The cop operon is a key element of copper homeostasis in Enterococcus hirae. It encodes two copper ATPases, CopA and CopB, the CopY repressor, and the CopZ metallochaperone. It was previously shown that the transcription of the operon is induced by copper. The concomitant increase in the levels of Cop proteins, particularly the CopB copper export ATPase, allows uncompromised growth of E. hirae in up to 5 mM ambient copper. We here show by Western blotting that the steady-state level of CopZ was increased only up to 0.5 mM copper. At higher copper concentrations, the level of CopZ was decreased and became undetectable at 5 mM media copper. When CopZ was overexpressed from a plasmid, the cells exhibited increased sensitivity to copper and oxidative stress, suggesting that high CopZ expression could become toxic to cells. In wild-type cells, the level of mRNA transcripts from the cop operon remained high in up to 5 mM copper, suggesting that CopZ was proteolyzed. Cell extracts were found to contain a copper-activated proteolytic activity that degraded CopZ in vitro. In this assay, Cu-CopZ was more susceptible to degradation than apo-CopZ. The growth of E. hirae in copper increased the copper-inducible proteolytic activity in extracts. Zymographic studies showed the presence of a copper-dependent protease in crude cell lysates. Thus, copper-stimulated proteolysis plays an important role in the regulation of copper homeostasis in E. hirae.

Although copper is an essential cofactor of many enzymes, such as superoxide dismutase, cytochrome c oxidase, or lysyl oxidase, toxicity arises by excess accumulation in the cell (1). The two oxidation states of copper, copper(I) and copper(II), not only allow its participation in essential redox reactions but can also form reactive oxygen species that cause cellular damage. Hence, the maintenance of copper homeostasis by controlling uptake, accumulation, detoxification, and removal of copper is critical to living organisms. Many proteins involved in copper homeostasis have thus far been identified in prokaryotes and eukaryotes (for review see Refs. 2–5).

In the Gram-positive bacteria, Enterococcus hirae, copper homeostasis has been extensively studied and is known in some detail. The regulation of intracellular copper levels under the stress conditions of copper depletion as well as copper overload is achieved by the cop operon. The operon encodes four proteins: 2 copper ATPases (CopA and CopB), 1 repressor (CopY), and 1 metallochaperone (CopZ) (6, 7). CopA and CopB are transmembrane ATPases, which appear to be responsible for transporting copper ions into and out of the cell, respectively (8, 9). They belong to a family of heavy metal CPx-type ATPases that includes enzymes transporting Cu\(^{2+}\), Ag\(^{+}\), Co\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Hg\(^{2+}\), and Pb\(^{2+}\) (10–15).

Expression of the cop operon is induced by copper (7). This regulation is accomplished by the CopY repressor, which also regulates its own expression. Under normal growth conditions in complex media, expression of the cop operon is repressed by the binding of CopY to the promoter (16, 17). If copper levels are increased, the Zn\(^{2+}\) cofactor of CopY is replaced by two Cu\(^{+}\) ions, thereby releasing CopY from the promoter and allowing transcription to proceed (18). The Cu\(^{+}\) ions are delivered to CopY by the CopZ copper chaperone, a member of a family of metallochaperones that are conserved from bacteria to man. These proteins are ~70 amino acids in length and feature a copper(I) binding site involving an exposed CXpx motif (19–22).

It was observed that CopZ of E. hirae is more labile under high copper stress. In a number of systems, proteolysis provides cells with an additional means to modulate protein levels in response to changes in cellular physiology and external stress. However, these processes have not been studied as extensively as transcriptional and translational regulation, particularly in regard to copper (for review see Refs. 23 and 24). Copper-induced degradation of the copper transporter Ctr1p of Saccharomyces cerevisiae has been described previously (25).

The yeast transcription factor Mac1, which is involved in high affinity copper uptake, is also degraded under high copper concentrations (26). Conversely, in the green algae Chlamydomonas reinhardtii, apo-plastocyanin, a copper-binding protein that catalyzes electron transfer during photosynthesis, is rapidly degraded under copper-deficient conditions. This presumably serves to ensure that copper is available to indispensable cuproenzymes (27). However, the proteases involved in these processes have not been identified or characterized in biochemical terms.

In this study, we examined the expression and stability of the CopZ protein and assessed mRNA levels under varying copper conditions. At high copper concentrations, increased proteolysis of CopZ was observed, whereas mRNA levels were maintained. If proteolysis of CopZ was saturated by overexpression, toxic effects ensued. Our results show that copper-activated proteolysis is an additional mechanism of E. hirae to fine-tune the response to copper.

**EXPERIMENTAL PROCEDURES**

*Strains and Growth Conditions—*Wild-type E. hirae (ATCC9790) was obtained from the American Type Culture Collection. The copZ null

---

* This work was supported by Grant 32-56716.99 from the Swiss National Foundation and by a grant from the Novartis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 41-31-632-3268; Fax: 41-31-632-4997; E-mail: marc.solioz@ikp.unibe.ch.

1 Lu and Solioz, unpublished observations.
mutant of this strain and its complementation with a CopZ-overexpressing plasmid, pC6C6, were described previously (7). Logarithmically growing cells, frozen in 17% glycerol at −70 °C, were used to inoculate cultures in all growth experiments. The growth response of cells to different agents was monitored in N-media (1% Na2HPO4·2H2O, 1% tryptone, 0.5% yeast extract, 1% glucose) in 1-ml cultures in capped cuvettes. The cultures were inoculated with 1/50 volume of frozen log cells and grown at 37 °C for 1 h before the addition of the additive. Growth was followed by measuring the A560 with a Lambda 16 Spectrophotometer (PerkinEimer Life Sciences). Chemicals for growth media were purchased from Life Technologies, Inc.

CopZ Expression and Western Blotting—Expression of CopZ was monitored from total lysates of E. hirae 11003 in 5 ml of N-media were grown to an A546 of 0.4–0.6 by induction with different CuSO4 concentrations for 90 min. Cell lysates were prepared by centrifuging 2-ml aliquots and treating the cell pellets with 50 μl of 10 mg/ml lysozyme, 1 mM EDTA, 10 mM Tris-Cl, pH 8.0, at room temperature for 10 min. After a freeze-thaw cycle, the lysates were treated with 10 μl of DNase I in 100 mM MgCl2 for 5 min at room temperature. Proteins were then finally denatured in SDS sample buffer at 37 °C for 10 min. Amounts of extracts corresponding to the same number of cells were resolved by tricine-SDS-polyacrylamide gel electrophoresis (28). Western blots were prepared as described previously (29) using a rabbit polyclonal antibody against CopZ and a horseradish peroxidase-coupled donkey anti-rabbit IgG secondary antibody (Pierce). Bands were visualized by chemiluminescent detection using the ECL kit (Amersham Pharmacia Biotech) following the instructions of the manufacturer. Chemiluminescent signals were captured and quantified with the Fujifilm LAS-1000 imaging system (Fujifilm Photo Film Tokyo, Japan). The overexpression of CopZ was accomplished in a ΔcopZ strain harboring plasmid pC6C6 as described previously (7).

Determination of Cellular Copper Content—Frozen log cells were grown to mid-log phase (A560 = 0.7) followed by induction for 75 min with the CuSO4 concentrations specified in the experiment. A 1-ml aliquot of each culture was then harvested and dried for 1 h at 120 °C to determine the dry weight. Another 1-ml aliquot was washed three times with 50 mM Tris-Cl, 50 mM NaCl, 1 mM CaCl2, 3 mM KCl, 1 mM MgCl2, pH 7.0, resuspended in 1 ml of 10% perchloric acid, 0.05%, and hydrolyzed at 95 °C for 30 min, and the copper content was determined by flame atomic absorption spectroscopy on a Perkin-Elmer 2380 instrument.

RNA Extraction—Wild-type E. hirae cells were grown to an A546 of 0.4–0.6 in N-media followed by induction with the respective CuSO4 concentration for 90 min. From 6 × 108 of these cells, mRNA was isolated with the Qiagen RNeasy Mini kit (Hilden, Germany). Contaminating DNA was removed by incubation with RNA samples (50 μl) with DNase I reaction buffer (10 μl of 100 μg/ml MgCl2, 10 mM dithiorthiostil, 10 mM Tris-Cl, 1 mM EDTA, 40 units of RNase-free DNase I (Roche Molecular Biochemicals), 1 unit of Rnasin (Promega, Madison, WI), pH 7.4) at 37 °C for 1 h. The reaction was stopped with 25 μl of DNase I stop mix (50 mM EDTA, 1.5 mM sodium acetate, 1% SDS). RNA samples were then cleaned a second time with the RNeasy kit. Concentration and integrity of RNA samples were checked on 1.2% formaldehyde-agarose gels. To look at variations in mRNA levels at high copper concentrations over time, wild-type E. hirae was grown to an A546 of 0.4–0.6 in N-media containing 0.25 mM copper (preinduction), followed by growth in 5 mM copper for the indicated times.

Reverse Transcription-Polymerase Chain Reaction—Reverse transcription of 250 ng of RNA was conducted with the SuperScript Pre amplification System (Life Technologies, Inc.) and the random hexamers supplied with this kit, according to the protocol of the manufacturer. A control without reverse transcriptase was run for each sample. To verify the absence of DNA contamination, reverse transcription products (2 μl) were amplified by polymerase chain reaction (PCR)2 using the primers 5′-GGCTGTCATTGGATGAGAG and 5′-CTGTTAGATC TCTTGCGTTGTG designed to amplify a 521-base pair region encompassing the 3′ end of copZ and the beginning of copA. Thermal cycling was performed as follows: 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 45 s, and extension at 72 °C for 60 s.

Real-Time Quantitative PCR—The PCR mixtures consisted of 2 μl of 50-fold diluted reverse transcription product prepared as described above and a mixture of 1 μl of the primer set above, 4 mM MgCl2, 2 μl of master mix (FastStart DNA Master SYBR Green I kit, Roche Molecular Biochemicals). Primers for the internal control (5′-AGGCTTATTGGAACATGGAG and 5′-CTGTTAGATCTCTTGCGTTGTG) were designed based on the 16S rRNA of E. hirae (ATCC9403) (GenBank™ accession number AF061011) and amplified in a 359-base pair region. Reactions were run in a LightCycler (Roche Molecular Biochemicals) under the following conditions: hot start denaturation at 95 °C for 10 min; 52 cycles of amplification with 95 °C for 15 s, 59 °C for 5 s, and 72 °C for 15 s. Melting curve analysis was conducted by heating to 95 °C followed by 65 °C for 15 s. Fluorescence was then continuously measured from 65–95 °C followed by final cooling to 40 °C for 30 s. Levels of the cop operon RNA transcription were corrected for variations of the internal control.

Induction of CopZ overexpression by the ΔcopZ strain harboring the pC6C6 CopZ expression plasmid was as described in A.S., standard of 1 μg of purified CopZ.

In Vitro Degradation of CopZ—CopZ was purified as described previously (19). Degradation was measured at 37 °C under anaerobic conditions in a total volume of 20 μl of 50 mM K-HEPES, pH 7.0, containing 80 ng of CopZ and 20 μg of crude cell lysate of a ΔcopZ mutant. Other additions were as detailed under “Results.” Degradation was assessed by Western blotting and quantitative chemiluminescent measurements as described above. Lysates were prepared as described under “CopZ expression and Western Blotting” but without the addition of SDS sample buffer. Where required, cells were induced with 3 mM CuSO4, the copper chelators bicinechonic acid (0.1 mM), or o-phenanthroline (0.25 mM) for 1 h.

Yzymography Detection of Proteolytic Activities—Zymography was conducted essentially as described previously (30). 7.5% SDS gels were polymerized with 130 μg/ml purified CopZ or bovine serum albumin. Crude lysates (50 μg) from uninduced wild-type E. hirae and from cells induced with 3 mM copper were resolved electrophoretically. Gels were then soaked in 2.5% Triton X-100 at room temperature for 1 h followed by the proteolytic reaction overnight at 37 °C in 50 mM K-HEPES, pH 7.0, 2% acetonitrile. Gels were rinsed with distilled H2O and proteolytic clearing zones visualized by staining in 0.1% Coomassie Blue, 7.5% acetic acid, 10% ethanol at room temperature for 105 min.

RESULTS

It has previously been shown that the growth of wild-type E. hirae is not significantly affected by up to 5 mM copper in the growth media (31). This copper resistance is accomplished by the induction of the CopB copper exporting ATPase encoded by the cop operon. The expression of CopB (and also CopA) increases with rising copper concentrations up to a maximum at 2 mM copper (31). Here we observed that the expression of the CopZ copper chaperone encoded by the same polycistronic message as CopA and CopB only increased up to 0.5 mM copper and declined again at higher copper concentrations to become nearly undetectable at 3 mM copper (Fig. 1A). The disappearance of CopZ under high copper conditions raised two questions: 1) does CopZ damage to cells under high copper conditions, and 2) does down-regulation proceed as CopA and CopB only increased up to 0.5 mM copper and declined again at higher copper concentrations to become nearly undetectable at 3 mM copper (Fig. 1A). The disappearance of CopZ under high copper conditions raised two questions: 1) does CopZ damage to cells under high copper conditions, and 2) does down-regulation proceed.

The induction of possible effects of high levels of CopZ under high copper conditions was addressed with a strain strongly overexpressing CopZ from plasmid pC6C6 in a ΔcopZ background. Even when not induced by copper, these cells showed strong CopZ expression, and expression was further induced by copper (Fig. 1B). At all copper concentrations employed, expression levels were 10–20 times higher in the CopZ-
overexpressing strain compared with wild-type *E. hirae* as assessed by quantitative evaluation of chemiluminescence on Western blots.

Overexpression of CopZ led to copper sensitivity. Growth of the CopZ-overexpressing strain was inhibited by ambient copper in excess of 0.1 mM and virtually ceased to grow in 1.5 mM copper (doubling time $\approx 10$ h, Fig. 2A). In contrast, the doubling time of the wild-type and the $\Delta$copZ parent strains were not affected by these copper concentrations. All three strains were normal in terms of the expression of the other three Cop proteins, CopA, CopB, and CopY. Thus, it appeared that CopZ overexpression was toxic to cells. Total cellular copper increased with rising ambient copper (Fig. 2B). Although a significant portion of this copper was bound to the cell wall, at least a part of the increase can be ascribed to an increase in cytoplasmic copper. This is supported by the observation that cells devoid of the CopB ATPase, the chief copper export route, accumulated considerably larger amounts of copper than cells expressing CopB (31). CopZ overexpression also made the cells more sensitive to $H_2O_2$ and paraquat (Fig. 3), although these effects were not as marked as the sensitivity to copper. Taken together, these findings suggest that the combination of increased cytoplasmic copper and high CopZ levels induced artificially here are detrimental to the cell. This would provide a rationale for the efficient down-regulation of CopZ in wild-type under high copper conditions.

To test whether the biphasic expression patterns of CopZ was accompanied by corresponding changes in mRNA levels, cop mRNA was measured by reverse transcription-quantitative real-time PCR. Steady-state levels of cop mRNA were found to rise with increasing copper concentrations, reaching a plateau at 0.25 mM ambient copper (Fig. 4). This level was maintained in up to 5 mM copper. At higher copper concentrations, cop mRNA levels dropped, probably because of the significant growth inhibition under these conditions. When cop mRNA levels were analyzed as a function of time, only a marginal change could be seen over 3 h (Fig. 4, inset). Clearly, the reduced CopZ expression observed under high copper conditions was not the result of decreased steady-state mRNA levels.

We thus investigated proteolysis as a mechanism for the disappearance of CopZ at high copper concentration. To this end, the degradative activity of crude cytosolic extracts on purified CopZ was investigated *in vitro*. These experiments were carried out under anaerobic conditions to mimic the reducing conditions prevailing inside the cell. A $\Delta$copZ mutant was used for the preparation of cytosolic extracts to prevent interference from endogenous CopZ. When purified Cu-CopZ was mixed with extract prepared from cells grown in 3 mM copper, it was rapidly degraded (Fig. 5A). apo-CopZ was significantly more resistant to degradation than Cu-CopZ. The half-times of degradation for apo-CopZ and Cu-CopZ were 7 and 22 min, respectively (Fig. 5B). The 3-fold higher resistance to proteolysis of apo-CopZ compared with Cu-CopZ was unexpected, because copper interacts strongly with two cysteines on CopZ, which would be expected to stabilize the protein struc-

---

**Fig. 2.** Copper sensitivity of growth and cellular copper content. A, wild-type (●), a ΔcopZ strain (○), and a ΔcopZ strain complemented with the CopZ overexpressing plasmid pCC6 (▼) were grown in 1-ml cuvettes containing 0–1.5 mM CuSO$_4$. Doubling times were monitored by following $A_{546}$ readings. B, mid-log cells were induced with the respective copper concentrations for 75 min, and the copper content was measured by atomic absorption spectroscopy as detailed under “Experimental Procedures.” ●, *E. hirae* wild-type; ○, ΔcopZ mutant.

**Fig. 3.** Effect of CopZ overexpression on sensitivity to oxidative stress. Cells were grown in 1-ml cuvettes containing $N$-media with increasing concentrations of either $H_2O_2$ (A) or paraquat (B), and growth was monitored by following the $A_{546}$. Growth rates were determined as 1/doubling time. ●, *E. hirae* wild-type; ○, ΔcopZ mutant; ▼, ΔcopZ mutant complemented with a pCC6 plasmid bearing the copZ gene.
Expression of cop mRNA in response to ambient copper. Wild-type *E. hirae* was induced for 90 min with varying CuSO₄ concentrations. RNA was extracted and reverse-transcribed, and *cop* mRNA was quantified by real-time quantitative PCR using the Light-Cycler. 10⁻⁷ m copper corresponds to no added copper, and the corresponding basal *cop* mRNA level was set to one. Inset, change of cop mRNA level over time in fully induced *E. hirae* wild-type cells. Other details are described under “Experimental Procedures.”

To test whether the observed proteolytic activity was inducible, cytosolic extracts were prepared from cells grown under a variety of conditions: in unsupplemented N-media or in N-media supplemented with 3 mM CuSO₄, 0.1 mM copper(I) chelator bicinechonic acid or 0.25 mM copper(II) chelator o-phenanthroline. Proteolytic activity was tested on apo-CopZ in the presence and absence of copper. This experiment is complicated by the fact that the different extracts are “contaminated” with either copper or copper chelators. However, it is apparent that the extracts prepared from cells under all growth conditions contained a copper-stimulated proteolytic activity (Fig. 6A).

The inhibition of protein synthesis with chloramphenicol 1 h before the preparation of cell extracts also did not suppress the proteolytic activity (data not shown). For the *in vitro* proteolysis of CopZ, it made no difference whether copper(I)-acetoni-trile or copper(II)-sulfate was added for the incubations of lysates with CopZ, presumably because copper(II) was reduced to copper(I) under the strictly anaerobic conditions of the experiment. Further support for copper(I) being the active species stems from the observation that Ag(I), a potential Cu(I) mimic, also stimulated proteolysis (data not shown). These results suggest the presence of a constitutive copper(I)-stimulated proteolytic activity in cell extracts.

To obtain a handle on the protease acting on CopZ, the susceptibility of the proteolytic activity to protease inhibitors was tested. The two serine protease inhibitors p-phenylmethy-lysulfonyl fluoride (PMSF) and p-aminobenzamidine were found to inhibit the degradation of CopZ, whereas TLCK and TPCK, which are also serine protease inhibitors, had no marked effect (Fig. 6B). Similarly, the metalloprotease inhibitor o-phenanthroline did not inhibit CopZ degradation. It can thus be concluded that the protease-degrading CopZ is a serine-type protease.

Zymography has been widely used to associate enzymatic activities with protein bands on gels. We here used this method to visualize the Cop-protein-degrading activity in crude cell extracts. The zymograms revealed a protease activity of 58 kDa that exhibited the expected properties, namely the activation by copper and inhibition by p-aminobenzamidine (Fig. 7, arrow). Maximal activation of proteolysis was observed at 3 μM copper(I)-acetoni-trile. Silver(I) also activated but to a lesser extent (data not shown). This protease did not degrade bovine serum albumin and thus exhibited substrate specificity. The copper-treated samples always displayed fewer protein bands by Coomassie Blue staining. We ascribe this result to aggregation and trapping in the stacking gel of a selected population of proteins by copper. This tentative zymographic identification of the protease could form the basis for its biochemical characterization and the isolation of knock-out strains.

**DISCUSSION**

Previous studies demonstrated that the *cap* operon, encoding the four genes CopY, CopZ, CopA, and CopB, is regulated at the transcriptional level by copper (7, 31). The copper-responsive repressor, CopY, is released from the promoter by increased copper levels, thereby allowing transcription to proceed (17). In this work, we report a second mechanism that controls the
proteolytic degradation of CopZ under high copper conditions.

CopZ was maximally induced at 0.5 mM extracellular copper and was reduced at higher copper concentrations to eventually become undetectable above 3 mM media copper. The involvement of proteolysis under these conditions is supported by several lines of evidence. At a copper concentration of 0.5 mM, CopZ expression and the level of cop mRNA both reached a maximum. Thereafter, cop mRNA transcription remained high, whereas CopZ levels decreased and eventually became undetectable. Because the primers used to measure mRNA levels by real-time quantitative PCR covered the 3’ end of the copZ gene, it appears improbable that the specific partial degradation of the cop message is the cause for decreased CopZ expression. Rather, CopZ levels are apparently copper-regulated by two distinct mechanisms, regulation at the level of transcription and posttranslational control by proteolysis. The degradation of CopZ could also be demonstrated in vitro using purified CopZ and cell extracts. Zymographic studies revealed a protease with the anticipated properties.

The proteolytic degradation of CopZ in wild-type cells grown in high copper not only implies that the chaperone is dispensable under these conditions but suggests that it becomes toxic to the cell. The chief cause or at least a contributing factor to this could be a rise in cytoplasmic copper in cells grown at elevated ambient copper. Increased intracellular copper could result in excessive and thus detrimental Cu-CopZ concentrations. The toxic effects of CopZ overexpression from a plasmid strongly support this notion. Elevated Cu-CopZ levels could compromise the specificity of the intracellular copper delivery process, which would lead to the transfer of copper to unqualified sites. Alternatively, the exposed copper in Cu-CopZ (19) could participate in radical formation. In support for the latter mechanism, we observed that the overexpression of CopZ led to increased sensitivity of cells to copper, hydrogen peroxide, and paraquat, a generator of superoxide anions. In light of this, the 3× faster degradation of Cu-CopZ versus apo-CopZ that we observed in our in vitro assay would be biologically meaningful. With priority, the toxic Cu-CopZ has to be removed.

The down-regulation of CopZ under high copper conditions would preclude an essential role of this chaperone in the secretion of excess copper, which proceeds by the CopB copper ATPase in E. hirae. Conceivably, copper complexed to glutathione or other biomolecules is the substrate for secretion byCopB. On the other hand, interaction studies using surface plasmon resonance analysis revealed that CopZ interacts with the putative copper import ATPase, CopA (32). These observations would suggest that CopZ picks up copper from CopA under copper-limiting conditions but is not involved in the delivery of copper to CopB for export in high copper states.

In yeast, the CopZ homologue, Atx1, serves in the delivery of copper to CCC2, the sole copper ATPase in this organism (for review see Ref. 3). CCC2 is located in the trans-Golgi network and provides copper for the synthesis of Fet3, an oxidase required for high affinity iron acquisition. This creates an interesting copper-iron connection, i.e. copper-deficient cells display iron deficiency. Atx1 has been shown to deliver copper to the CCC2 copper ATPase, and Atx1-deficient yeast mutants display iron deficiency. However, this phenotype is "leaky" and can be suppressed by raising copper media levels, suggesting that a parallel pathway for copper delivery to CCC2 exits (33). Similarly, in E. hirae, copper transfer by CopZ can be bypassed by other mechanisms under high copper conditions.

Yeast Atx1 was originally isolated by complementation of auxotrophies for lysine and methionine, which are phenotypic to superoxide dismutase deficiency. Overexpressing Atx1 from a plasmid also relieved the sensitivity of a sod1Δ sod2Δ yeast strain to paraquat and was thus named antioxidant protein (34). It only later became clear that the primary function of Atx1 is that of a copper chaperone, which delivers copper to the copper ATPases (35). It was postulated that copper-trafficking and antioxidant functions of Atx1 arise from chemically and structurally distinct attributes of the protein, and it was shown that purified Cu-Atx1 can react with superoxide anions in vitro (36). However, this process was noncatalytic, and the in vivo antioxidant function of Atx1 required overexpression, suggesting that it may not be physiologically relevant (37). The situation for CopZ in E. hirae differs from that of Atx1 in yeast, the overexpression of CopZ increased the sensitivity of E. hirae to oxidative stress rather than offering protection as was observed for Atx1 (overexpression levels in yeast and E. hirae were both approximately 10-fold). The toxicity of high CopZ levels may thus be the raison d’être for the proteolytic degradation mechanism described here. In the absence of such a proteolytic control, wild-type CopZ expression could probably attain the (toxic) levels observed in the overexpressing strain. This conjecture gains support from the copper-induced rise in cop mRNA by three orders of magnitude and the increase in CopB protein by 50–100-fold.

Whether copper delivery to the trans-Golgi network in yeast also provides a route for copper secretion remains to be shown. In mammalian cells, this is clearly the case. The trans-Golgi copper ATPases homologous to CCC2, the Menkes and Wilson proteins, are directly involved in the secretion of excess copper but also in delivery of copper to the trans-Golgi network for the synthesis of cuproenzymes like ceruloplasmin, a homologue of yeast Fet3 (5). The human CopZ homologue, HAH1, has been shown to also deliver copper to the Menkes and Wilson ATPases, establishing a role for human HAH1 analogous to that of yeast Atx1 (38). Knock-out mice that are deficient in the Atx1 homologue, Atox1, exhibited high mortality and growth failure and displayed many symptoms typical of copper deficiency, such as impaired tissue integrity, temperature regulation, and pigment formation (39). Atox1 was shown to be widely expressed in rat tissues, including brain, with the highest expres-
sion observed in neuronal subtypes that are also characterized by their high levels of metals like copper, iron, and zinc. These findings suggest that in higher organisms unlike in yeast or bacteria, the role of the copper chaperone is a vital one and cannot be compensated by other mechanisms. Metallochaperones may be particularly important in maintaining the functionality of certain neuronal cells (40). Indeed, the overexpression of Atox1 in rat neuronal cell lines offered significant protection against stress induced by serum deprivation or oxidative injury (41). The interesting question of how Atox1-overexpression cells to 10 nM copper is sufficient to repress transcription and to identify the proteolytic process involved.

In heavy metal homeostasis and provide a lead toward the controlled proteolysis as an additional safeguarding mechanism that extends to other cytoplasmic proteins and whether it also exists in other cells.

REFERENCES

1. Linder, M. C., and Hazegh Azam, M. (1996) *Am. J. Clin. Nutr.* 63, S797–S811
2. Camakaris, J., Vokoboinik, I., and Mercer, J. F. (1999) *Biochem. Biophys. Res. Commun.* 261, 225–232
3. Pena, M. M., Lee, J., and Thiele, D. J. (1999) *J. Nutr.* 129, 1251–1260
4. Horn, N., and Tumer, Z. (1999) *J. Trace Elem. Exp. Med.* 12, 297–313
5. Mercer, J. F. (2001) *Trends Mol. Med.* 7, 64–69
6. Odermatt, A., Suter, H., Krapf, R., and Soliz, M. (1992) *Ann. N. Y. Acad. Sci.* 671, 484–486
7. Odermatt, A., and Soliz, M. (1995) *J. Biol. Chem.* 270, 4349–4354
8. Odermatt, A., Suter, H., Krapf, R., and Soliz, M. (1990) *J. Biol. Chem.* 265, 12775–12779
9. Soliz, M., and Odermatt, A. (1995) *J. Biol. Chem.* 270, 9217–9221
10. Soliz, M., and Vulpe, C. (1996) *Trends Biochem. Sci.* 21, 237–241
11. Gupta, A., Matsui, K., Le, J. F., and Silver, S. (1999) *Nat. Med.* 5, 183–188
12. Rutherford, J. C., Cavet, J. S., and Robinson, N. J. (1999) *J. Biol. Chem.* 274, 25827–25832
13. Sharma, R., Rensing, C., Rosen, B. P., and Mitra, B. (2000) *J. Biol. Chem.* 275, 3873–3878
14. Silver, S., Nucifora, G., and Phung, L. T. (1993) *Mol. Microbiol.* 10, 7–12
15. Rensing, C., Ghosh, M., and Rosen, B. P. (1999) *J. Bacteriol.* 181, 5891–5897
16. Strausak, D., and Soliz, M. (1997) *J. Biol. Chem.* 272, 8092–8096
17. Wunderli-Ye, H., and Soliz, M. (1999) *Biochem. Biophys. Res. Commun.* 259, 443–449
18. Cobine, P., Wickramasinghe, W. A., Harrison, M. D., Weber, T., Soliz, M., and Dameron, C. T. (1999) *FEBS Lett.* 445, 27–30
19. Wimmer, R., Herrmann, T., Soliz, M., and Wüthrich, K. (1999) *J. Biol. Chem.* 274, 22597–22603
20. Harrison, M. D., Jones, C. E., Soliz, M., and Dameron, C. T. (2000) *Trends Biochem. Sci.* 25, 29–32
21. Huffman, D. L., and O’Halloran, T. V. (2001) *Annu. Rev. Biochem.* 70, 677–701
22. Rosenzweig, A. C. (2001) *Acc. Chem. Res.* 34, 119–128
23. Gottesman, S., and Maurizi, M. R. (1992) *Science* 259, 278–283
24. Gottesman, S. (1996) *Annu. Rev. Genet.* 30, 465–506
25. Ott, C. E., Rahmovich, E., Danilo, A., Bonface, J. S., and Klausner, R. D. (1996) *EMBO J.* 15, 3515–3523
26. Zhu, Z., Labbe, S., Pena, M. M., and Thiele, D. J. (1998) *J. Biol. Chem.* 273, 1277–1280
27. Li, H. H., and Merchant, S. (1998) *J. Biol. Chem.* 273, 23504–23510
28. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 368–379
29. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350–4354
30. Leber, T. M., and Balkwill, F. R. (1997) *Biochem. Biophys. Res. Commun.* 202, 44–48
31. Odermatt, A., Krapf, R., and Soliz, M. (1994) *Biochem. Biophys. Res. Commun.* 202, 44–48
32. Muthuap, G., Strausak, D., Bissig, K.-D., and Soliz, M. (2001) *Biochem. Biophys. Res. Commun.* 288, 172–177
33. Lin, S. J., Pufahl, R. A., Dancis, A., O’Halloran, T. V., and Culotta, V. C. (1997) *J. Biol. Chem.* 272, 9215–9220
34. Lin, S. J., and Culotta, V. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 3774–3788
35. Pufahl, R. A., Singer, C. P., Pearson, R. L., Lin, S., Schmidt, P. J., Fahrni, C. J., Culotta, V. C., Penner-Hahn, J. E., and O’Halloran, T. V. (1997) *Science* 276, 855–860
36. Portnoy, M. E., Rosenzweig, A. C., Rae, T., Huffman, D. L., O’Halloran, T. V., and Culotta, V. C. (1999) *J. Biol. Chem.* 274, 15041–15045
37. O’Halloran, T. V., and Culotta, V. C. (2000) *J. Biol. Chem.* 275, 25052–25060
38. Hamza, I., Faist, A., Prohaska, J., Chen, J., Gruss, P., and Gitlin, J. D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 6848–6852
39. Naeva, G. S., Vana, A. M., Eggold, J. R., Kelner, G. S., Maki, R., Dessouza, E. B., and Foster, A. C. (1999) *Neuroscience* 93, 1179–1187
40. Kelner, G. S., Lee, M., Clark, M. E., Maciejewski, D., McGrath, D., Rabizadeh, S., Lyons, T., Breiden, D., Jenner, P., and Maki, R. A. (2000) *J. Biol. Chem.* 275, 580–584
41. Gilman, R. S., Luo, H., Rodgers, J., Broderius, M., and Eide, D. (1998) *J. Biol. Chem.* 273, 28617–28624
42. Willett, W. S., Brinen, L. S., Fletterick, R. J., and Craik, C. S. (1996) *Biochemistry* 35, 5992–5998