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Communication

Copper Differentially Regulates the Activity and Degradation of Yeast Mac1 Transcription Factor*

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Copper is an essential metal ion that is toxic when accumulated to high intracellular concentrations. The yeast Mac1 protein is a copper-sensing transcription factor that is essential for both the activation and inactivation of genes required for high affinity copper ion transport. Here we demonstrate that in response to low copper ion concentrations Mac1 protein is rendered inactive for copper transporter gene transcription. Under high copper ion concentrations Mac1 is degraded in a rapid, copper-specific manner. This degradation is critical to prevent copper toxicity that would otherwise result from sustained expression of the copper transport genes. These results demonstrate that nutritional and toxic copper concentrations elicit distinct fates for the Mac1 copper-sensing transcription factor and establish a new mechanism by which trace metals regulate gene expression.

Copper is an essential trace metal that serves as a critical co-factor for a number of enzymes, including cytochrome oxidase for respiration and Cu,Zn-superoxide dismutase for oxidative stress protection (1, 2). When accumulated to excessive levels, copper is highly toxic due to its ability to engage in redox chemistry resulting in the generation of the destructive hydroxyl radical (3). Therefore, a delicate balance must be established and maintained between the accumulation of sufficient copper ion levels for biochemical reactions and the elevation of copper ions to toxic levels. High affinity Cu(I) transport into yeast cells requires the action of the integral membrane Cu(I) transport proteins encoded by the *CTR1* and *CTR3* genes and the Fe(III)/Cu(II) reductase encoded by the *FRE1* gene (4–6). Transcription of the *CTR1*, *CTR3*, and *FRE1* genes is activated under copper starvation conditions, while expression of these genes is potently and rapidly extinguished by exogenous Cu ion concentrations in the picomolar to nanomolar range (8). The transcriptional activation and inactivation of the yeast Cu(I) transport genes requires conserved cis-acting copper-responsive (CuRE)[1] promoter elements and the Mac1 nuclear protein. *In vitro* the CuRE elements are specifically bound by Mac1p, while *in vivo* the *CTR3* CuREs are occupied under copper starvation conditions and not bound when *CTR3* expression is extinguished by the addition of 10 nM copper (7–11). In this work, we have investigated the mechanisms by which copper ions regulate expression of the yeast Cu(I) transport genes through Mac1p. We demonstrate that Mac1 is a stable protein in cells grown in low copper ion concentrations; however, Mac1 is degraded in a rapid, metal-specific fashion when cells are grown under high copper ion concentrations. Although Mac1 degradation is not obligatorily coupled to target gene regulation, the rapid turnover of Mac1 is essential to prevent copper ion toxicity as a consequence of sustained Cu(I) transporter gene expression. Copper ion activated degradation of the Mac1 transcription factor represents a new mechanism by which trace metals play important cellular regulatory roles.

EXPERIMENTAL PROCEDURES

Yeast cells were grown under low copper ion conditions as described (8). All molecular biology manipulations were carried out as described (13). A coding region for a nine-amino acid epitope from the influenza hemagglutinin HA protein (YPYDVPDYA) was inserted in frame at the carboxyl terminus of the MAC1 or MAC1^{up1} open reading frame by polymerase chain reaction from genomic DNA of a wild type (DTY1) or MAC1^{up1} (DTY205) strain, respectively. The sequence of the gene was verified and the HA epitope-tagged allele, under the control of the MAC1 promoter, was subcloned via *Hin*dIII (Klenow) and *Bam*HI sites into the *Sal*I (Klenow) and *Bam*HI sites of the centromeric yeast plasmid pRS313 to generate pRSMac1(HA). The epitope-tagged *MAC1* and *MAC1*^{up1} alleles accurately reflected the function of the unadulterated *MAC1* or *MAC1*^{up1} alleles as ascertained by equivalent growth rates on YPE medium (1% yeast extract, 2% bactopeptone, and 3% ethanol) and copper-responsive regulation of the *CTR3* gene that was indistinguishable from the parental alleles, as assessed by RNase protection experiments when transformed into the *mac1*Δ strain SLY2 (*MATa a gal1 trp1-1 his3 ade8 ura3::Kn^r mac1::URA3*). Plasmids pRSMac1-REP-I and pRSMac1-REP-II were constructed by site directed mutagenesis to generate the corresponding allele, followed by the exchange of a 544-base pair *Bsp*EI fragment encompassing the mutations in the MAC1 REP-I and REP-II alleles, with the same fragment from the wild type MAC1HA gene in plasmid pRSMac1(HA). All mutations and fusion junctions were verified by DNA sequencing. Point mutations in *MAC1* were isolated based on the inability of cells to repress the expression of a fusion between the *CTR3* promoter to the *URA3* gene in the presence of 20 μM copper. One category of uracil prototrophs mapped to the *MAC1* gene and recombination mapping and sequence analysis demonstrated the presence of single amino acid substitutions in REP-I designated ^{up3} and ^{up4}. The ^{up3} and ^{up4} alleles regulate *CTR3* mRNA levels in response to copper ions in a manner indistinguishable from the ^{up1} allele.[2]

RESULTS AND DISCUSSION

Mac1 Degradation Occurs Only in the Presence of High Copper Ion Concentrations—To investigate the mechanisms by which Mac1p senses copper levels to control transcription of the high affinity Cu(I) transport genes, both Mac1 protein and mRNA levels were measured (12, 13). Mac1p-HA contains a

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[1] The abbreviations used are: CuRE, copper-response element; HSF, heat shock factor.

[2] S. Labbé and D. J. Thiele, unpublished data.

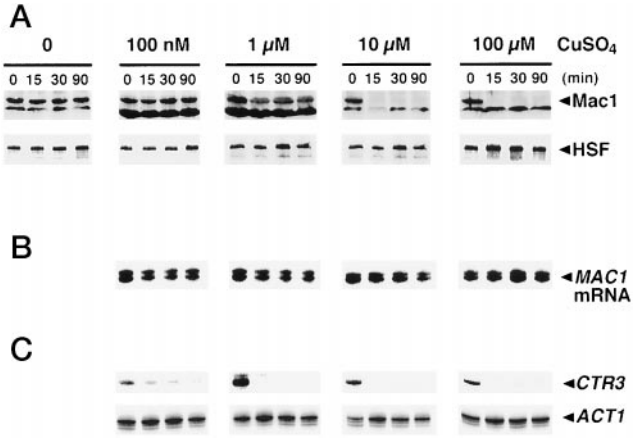

FIG. 1. **Copper ion concentration-dependent Mac1p degradation.** *Saccharomyces cerevisiae* strain SLY2 (*MATa gal1 trp1–1 his3 ade8 ura3::Kn*^{r} *CTR1 CTR3 mac1::URA3*) transformed with pRSMac1(HA) was grown under low copper ion conditions and cultures untreated or treated with $CuSO_4$ at final concentrations of 100 nM, 1 μM, 10 μM, or 100 μM for the indicated times in minutes (*min*). Whole cell protein extracts and total RNA were prepared from culture aliquots. *A*, Mac1p-HA is degraded at high copper ion concentrations. Mac1p-HA was detected by immunoblotting using anti-HA antibody 12CA5 (1:1000 dilution) and goat anti-mouse IgG conjugated to horseradish peroxidase (1:1000), and HSF was detected using polyclonal rabbit anti-HSF antiserum (14). *0 lanes* represent untreated cells. Mac1p-HA (*Mac1*) and heat shock factor (*HSF*) are marked with an *arrowhead*. Mac1p-HA was conclusively identified by co-electrophoresis of Mac1p-HA produced in *Escherichia coli*. The lower ~45-kDa band is commonly observed to cross-react with the 12CA5 antibody and is not a Mac1p-HA degradation product. *B*, the steady state levels of MAC1 mRNA do not change upon copper ion treatment. *MAC1* mRNA (*arrowhead*) levels were detected using RNase protection assays. *C*, Mac1p degradation is independent of copper ion-dependent transcriptional regulation of *CTR3*. *CTR3* and *ACT1* mRNAs (*arrowheads*) were detected using RNase protection assays.

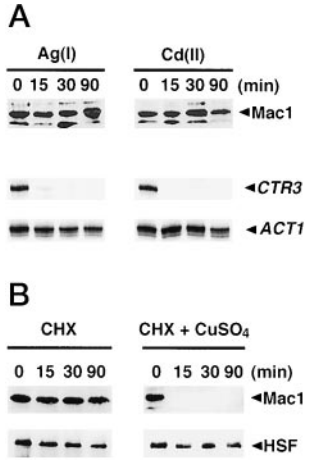

FIG. 2. **Mac1p degradation is uncoupled from Cu(I) transporter gene regulation.** *A*, Mac1p degradation is copper ion-specific. Strain SLY2 transformed with pRSMac1(HA) was grown in low copper medium, treated with 10 μM $AgNO_3$ or 10 μM $CdSO_4$ for the indicated times, and whole cell protein extracts and total RNA were prepared from culture aliquots. Mac1p-HA protein and *CTR3* mRNA levels were detected as described in the legend to Fig. 1. Mac1p-HA protein and *CTR3* and *ACT1* mRNAs are indicated with an *arrowhead*. *B*, copper ion-dependent Mac1p degradation does not require new protein synthesis. Strain SLY2 transformed with pRSMac1(HA) was treated with cycloheximide (*CHX*) at 100 μg/ml for 30 min to inhibit protein synthesis, followed by $CuSO_4$ treatment (100 μM) for the indicated times. Mac1p-HA and HSF were detected by immunoblotting as described in Fig. 1.

single HA epitope at the Mac1p carboxyl terminus and completely functionally complements a *mac1* deletion strain. The analysis of Mac1p-HA from cells grown under low copper ion concentrations, and treated with increasing copper concentrations for up to 90 min, demonstrated that Mac1p-HA is stable at low copper concentrations. However, Mac1p-HA steady state levels are rapidly and nearly completely extinguished at or above 10 μM $CuSO_4$ (Fig. 1*A*). The steady state levels of another yeast nuclear transcription factor, heat shock transcription factor (HSF; Ref. 14), remained constant at all copper ion concentrations over the course of these experiments (Fig. 1*A*). In contrast to Mac1p-HA protein levels, MAC1-HA mRNA levels were not responsive to copper ions and were constant for all samples (Fig. 1*B*). To ascertain if the reduction in Mac1p-HA levels parallels the inactivation of high affinity Cu(I) transporter gene expression, RNA was prepared from aliquots of the same cell cultures, and *CTR3* mRNA levels were measured by RNase protection assays. The data in Fig. 1*C* show that, as observed previously, *CTR3* mRNA levels were rapidly reduced in response to nanomolar copper ion concentrations. These data demonstrate that the loss of Mac1p-HA occurs at copper ion concentrations that exceed those required for normal inactivation of *CTR3* transcription. We observed the same copper ion-mediated extinction of Mac1p steady state levels in response to high copper ion concentrations by using a single or a double MYC epitope tag at the carboxyl terminus of Mac1 (data not shown). Taken together, these results suggest that loss of Mac1p in response to high copper ion concentrations can be uncoupled from low copper ion-mediated Mac1-dependent extinction of Cu(I) transport gene transcription.

Mac1p Degradation Is Uncoupled from Target Gene Regulation—The inactivation of yeast Cu(I) transporter gene transcription is highly specific for the metal ions copper and Ag(I), with responses to Cd(II) and Hg(II) only at metal concentrations 1000 times that of copper or Ag(I) (8). To further test whether the dramatic reduction in Mac1p-HA levels observed with high copper ion concentrations is obligatorily coupled with Cu(I) transporter gene regulation, Mac1p-HA and *CTR3* mRNA levels were measured from cells treated with 10 μM Ag(I) or Cd(II). The data in Fig. 2*A* clearly demonstrate that while *CTR3* expression was inactivated by Ag(I) and Cd(II) as observed previously (8), steady state levels of Mac1p-HA were not affected by either metal. Therefore, although Cu(I) transporter gene expression is inactivated by Cu and Ag(I) ions with equal efficacy, and by Cd(II) at much higher concentrations, the dramatic reduction of Mac1p-HA levels is highly specific to copper ions and is not essential for regulation of Cu(I) transport gene expression at low copper ion concentrations. Since high Cu(I) ion levels are known to activate transcription of genes encoding proteins such as the yeast metallothioneins, which protect cells from copper toxicity and Cu,Zn-superoxide dismutase (15–17), an experiment was conducted to ascertain whether the reduction in Mac1p-HA steady state levels depends on the copper ion-dependent synthesis of new proteins. Yeast cells expressing the Mac1p-HA protein were pretreated with 100 μg/ml cycloheximide for 30 min to inhibit new protein synthesis, followed by either no addition or the addition of 100 μM $CuSO_4$. As shown in Fig. 2*B*, Mac1p-HA levels were stable when cells were grown under low copper ion concentrations and in the presence of cycloheximide. However, Mac1p-HA levels were dramatically reduced when cells were incubated with copper ions under conditions where protein synthesis was inhibited. The levels of yeast HSF protein remained constant under both conditions over the course of the experiment (Fig. 2*B*). Therefore, the reduction in Mac1p-HA levels in response to high copper ion concentrations is not dependent on the synthesis of new proteins. Furthermore, these data demonstrate that high, but not low, copper ion concentrations rapidly and specifically trigger the degradation of Mac1p-HA.

Mac1p Degradation Is Critical for Copper Resistance—A dominant allele, *MAC1*^{up1}, encodes a Mac1 protein that is only partially responsive to copper and therefore drives robust transcription of the *CTR1*, *CTR3*, and *FRE1* genes even in the presence of high copper ion concentrations (7–11). The *MAC1*^{up1} mutation resides in one of two repeats that are located in the carboxyl-terminal half of the protein, designated REP-I and REP-II, each containing 5 cysteines and 1 histidine residue (Fig. 3*A, WT*). To ascertain the role of the REP-I and REP-II elements in Cu(I) transporter gene regulation and to determine whether they are involved in copper ion-dependent degradation of Mac1p, all of the Cys and His residues were mutated in each independent REP element (Fig. 3*A, REP-I* and *REP-II*). The analysis of *CTR3* mRNA levels in a strain expressing the wild type Mac1p (Fig. 3*B, WT*) gave typical rapid and complete inactivation of *CTR3* expression. In contrast, isogenic cells with the *MAC1*^{up1} allele (^{up1}) exhibited elevated basal *CTR3* mRNA levels that were only transiently repressed by copper ions. Longer exposure of cells to copper ions resulted in sustained *CTR3* expression as compared with wild type cells (Fig. 3*B*, ^{up1}). The comparison of cells expressing either the REP-I or REP-II *MAC1* allele demonstrated strikingly distinct phenotypes with respect to *CTR3* gene expression. REP-I cells displayed the transient but largely refractile response to copper ion levels similar to that observed with *MAC1*^{up1} cells (Fig. 3*B*) or in *Mac1*^{up2} (11) and *Mac1*^{up3} and *Mac1*^{up4} cells.[2] Although we have ascertained that the copper ion-dependent turnover of Mac1p is not obligatory for *CTR3* mRNA regulation, we observed in fact increased *CTR3* mRNA in strains with the Mac1^{up1} and REP-I *MAC1* alleles. The fact that *CTR3* expression in REP-I cells was slightly lower than in *MAC1*^{up1} cells may be due to the multiple mutations in REP-I rather than a single amino acid substitution in the Mac1^{up1} protein. In yeast cells expressing the Mac1 REP-II protein, *CTR3* mRNA levels were undetectable (Fig. 3*B*, compare *REP-I* and *REP-II*). These data demonstrate that, although identical mutations were made in the Mac1 REP-I and REP-II elements, these elements are not functionally equivalent with respect to their role in expression of the high affinity Cu(I) transport genes. This conclusion is strongly supported by additional experimental observations. First, the *in vivo* selection of yeast mutations linked to the *MAC1* gene that exhibit high constitutive expression of the Cu(I) transport genes gave rise to single amino acid substitutions in REP-I, but not REP-II (Fig. 3*A* and Refs. 7, 8, and 11). Second, the kinetics of copper ion-dependent degradation of the protein expressed from the *MAC1* REP-I allele were much slower than the wild type, with Mac1p-HA REP-I levels still detectable 90 min after copper ion treatment (Fig. 3*C*). In contrast, Mac1p expressed from the *MAC1* REP-II allele exhibited copper ion-dependent degradation kinetics indistinguishable from that of the wild type Mac1p (compare Figs. 1*A* and 3*C*). Therefore, although the Mac1p-HA expressed from the REP-II allele is stable under low copper ion concentrations, it is incapable of activating *CTR3* expression and is similar to a strain harboring a disrupted *mac1::URA3* allele (8, 10, 11). The observation that Mac1 REP-II protein, which is defective in activation of copper transporter gene expression, is degraded as well as the wild type protein, is likely due to the fact that copper ions (100 μM) are transported via the low affinity copper transport system. It should be noted that single or clustered point mutations in REP-II, in the context of a Gal4-Mac1 fusion protein, failed to exhibit a defect in regulating a GAL UAS-driven *lacZ* gene in response to 100 μM copper (9). We suggest two possible explanations. First, the fusion of Mac1 to the Gal4 DNA binding domain would mask any requirement for REP-II in Mac1 sequence-specific DNA binding to the CuREs (11).

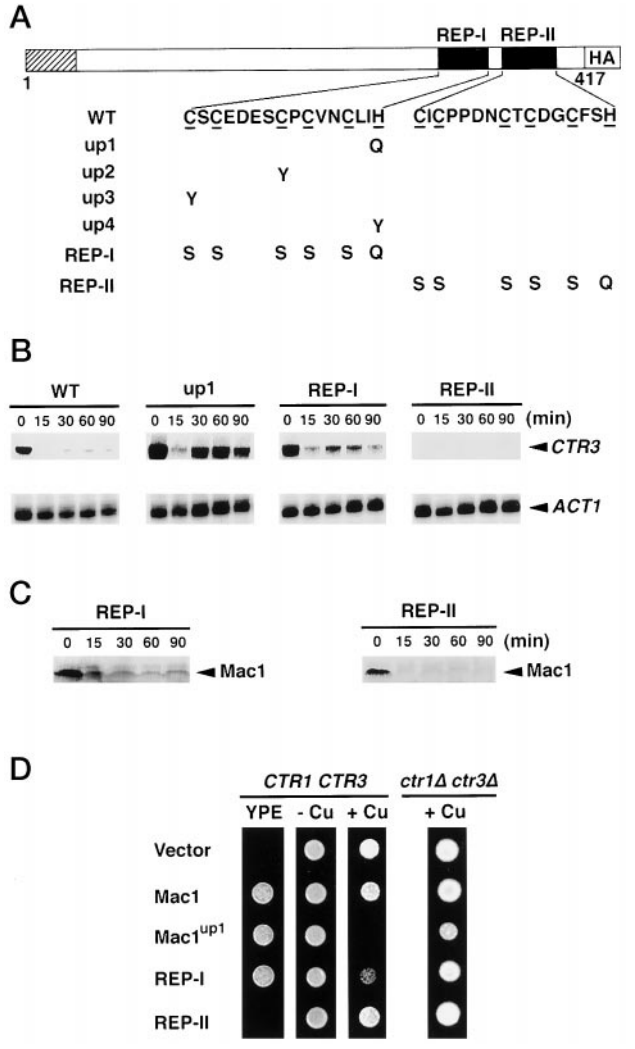

FIG. 3. **Mac1 Cys-His repeats play distinct roles in Mac1p function and regulation.** *A*, schematic representation of the Mac1 protein tagged with a single copy of the HA epitope. The *hatched rectangle* represents the putative amino-terminal minor groove DNA-binding domain, which shows sequence homology to the two known copper ion sensing DNA-binding transcription factors Ace1p and Amt1p (12). The *filled rectangles* in the carboxyl-terminal half represent two Cys-His motifs (REP-I and REP-II). The primary sequences of REP-I and REP-II are shown below, and putative metal ion-binding ligands are *underlined*. The sequence of the Mac1^{up1}, Mac1^{up2}, Mac1^{up3}, and Mac1^{up4} alleles and the mutations in REP-I and REP-II alleles are shown corresponding to the residues in the wild type Mac1p. *B*, the Mac1 REP-I and REP-II alleles are differentially functional in copper ion-responsive transcription of *CTR3*. SLY2 transformed with pRSMac1(HA) (*WT*), pRSMac1^{up1}(HA) (^{up1}), pRSMac1(HA)REP-I (*REP-I*), and pRSMac1-(HA)REP-II (*REP-II*) were treated with 100 μM $CuSO_4$ for the indicated times and whole cell protein extract and total RNA prepared. The steady state levels of *CTR3* and *ACT1* mRNA were detected as described in the legend to Fig. 1. *C*, Mac1p-HA was detected by immunoblotting using extracts derived from SLY2 containing pRSMac1(HA)-REP-I (*REP-I*) and pRSMac1(HA)REP-II (*REP-II*) as described in the legend to Fig. 1. *D, MAC1* REP-I and REP-II alleles display distinct copper ion homeostatic defects. Isogenic strains SLY2 (*CTR1 CTR3 mac1::URA3*) and MPY18 (*ctr1*Δ *ctr3* Δ *mac1::URA3*) harboring the plasmids pRS313 (*Vector*), pRSMac1(HA) (*WT*), pRSMac1^{up1}(HA) (^{up1}), pRSMac1(HA)REP-I (*REP-I*), and pRSMac1(HA)REP-II (*REP-II*) were spotted onto yeast medium containing the nonfermentable carbon source ethanol (*YPE*) and synthetic complete medium lacking histidine without (−*Cu*) or with exogenous $CuSO_4$ at 400 μM (+*Cu*). Cells were incubated for 48 h at 30 °C and photographed.

Second, since the Mac1 REP-II mutant is subject to degradation when cells are incubated in the presence of high copper ion concentrations (Fig. 3*C*), it is possible that regulation of the GAL UAS-*lacZ* fusion gene was due to copper ion-dependent

degradation of the Gal4-Mac1 fusion protein. Although the REP-I and MAC1^{up1} cells are capable of high affinity Cu(I) transport and respiration, REP-II mutants are incapable of respiration, presumably because they are completely defective in expression of the high affinity Cu transport genes (Fig. 3*D*). Finally, both the MAC1^{up1} and REP-I mutants are sensitive to copper in a manner that parallels the magnitude of sustained expression of the *CTR3* gene, while the REP-II mutant is as resistant to copper as the parental wild type strain. Under the conditions used (Fig. 3*D*) the high affinity Cu(I) transport genes contributes only modestly to the copper ion sensitivity of the Mac1^{up1} and REP-I strains. Nonetheless, when the copper transport genes are deleted (*ctr1*Δ *ctr3*Δ) in the Mac1^{up1} and REP-I strains, significant copper resistance is restored (Fig. 3*D*).

It is intriguing that Mac1p, a key transcription factor required for both the activation of Cu(I) transport genes as a consequence of copper ion starvation, and copper ion-dependent inactivation of these genes, is regulated at two distinct levels by distinct copper ion concentrations. Since Mac1p binds the CuREs *in vitro* (11), and occupation of the CuREs *in vivo* directly correlates with *CTR3* gene expression (8), the following model is consistent with the requirement for Mac1 in copper homeostasis. Under copper ion starvation conditions, Mac1p is stable and bound to the CuREs of *CTR1*, *CTR3*, and *FRE1*, strongly activating gene transcription. Under copper ion concentrations that are low, but sufficient for growth, Mac1p DNA binding is reversibly inhibited, perhaps through the direct binding of copper ions to the REP-I or REP-II elements or indirectly via another copper ion sensor. Mac1p, however, is stably present to constantly survey the cellular copper status. Under conditions of copper ion excess (outside of the useful range of the high affinity Cu(I) transport system and within the range of metallothionein gene expression), Mac1p is rapidly degraded to completely eliminate expression of the Cu(I) transport machinery. Two observations support this model. First, the K_m values for Ctr1p and Ctr3p in Cu(I) transport are approximately 2.5 and 5 μM, respectively (5, 6); therefore, Mac1p degradation occurs at copper ion concentrations (10 μM) above the range in which Ctr1p and Ctr3p are useful to the cell. Second, *MAC1* mutants that are defective in copper ion-dependent transcriptional inactivation of the high affinity Cu(I) transport genes, and copper ion-dependent degradation, exhibit increased sensitivity to copper toxicity in a manner dependent on the presence of the *CTR1* and *CTR3* genes. An elegant study has recently shown that Ctr1p is also degraded when yeast cells are grown in the presence of high, but not low, copper concentrations and that this degradation occurs at the plasma membrane and in the absence of new protein synthesis (18). Therefore, the copper ion-dependent degradation of Mac1p and Ctr1p is likely to serve as an important cellular defense mechanism to minimize copper ion toxicity under conditions of copper excess.

The regulated degradation of proteins provides a facile means of establishing homeostatic control in response to changes in cellular physiology. For example, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a critical enzyme in the mevalonate pathway, is degraded via feedback control of the mevalonate pathway (19). The yeast Gcn4p, a key transcription factor required for expression of amino acid biosynthetic genes, is degraded during amino acid repletion via the ubiquitin conjugation pathway (20). Interestingly, the half-life of the mammalian iron-responsive element-binding protein-2 is dramatically decreased in iron replete, as compared with iron-starved cells, demonstrating that iron levels can regulate translation via modulation of the stability of this translational repressor protein (21–23). Of particular relevance to the results presented here, the coordinate regulation of plastocyanin and cytochrome c_6, two reciprocal photosynthetic electron transfer proteins in *Chlamydomonas reinhardtii*, is accomplished by copper ion starvation-mediated transcription of the cytochrome c_6 gene and stabilization of the holoplastocyanin protein as a consequence of copper ion repletion (24). Interestingly, the protease activity that degrades apoplastocyanin appears to be active only in copper ion-starved *C. reinhardtii* cells.

What is the mechanism by which Mac1p is degraded by high, but not low, copper ion concentrations? One possibility, supported by the Mac1 mutational analysis, is that the binding of copper, at least in part via the REP-I element, may allow Mac1p to adopt a conformation or distinct subcellular localization that renders it susceptible to a pre-existing constitutively active protease. Alternatively, the REP-I region may be part of an important domain for recognition by an as yet unidentified copper ion-activated cellular protease. Although no copper-dependent proteases are currently known, the observation that trypsin can be engineered to a form that is highly stimulated by copper ion coordination (25) suggests that nature could adopt similar mechanisms to target-specific proteins for degradation in the presence copper ions. The demonstration that the Mac1 transcription factor is specifically degraded in the presence of high copper ion concentrations represents a new mechanism by which trace metals play important roles in cellular regulation.

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