) of the apical and basolateral surfaces.

**Fig. 4.** Effect of intracellular copper on steady-state hephaestin. (A) Immunoblot analysis of lysates (45 µg) from three sets of polarized, differentiated T84 cells grown on Transwell filter supports for 4 days in control media (lanes 1-3), 100 µM CuCl2 (lanes 4-6) or 200 µM BCS (lanes 7-9). Lysates were analyzed for hephaestin, then the membrane stripped and reprobed with antibodies against calnexin. (B) Lysates (100 µg) from T84 cells grown in control media for 4 days (lane 1), 100 µM CuCl2 for 1-4 days (lanes 2-5) or 200 µM BCS for 1-4 days (lanes 6-9) were analyzed by immunoblotting for hephaestin and calnexin as indicated in Experimental Procedures.

**Fig. 5.** Limited trypsin proteolysis of hephaestin. T84 cells were grown in media alone (top panel), 100 µM CuCl2 (middle panel) or 200 µM BCS for 4 days prior to pulse-labeling. Cells were then either harvested immediately following the pulse (lanes 1-3) or chased for 4 hr (lanes 4-6), lysates prepared, hephaestin immunoprecipitated and following incubation with indicated concentration of trypsin at 37°C for 1 hour, hephaestin examined as indicated in Experimental Procedures. Arrows indicate positions of hephaestin species. Additional lysate was used from BCS-treated cells to allow visualization of the protein after 4 hr.

**Fig. 6.** Effect of intracellular copper on biosynthesis and turnover of hephaestin. T84 cells grown in control media, 100 µM CuCl2 or 200 µM BCS for 2 days were pulse-labeled for 20 min, and chased for the indicated times. Lysates were prepared and hephaestin immunoprecipitated and analyzed as indicated in Experimental Procedures. The bottom two panels contain an additional lane (lane 1) with hephaestin
immunoprecipitated from a control plate grown in media alone to directly allow comparison of initial rates of biosynthesis under each condition.

**Fig. 7.** Effect of intracellular copper on the half-life of hephaestin. Autoradiographs from Fig. 5 were quantitated following scanning using UnScanIt imaging software (Silk Scientific) and data were fitted to the curve using Sigma Plot (SPSS). The data are plotted to indicate the half-life of the 161-kDa mature hephaestin under different conditions where the amount of this protein at the 2 hr time point was calculated to represent 100% of total hephaestin in each case.

**Fig. 8.** Effect of copper on stability of cell-surface hephaestin. (A) Effect of copper on the half-life of cell-surface hephaestin. T84 cells grown on Transwell filter supports were incubated in control media or 200 μM BCS for 4 days prior to treatment with biotin. After quenching the biotinylation reaction, cells were washed and incubated in control media or 200 μM BCS for the indicated time points. Following solubilization and streptavidin-agarose precipitation, biotinylated proteins were separated on 7.5% gels and hephaestin identified by immunoblotting. Arrow indicates 161-kDa mature hephaestin. (B) Stability of cell-surface hephaestin to copper chelation. T84 cells were treated with biotin and then incubated in control media (lanes 1,2) or 200 μM BCS (lanes 3,4) for 4 hours. Biotinylated hephaestin (bottom panel) was detected as described above.

**Fig. 9.** Hephaestin turnover under copper limiting conditions. (A) Effect of lysosomal inhibitors on stability of hephaestin. Two independent dishes of T84 cells grown for 2 days in control media (lanes 1,2) or 200 μM BCS (lanes 3-6) were pulse-labeled and then treated with vehicle alone (DMSO) (lanes 1-4) or lysosomal inhibitor mixture (lanes 5-6), and analyzed for hephaestin or cathepsin D as described in Experimental Procedures. (B)
Effect of proteosome inhibitors on stability of hephaestin. Two independent dishes of T84 cells grown in control media (lanes 1, 2 and 7, 8) or 200 µM BCS (lanes 3-6, 9-14) were pulse-labeled and then treated with vehicle alone (DMSO) (lanes 1-4, 7-10) or indicated proteosome inhibitors and analyzed for hephaestin as described in Experimental Procedures.

Fig. 10. Ubiquitin conjugation to hephaestin. Hephaestin was in vitro translated for 1 hr at 30°C and ubiquitin conjugation reactions performed with rabbit reticulocyte lysate (lysate) as described in Experimental Procedures. The reaction was then divided into two aliquots and analyzed directly (lanes 1-4) or following immunoprecipitation (IP) of hephaestin (lanes 5-8). Arrows indicate the in vitro synthesized hephaestin and the high molecular mass ubiquitin conjugates.
Fig. 1

A

Mr x 10^3

B

Mr x 10^3

C

Pulse (20 min) Chase (2 hr)

Mr x 10^3
Fig. 3

**CuCl₂**  
**BCS**

Heph

XY

A

B

C

XZ

D

E

F

TLR4

XY

XZ
Fig. 5

Trypsin mg/ml

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Fig. 7

% Hephaestin Remaining vs. Chase time (hours)

- CuCl₂
- BCS
Fig. 8

A

BCS

B

BCS
Fig. 9
Fig. 10

ATPγS - + - +
Lysate - - + +

Hephaestin

IP

UB conj

1 2 3 4
5 6 7 8
Role of copper in the proteosome-mediated degradation of the multicopper oxidase hephaestin
Thalia Nittis and Jonathan D. Gitlin

J. Biol. Chem. published online April 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401151200

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