Role of Copper in the Proteosome-mediated Degradation of the Multicopper Oxidase Hephaestin*

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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 proteasome, and consistent with this finding, 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 posttranslational 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.

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 permits the precise and sensitive mechanism for the synthesis of this cuproprotein under the circumstances of limited copper availability. Although 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 anemia (4) because of 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 is 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 this 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) and inserted into pcDNA3.1 (Invitrogen) according to the manufacturer’s instructions. Madin Darby canine kidney, Chinese hamster ovary (CHO), 1 and T84 cell lines were obtained from the ATCC and cultured as described previously (5). Polarized T84 cells were grown on Transwell filters (Costar, Corning, NY) and maintained in a 25696 This paper is available on line at http://www.jbc.org

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1 The abbreviations used are: CHO, Chinese hamster ovary; BCS, bathocuproine disulfonic acid; TLR4, toll-like receptor 4; PBS, phosphate-buffered saline; Endo H, endoglycosidase H; ATP-β-S, adenosine 5’-O-(thiotriphosphate).

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Hephaestin Biosynthesis

In Vitro Ubiquitination Assay—Hephaestin cDNA was translated in the presence of [35S]methionine using the T7R 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, 10 mM creatine phosphate, 100 µg/ml creatine phosphokinase) or an ATP-depleting system (0.25 µg of hexokinase, 10 mM 2-deoxy-D-glucose) as described previously (16). Reactions were stopped by the addition of Laemmli sample buffer, heated at 72 °C for 15 min, and subjected to 7.5% SDS-PAGE, and proteins were visualized by PhosphorImager (Typhoon 9410, Amersham Biosciences).

RESULTS

Immunoblot analysis of lysates from Madin Darby canine kidney cells transfected with a cDNA encoding human hephaestin identified two specific bands of 144 and 161 kDa, and an 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 1–7). Heparinase digestion of the mature 161-kDa bands yielded 150 kDa product (Fig. 1C, lanes 8 and 9), suggesting the presence of tunicamycin was smaller than the products that resulted from Endo H or peptide N-glycosidase F (PNGase) prior to analysis. Some cells were treated with tunicamycin 3 h prior to the pulse and during the chase (lanes 4 and 8).

Cell Surface Biotinylation and Immunofluorescence Microscopy—For biotinylation, polarized T84 monolayers were washed with PBS on ice, 0.5 mg/ml sulfo-NHS-S-biotin (Pierce) was added for 30 min at 4 °C, then monolayers were washed with PBS containing 100 mM glucose, filters were excised, and cells were 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, and rinsed with PBS, and following blocking with PBS containing 3% nonfat dry milk and 2% BSA for 1 h, the monolayers were 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) and Alexa 555 (Molecular Probes), and analyzed on a laser-scanning microscope (BX61WI FV500, Olympus) as described previously (12).

The paracellular flux of [14C]inulin (11) and CuCl₂ and bathocuproine disulfonic acid (BCS) were made fresh for each experiment and used at the indicated concentrations in appropriate media containing 1% bovine serum. Lysosomes 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, samples were heated at 72 °C for 15 min, split into two aliquots, and incubated at 37 °C overnight in the presence or absence of 0.1 milli-unit/µl endoglycosidase H (Endo H) or peptide N-glycosidase F (13). Some cells were incubated for 3 h in medium containing 10 µg/ml tunicamycin prior to pulse-chase experiments.

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 × g at 4 °C. Protein concentration for all of the samples was determined by the Bradford method (Bio-Rad). 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 [35S]methionine and [35S]cysteine and chased with regular medium for the indicated times. Lysates were prepared, and hephaestin immunoprecipitated and analyzed as described under “Experimental Procedures.”

For lysosome and proteasome inhibition studies, T84 cells were grown in the presence or absence of BCS for 2 days, pulse-labeled with [35S]methionine and [35S]cysteine, and chased with regular medium for the indicated times.

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brane, exclusively localized to the basolateral surface (Fig. 2). To determine whether this localization is altered by intracellular copper concentration, experiments were performed following treatment of polarized T84 cells with either CuCl$_2$ 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 reduced greatly 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 $\mu$M CuCl$_2$ or 200 $\mu$M BCS, the abundance of hephaestin was affected dramatically with the amount of plasma membrane signal detected directly proportional to the intracellular copper content (Fig. 3, panels B and 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 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 $\mu$M CuCl$_2$ or 200 $\mu$M 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. The time course experiments revealed that these effects were maximum by 24 h for BCS and within 48 h for added CuCl$_2$ (Fig. 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 Madin Darby canine kidney cells transfected with hephaestin (data not shown). Variation in the abundance of hephaestin in cells grown in media alone is the result of 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 apoprotein 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, whereas the 161-kDa hephaestin present after a 4-h chase was much more resistant to proteolysis than the immature species (Fig. 5, upper panel, lane 3 versus lane 6), the 161-kDa hephaestin synthesized under copper-limiting conditions remained sensitive to proteolysis (Fig. 5, lanes 5 and 6, upper versus 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 and 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 $t_\text{1/2}$ 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, because 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, the treatment of T84 cells with BCS for 4 h 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 h in the presence or absence of proteasome- 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 and 4 versus 5 and 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 proteasome inhibitors (Fig. 9B). This effect was specific to proteasomal inhibition, because no change in the abundance of cathepsin D was observed under these conditions (data not shown). Moreover, the treatment of cells with either brefeldin A or bafilomycin A1, two agents known to disturb the Golgi apparatus and inhibit retrograde Golgi to endoplasmic reticulum transport (19), also abrogated the increase in hephaestin turnover observed in the presence of BCS (Fig. 9C). This effect of copper was direct, because the analysis...
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 (Figs. 6 and 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 apoprotein 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 (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. Importantly, although 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.
under each condition.

alone to directly allow a comparison of initial rates of biosynthesis

of hephaestin.

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The bottom two panels contain an additional lane (lane 1) with

mature hephaestin under different

indicate the half-life of the 161-kDa mature hephaestin. 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 and 2) or 200 μM

BCS (lanes 3 and 4) for 4 h. Biotinylated hephaestin (bottom panel) was detected as described above.

Although the intracellular copper content does affect the steady-state abundance of membrane-localized hephaestin (Fig. 4), this is most probably 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, proteasomal inhibition prevents the copper-dependent turnover of hephaestin, and it is the mature glycosylated apoprotein that accumulates under such conditions (Fig. 9, A and 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 apohphaestin 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 apohphaestin demonstrates polyubiquitination (Fig. 10), a finding consistent with the proteasomal 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 the analysis of ubiquitination of hephaestin in T84 cells in vivo under conditions of BCS treatment and proteasomal 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 proteasomal degradation. Although 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 probably is to be utilized widely because 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-operate an existing quality control mechanism for the retrieval of misfolded proteins from the secretory pathway to the endoplasmic reticulum.

The biotinylation and immunofluorescent data (Figs. 2 and
membrane hephaestin in transfected CHO cells regardless of intracellular copper content and previous studies that have localized transfected hephaestin to the endolysosomal pathway in yeast and mammalian cells (9) indicate that copper is necessary but not sufficient for newly synthesized mature holohphaestin to reach the plasma membrane and that additional factors must be required for basolateral membrane localization in enterocytes. In S. 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 are 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 a broad significance for cell biology. In terms of iron homeostasis, previous work (7, 8, 35) has revealed that the abundance of hephaestin is increased as systemic iron levels decrease. Although 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 because absorbed iron only would be sequestered further 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 proteasome-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.

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