Copper Ion-sensing Transcription Factor Mac1p Post-translationally Controls the Degradation of Its Target Gene Product Ctr1p*

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Copper ion uptake must be regulated to avoid both deficiency and excess because its essential yet toxic biological nature depends on the concentration. Yeast copper uptake is controlled at both the transcriptional and post-translational levels. The transcription of CTR1 and CTR3, encoding high affinity copper transporters, is regulated by the copper ion-sensing transcription factor Mac1p through the cis-acting copper ion-responsive elements in CTR1 and CTR3 promoters. Ctr1p is known to undergo degradation in cells exposed to high copper levels. We report that Mac1p is also required for copper-dependent Ctr1p degradation. Both mutations within a conserved copper ion binding motif, the “Cuf3” in the Mac1p DNA-binding domain, and within a metal ion binding motif, REP-III located in the cytosolic domain of Ctr1p, cause defects in Ctr1p turnover. Furthermore, we show that the Mac1p limits intracellular copper accumulation likely by controlling Ctr1p degradation. The findings have uncovered an unprecedented mechanism by which a transcription factor not only regulates its target gene transcription but also controls the degradation of its target gene product.

The entry of redox-active copper into cells must be tightly controlled due to its toxic nature when present in excess (1–3). Yet the physiological level of copper ions must be maintained to ensure the activities of those enzymes dependent on copper as their cofactor. This concentration-governed biological nature of copper is reflected in two human diseases. One, Menkes’ disease, is caused by copper deficiency. The other, Wilson’s disease, is caused by copper excess (4). Several recent studies have demonstrated that copper uptake through high affinity transporters is critical for copper homeostasis. Studies of transgenic mice show that homozygous mutations of copper transporters, Ctr1<sup>-/-</sup>, are embryonic lethal, and the heterozygous mutation Ctr1<sup>+/−</sup> causes profound developmental abnormalities (5–8). These findings are reminiscent of earlier observations in the model system yeast Saccharomyces cerevisiae. Yeast has two known high affinity copper transporters encoded by CTR1 and CTR3 (9, 10). The transcription of CTR1 and CTR3 is regulated by the copper-sensing transcription factor Mac1p (11, 12). Yeast cells of ctr1Δ ctr3Δ or mac1Δ genotypes show growth arrest under copper-demanding growth conditions (9–13). These studies support the idea that high affinity copper uptake is the mechanism by which eukaryotic organisms accumulate sufficient intracellular copper ions. A more recent study of Mac1p indicates that control of copper uptake is also critical for avoiding excessive accumulation and preventing copper toxicity. Taken together, the above studies show that control of copper uptake is at the center of copper homeostasis. Hence, understanding how copper uptake is controlled will shed light on the molecular mechanism by which eukaryotic cells maintain the physiological copper level.

The mechanism of copper uptake regulation in mammals is currently unknown. In yeast, copper uptake is regulated at both the transcriptional and post-translational levels (9, 10, 15). Transcription of CTR1 and CTR3 is activated by copper starvation and repressed by copper repletion (12). Mac1p regulates CTR1 and CTR3 transcription through the copper ion-responsive elements in their promoters (11–13, 15–20). Mac1p undergoes phosphorylation, and this modification is likely linked to the regulation of CTR1 and CTR3 transcription (19). DNA binding studies have indicated that Mac1p senses two different levels of copper, perhaps the physiological and toxic levels (19). Thus, elucidation of the molecular mechanism by which Mac1p senses copper ions will be important for understanding how copper uptake is controlled.

In addition to the transcriptional regulation, yeast copper uptake is also controlled at the post-translational level: Ctr1p undergoes degradation in response to high levels of copper ions (15). Interestingly, unlike the Ctr1p, the Ctr3p does not undergo turnover (21). The post-translational processing of Ctr1p is thought of as a defensive means for cells to avoid excessive copper accumulation (15). However, how Ctr1p degradation is controlled is currently unknown. In this study, we report that Mac1p is required for Ctr1p to undergo post-translational turnover in response to increases in copper ion concentrations. The discovery suggests that, in addition to its regulatory role in the CTR1 transcription, Mac1p may also regulate the Ctr1p degradation.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmid Construction, Cell Growth, and Protein Analysis—The S. cerevisiae strains used in this study were SLY2 (MATa gal1 trp1 his3 ade8 lys2–801 ura3–52 mac1::URA3) (13) and ZY60 (MATa gal1 ade8 ctr1::ura3::Kan<sup>R</sup> ctri::TRP1::hisG his3 lys2–801 mac1::ura3). Strain ZY60 was generated by disrupting the TRP1 locus in strain ZY59 (MATa gal1 ade8 ctr1::ura3::Kan<sup>R</sup> ctri::TRP1::hisG his3 lys2–801 mac1::ura3) using the TRP1 deletion plasmid pNY1009 (purchased from ATCC). The plasmid was digested with EcoRI and BglII; URA3 integrants were selected on growth medium lacking uracil (SC-Ura<sup>−</sup>). The TRP1 deletion strain ZY60 was then generated by selection on growth medium containing 5-fluoro-orotic acid (22). Strain ZY59 was constructed using the URA3 deletion plasmid pJL164 (purchased from ATCC). The plasmid was digested with SpeI and XhoI and then transformed into yeast strain MPY18 (MATa gal1 ade8 ctr1::ura3::Kan<sup>R</sup> ctri::TRP1::hisG his3 lys2–801 mac1::URA3) (23) followed by selection on the 5-fluoro-orotic acid plates. The deletions were verified by PCR.

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A plasmid p414GAL-CTR1myc was constructed by cloning CTR1 flanked with BamHI (5' to the start codon) and PstI (3' to the stop codon) sites into vector p414GAL1 (a generous gift from Dennis Thiele). The BamHI and PstI restriction sites were introduced by PCR. The plasmid pCTR1-myc (generously provided by Andy Dancis) was used as template DNA for the PCR (9). Mutations of REP-III (C303S, C310S, C312S, and H315S) were introduced into CTR1 by oligo-directed mutagenesis using the QuikChange method (Stratagene). Mutations were verified by DNA sequencing. The expression plasmid for REP-III mutant Ctr1-Myc, p414GAL-REP-III-myc, was constructed in virtually the same manner as for the p414GAL-CTR1myc.

Mutations of mFist, REP-I, REP-IIA, and REP-I + IA were introduced into MAC1 by oligo-directed mutagenesis using the QuikChange method (Stratagene). Expression plasmids pRS313-mCuFist(HA), pRS313-REP-I(A-HA), pRS313-REP-IIA(HA), and pRS313-REP-I + IIA(HA) were constructed as described previously (13).

A plasmid p414GAL-CTR1myc was co-transformed with the above wild type or mutant Mac1-HAp expression plasmid into strain YZ60. Cells were grown in SC-His ‘Trp’ medium containing raffinose (2%), and Ctr1-Myc expression was induced by galactose (0.5%). The medium was either treated (copper-depleted) or not treated (copper-rich) with Chelex-100 resin (Bio-Rad). Yeast whole cell extracts were prepared, and Mac1-HA² and Ctr1-Myc were detected by Western blotting using anti-HA and anti-Myc antibodies as described previously (13, 15).

Copper Accumulation Analysis—Strain YZ60 harboring plasmids pRS313 and pGAL14, pRS313 and p414GAL-CTR1myc, or pRS313-mCuFist(HA) and pGAL-CTR1myc was grown in the SC-His ‘Trp’ medium containing raffinose (2%) and galactose (0.5%) to early log phase (A500nm of 1.0) and then was treated with CuSO₄ at 10 μM for 30 min or left untreated. Cells of 50 A500nm units were harvested and frozen at ~80°C. The cell pellets were thawed and washed twice with EDTA solution of 10 mM and subsequently washed twice with Nano-pure water. Then cells were lysed by resuspending them in the nitric acid solution (50%) and heating at 80°C. Five duplicates were prepared for each sample. Copper concentrations of the lysates were measured using inductively coupled plasma-mass spectroscopy (ICP-MS) and calculated according to standards of known concentrations.

RESULTS

Mac1p Is Required for the Ctr1p to Undergo Degradation in Response to Copper Ions—A previous study by the Klausner group (15) reported that Ctr1p undergoes degradation at high copper levels. We have recently found that Mac1p likely senses both physiological and toxic copper levels and that inactivation of CTR1 and CTR3 gene expression is critical for yeast to defend against copper toxicity (19). These findings prompted us to speculate that the Mac1p may also regulate Ctr1p degradation. We analyzed Ctr1p turnover in cells either expressing or not expressing Mac1p-HAp. To remove the Mac1p control over CTR1 transcription, expression of Ctr1p, tagged with one copy of the Myc epitope, was driven by the GAL1 promoter and detected by Western blotting using anti-Myc antibody 9E10 as described previously (9, 15). We first observed that when cells were grown in the standard yeast growth medium, the Ctr1-Myc protein level in cells expressing the Mac1-HAp was drastically lower than that in cells not expressing Mac1-HAp (Fig. 1A). We then repeated the experiment by growing the cells in medium either containing or not containing the copper-specific chelator bathocuproinedisulfonic acid (BCS). As shown in Fig. 1B, the Ctr1-Myc level in BCS-treated cells was much higher than in untreated cells and comparable to that of cells lacking Mac1p. These data suggest that the Ctr1-Myc level is modulated by Mac1p and that Mac1p modulates the Ctr1-Myc level in a copper-dependent fashion. We next characterized Ctr1-Myc degradation by first growing cells in copper-depleted medium and then treating them with CuSO₄. The data in Fig. 1C show that copper at 100 μM failed to induce Ctr1-Myc to undergo turnover in cells not expressing Mac1-HAp. In contrast, in cells expressing Mac1-HAp, the Ctr1-Myc was degraded upon exposure to the same concentration of copper ions. The data not only confirmed the previous finding by the Klausner group but also demonstrated that the Mac1p is required for Ctr1p to undergo degradation in response to high copper levels.

The Mac1p ‘Cu-fist’ Motif Plays an Important Role in the Ctr1p Degradation—To begin to explore how Mac1p may function in the turnover of Ctr1p, we assessed whether the known copper ion-sensing motifs in Mac1p, the N-terminal Cu-fist and C-terminal REP-I and REP-II motifs, are involved. The Cu-fist motif is conserved among known copper-sensing transcription factors such as Ace1p, Amt1p, Mac1p, and Cup1p (3); copper binding by this motif triggers DNA binding of Ace1p and Amt1p in the transcriptional activation of metallothionein genes (27). Cells expressing a dominant mutant Mac1p, Mac1p(H279Q), carrying a single mutation of H279Q within the REP-I motif are found to be hypersensitive to copper (11). We have recently found that the Cu-fist and REP-I motifs in the Mac1p likely sense two different copper levels during the transcriptional regulation of CTR1 and CTR3. In turn, we analyzed the turnover of Ctr1-Myc in the YZ60 strain expressing either wild type Mac1p or mutant Mac1p carrying mutations within the Cu-fist, REP-I, and REP-II motifs, respectively (Fig. 2A). Cells were grown in copper-rich medium as in Fig. 1A, and Ctr1-Myc was detected by immunoblotting. In cells expressing mutant Mac1-HAp, including Mac1p(H279Q), REP-IA, REP-IIA, and REP-I + IIA, the Ctr1-Myc levels were the same as in the wild type cells and markedly lower than in cells expressing no Mac1p (Fig. 2A). In contrast, in cells expressing the mutant Mac1-HAp with mutations within the Cu-fist motif, mFist, the Ctr1-Myc level was much higher than that in the wild type cells, indicative of defects in Ctr1p degradation. These results suggest that the Cu-fist motif, but not the REP-I and REP-II motifs, is important for Mac1p to function in the copper-induced degradation of Ctr1p.

A Metal Ion Binding Motif, REP-III, Located in the Cytosolic
Control of Copper Ion Uptake

How does Mac1p function in the degradation of Ctr1p? Ctr1p, controlled in response to high copper levels. We found that the copper-induced degradation of Ctr1p occurs only when yeast express Mac1p, a copper-sensing transcription factor known to regulate the transcription of CTR1 (12, 16). In this study, we have also provided evidence demonstrating that Mac1p limits intracellular copper accumulation. Thus, it seems that Mac1p plays a critical role in balancing intracellular copper concentration by regulating copper transporter gene expression at both transcriptional and post-translational levels.

How does Mac1p function in the degradation of Ctr1p? Ctr1p degradation occurs at the plasma membrane (15), while Mac1p is localized in the nucleus (18). One possible mechanism of Mac1p controlling Ctr1p turnover is that the Mac1p activates a transcription factor known to regulate the transcription of CTR1 mac1Δ. These data show that Mac1p limits intracellular copper accumulation possibly by controlling Ctr1p degradation.

**DISCUSSION**

We have investigated how the turnover of a high affinity copper transporter, Ctr1p, is important in Ctr1p degradation.
the inability of this mutant Mac1p, mFist, to express the necessary protease(s). We have found that the mutant fails to activate the transcription of CTR1 and CTR3. Currently the protease for Ctr1p degradation is unknown. DNA microarray studies by the Winge group (28) have identified two new genes, YFR055w and YJL217w, that are activated by Mac1p in addition to the previous known target genes CTR1, CTR3, FRE1, and FRE7. FRE1 and FRE7 encode plasma membrane reduc-tases important in iron uptake and are believed to be required for copper uptake as well (14, 29). YFR055w shares homology with a family of trans-sulfuration enzymes involved in cysteine biosynthesis; the function of YJL217w is currently unknown (28). Whether or not YFR055w and YJL217w are involved in Ctr1p degradation has yet to be determined.

Another possible scenario is that Mac1p helps to recruit a pre-existing protease(s) or that Mac1p acts as a protease itself. Since the Ctr1p degradation occurs at the plasma membrane, either mechanism would require Mac1p to be present in the cytosol. Currently there is no indication that Mac1p is localized in the cytoplasm. However, a truncated Mac1p (residues 1–70) was found in the cytosol (18), and Mac1p itself underwent degrada-tion in response to high copper levels (13). Previous work from our laboratory has shown that Mac1p undergoes phosphoryla-tion and that the phosphorylation is required for Mac1p to bind to DNA (19). This phosphorylation event may affect cellular localization of Mac1p, and the unphosphorylated Mac1p may be present in the cytosol to function in Ctr1p degradation. A precedent of such a mechanism is that the nuclear translocation of transcription factor NF-κB is regulated by phosphorylation (24). Currently whether or not phosphorylation affects Mac1p localization is not known, and there is no indication that Mac1p has proteolytic activity.

The finding that Mac1p is required for Ctr1p degradation represents an important advance in understanding copper homeostasis, especially how copper uptake is controlled in eu-karyotic cells. Since high affinity copper transporters are highly conserved (5–9), this work may serve as a framework for understanding how copper uptake is controlled in higher organisms such as humans. The current study also raises the possibility that Ctr1p degradation involves metal ion-Ctr1p interaction given that a mutation of the putative metal ion binding motif REP-III stabilized Ctr1p. This interaction may transfer copper signals in the Mac1p-dependent degradation of Ctr1p. Although the exact mechanism of Mac1p functioning in Ctrlp turnover has yet to be elucidated, the current work, nonetheless, uncovers an unprecedented mechanism in which a transcription factor functions at transcriptional as well as post-translational levels in the control of its target gene expression.

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