The EMBO Journal vol.15 no.14 pp.3515–3523, 1996

Copper-dependent degradation of the *Saccharomyces cerevisiae* plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis

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The cell surface protein repertoire needs to be regulated in response to changes in the extracellular environment. In this study, we investigate protein turnover of the *Saccharomyces cerevisiae* plasma membrane copper transporter Ctr1p, in response to a change in extracellular copper levels. As Ctr1p mediates high affinity uptake of copper into the cell, modulation of its expression is expected to be involved in copper homeostasis. We demonstrate that Ctr1p is a stable protein when cells are grown in low concentrations of copper, but that exposure of cells to high concentrations of copper (10 μM) triggers degradation of cell surface Ctr1p. This degradation appears to be specific for Ctr1p and does not occur with another yeast plasma membrane protein tested. Internalization of some Ctr1p can be seen when cells are exposed to copper. However, yeast mutant strains defective in endocytosis (*end3*, *end4* and *chc1-ts*) and vacuolar degradation (*pep4*) exhibit copper-dependent Ctr1p degradation, indicating that internalization and delivery to the vacuole is not the principal mechanism responsible for degradation. In addition, a variant Ctr1p with a deletion in the cytosolic tail is not internalized upon exposure of cells to copper, but is nevertheless degraded. These observations indicate that proteolysis at the plasma membrane most likely explains copper-dependent turnover of Ctr1p and point to the existence of a novel pathway in yeast for plasma membrane protein turnover.

Keywords: copper homeostasis/copper transporter/endocytosis/plasma membrane protein turnover/protein degradation

Introduction

Plasma membrane proteins provide a means of communication between the interior and exterior of a cell. The expression of these proteins may thus be modulated by changes in the extracellular environment as part of an adaptive response. Protein turnover provides an essential means of regulating the surface expression of these proteins. In mammalian cells, degradation of plasma membrane proteins such as the growth factor and cytokine receptors takes place in lysosomes following ligand-triggered internalization from the surface. Internalization is mediated via clathrin-coated vesicles, and transport to lysosomes occurs through endosomal intermediates (Gruenberg and Howell, 1989; Pearse and Robinson, 1990; Smythe and Warren, 1991; Trowbridge, 1991). An analogous pathway modulates the expression of two plasma membrane proteins of *Saccharomyces cerevisiae*, the mating pheromone a- and α-factor receptors. Binding of pheromone triggers endocytosis of the ligand–receptor complex, which is then delivered via membrane-bound intermediates to the vacuole for degradation, thus preventing unwanted continuous signaling (Jenness and Spatrick, 1986; Singer and Riezman, 1990; Davis *et al.*, 1993; Riezman, 1993; Schandel and Jenness, 1994). This mechanism for protein turnover is also utilized by another functionally distinct class of yeast plasma membrane proteins, which are the permeases for small molecules such as inositol and uracil. Rapid degradation of the inositol permease is triggered by exposure of cells to high concentrations of inositol in the growth medium, as would be expected for homeostatic control of inositol uptake (Lai *et al.*, 1995), while degradation of the uracil permease is induced by stress (e.g. nutrient starvation) (Volland *et al.*, 1994).

The use of yeast to study plasma membrane protein regulation and turnover enables one to take advantage of the power of yeast genetics. However, a more comprehensive exploration has not been possible due to the limited availability of yeast plasma membrane markers. We have identified recently a novel plasma membrane protein from *S.cerevisiae*, the copper transporter Ctr1p (Dancis *et al.*, 1994a). Ctr1p is the product of the *CTR1* gene, and is required for high affinity copper uptake into the cell. The 406 amino acid predicted protein comprises three domains (Dancis *et al.*, 1994a). The N-terminal domain contains multiple repeats of a methionine/serine-rich motif found in bacterial proteins that interact with copper (Cha and Cooksey, 1991; Odermatt *et al.*, 1993). The middle portion has three predicted transmembrane segments, and the C-terminal domain is hydrophilic. Electron microscopic analysis of epitope-tagged Ctr1p shows that Ctr1p is located at the plasma membrane with the C-terminal domain residing in the cytosol (Dancis *et al.*, 1994a). Thus, the N-terminal domain, which is likely to interact with copper, is predicted to be extracellular. The protein, predicted to have an M_r of 46 kDa, migrates on denaturing gels as a 105 kDa protein due to extensive *O*-glycosylation. *In vivo*, Ctr1p can form oligomers (Dancis *et al.*, 1994b). The phenotypes associated with altered Ctr1p expression (using either *CTR1* deletion strains or strains overexpressing CTR from a 2μ plasmid) indicate that Ctr1p is responsible for delivery of copper to the cytosol and represents the rate-limiting component for copper uptake in *S.cerevisiae* (Dancis *et al.*, 1994b).

Table I. Yeast strains used in this study

Strain	Genotype	Source
66	MATα *trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52 FRE1-HIS::LEU2*	a
64	MATα *trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52 FRE1-HIS::URA3Δctr1::LEU2*	a
GPY1100α	MATα *leu2-3,112 ura3-52 his4-419 trp1 can1*	b
GPY418	MATα *leu2-3,112 ura3-52 his4-419 trp1 can1 chc1-521*	b
GPY449	MATα *pep4::LEU2 leu2-3,112 ura3-52 his4-419 trp1 can1*	b
RH144-3D	MATa *his4 leu2 ura3 bar1-1*	c
RH266-1D	MATa *his4 leu2 ura3 bar1-1 end3*	c
RH268-1C	MATa *his4 leu2 ura3 bar1-1 end4*	c

[a]Dancis *et al.* (1994b).
[b]Tan *et al.* (1993).
[c]Raths *et al.* (1993).

Copper is required as a co-factor in several enzymes but is toxic when present in excess due to its ability to form reactive free radicals and to cause damage to membranes (Halliwell and Gutteridge, 1988; Oshumi *et al.*, 1988; Linder, 1991). Cellular copper levels must be homeostatically controlled to avoid toxicity. Modulation of surface expression of Ctr1p therefore constitutes an important part of the cellular response to changing copper levels in the environment. *CTR1* transcript abundance is regulated by copper, being induced by copper deprivation and repressed by copper excess (Dancis *et al.*, 1994b). However, even after transcription of *CTR1* has been fully repressed, copper uptake mediated by Ctr1p may continue as long as the protein remains on the cell surface. Control of Ctr1p expression at the biosynthetic level, therefore, may not be sufficient to effect the rapid changes of Ctr1p levels needed to avoid copper-mediated cell damage. Here, we investigate effects of copper in the growth medium on the presence of Ctr1p on the cell surface. We find that in cells grown in low copper conditions, Ctr1p is a stable protein, and that exposure of cells to high copper triggers degradation of Ctr1p. Surprisingly, this copper-induced degradation is not due to internalization and vacuolar hydrolysis but appears to occur at the plasma membrane.

Results

Degradation of Ctr1p following exposure of cells to copper

To assess the effect of copper on turnover of Ctr1p, we first examined the stability of Ctr1p in cells grown in low copper medium. Cells were transformed with a 2μ plasmid encoding Ctr1p with a c-myc epitope tag inserted near the C-terminus and under the control of its native promoter (plasmid 352-CTR-myc). Whole cell extracts were then examined by immunoblotting, using an anti-myc antibody. The presence of the epitope tag did not interfere with Ctr1p function, as judged by genetic complementation (Dancis *et al.*, 1994a), and the tagged protein was localized to the plasma membrane, as assessed by indirect immunofluorescence (see below) and immunoelectron microscopy (Dancis *et al.*, 1994a).

When strain 66 cells (see Table I) were incubated in low copper medium (see Materials and methods) in the presence of cycloheximide to prevent new protein synthesis, Ctr1p was stable for at least 4 h (Figure 1A, left panel). A similar result was obtained when Ctr1p was expressed in a different strain, GPY1100α, under the control of a heterologous *GAL1* promoter. Ctr1p that was synthesized upon galactose induction remained stable for at least 3 h when cells were in low copper medium, after addition of glucose to repress further synthesis (Figure

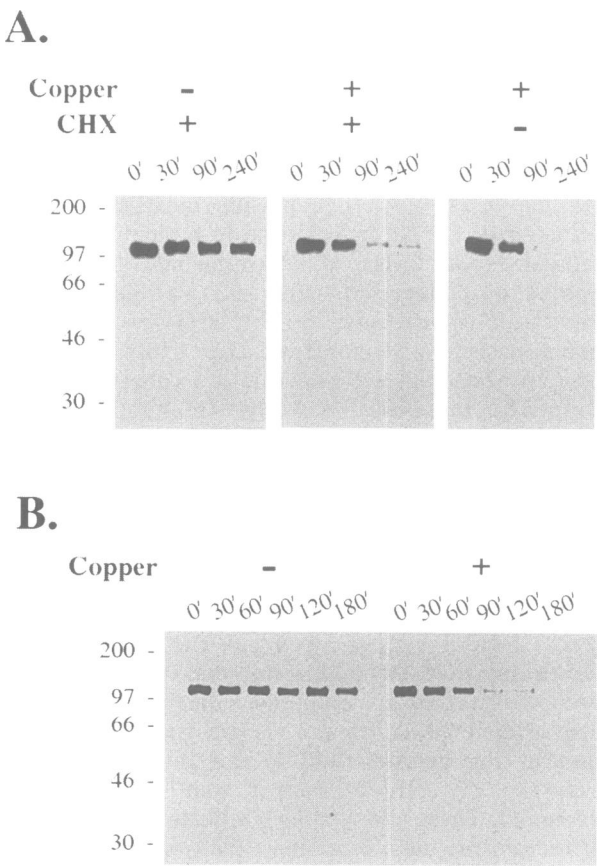

Fig. 1. Degradation of Ctr1p following exposure of cells to copper. (**A**) Strain 66 cells transformed with plasmid 352-CTR-myc were grown and harvested as described in Materials and methods. Cells were incubated at 30°C either in the absence (right panel) or presence (left and middle panels) of 100 μg/ml cycloheximide (CHX), and either in the absence (left panel) or presence (middle and right panels) of 10 μM copper sulfate. At 0, 30, 90 and 240 min after addition of copper, aliquots were removed for immunoblot analysis as described in Materials and methods. (**B**) Strain GPY1100α cells transformed with GAL-CTR-myc were grown and harvested as described in Materials and methods. Cells were incubated at 30°C in gluscose medium either in the absence (left panel) or presence (right panel) of 10 μM copper sulfate. At 0, 30, 60, 90, 120 and 180 min after addition of copper, aliquots were removed for immunoblot analysis.

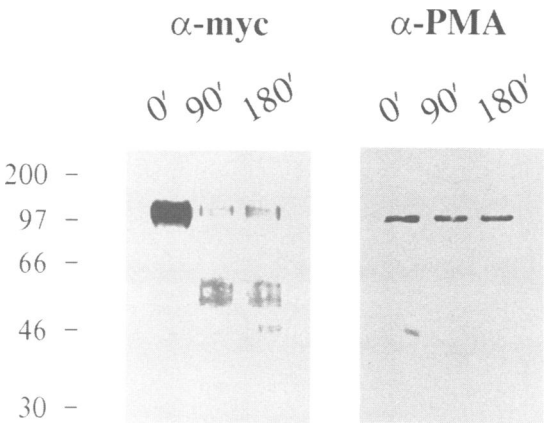

Fig. 2. Copper-induced degradation is specific for Ctr1p. Strain 66 cells transformed with plasmid 352-CTR-myc were grown and harvested as described in Materials and methods. Cells were incubated in the presence of 100 μg/ml cycloheximide and 20 μM copper sulfate. At 0, 90 and 180 min after addition of copper sulfate, aliquots were removed, cell lysates prepared and run on duplicate gels for SDS–PAGE. Immunoblotting was performed with either the 9E10 (left panel) or the anti-Pma1p antibody (right panel).

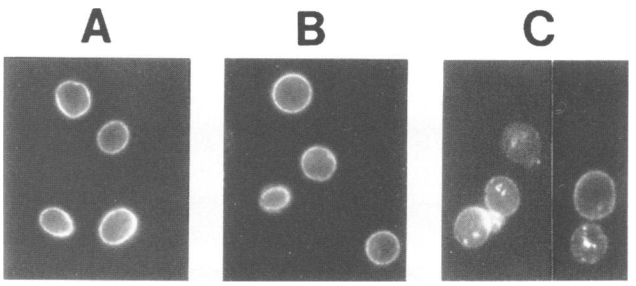

Fig. 3. Copper-dependent internalization of Ctr1p from the cell surface. Strain 66 cells transformed with plasmid 352-CTR-myc were grown and harvested as described in Materials and methods. Cells were pre-incubated at 15°C for 10 min in low copper media in the presence or absence of 10 μM BCS and then 10 μM copper sulfate was added to the sample without BCS. Cells were incubated at 15°C for another 60 min and then analyzed by immunofluorescence microscopy. (**A**) Cells before pre-incubation at 15°C; (**B**) cells incubated for 60 min at 15°C in the presence of BCS; (**C**) cells incubated for 60 min at 15°C in the presence of added copper sulfate.

1B, left panel). Thus, Ctr1p is a stable protein under conditions of low copper.

We next examined the effect of copper on the stability of Ctr1p. Strain 66 cells were starved for copper by growth in the presence of the copper chelator bathocuproinedisulfonate (BCS). The cells were harvested, washed and resuspended in the same low copper media without BCS, and copper sulfate (10 μM) was added to the medium. The amount of Ctr1p was analyzed in equal aliquots removed at different times after incubation. Significant loss of Ctr1p was observed after 90 min of exposure of cells to copper (Figure 1A). Comparable disappearance of Ctr1p was observed in the presence or absence of cycloheximide (Figure 1A), indicating that degradation of pre-existing protein was occurring, and that new synthesis of other proteins was not required for this event. Ctr1p degradation intermediates were frequently observed (see Figures 2, 5 and 6), although the amount of degradation intermediates varied between different experiments and could not be correlated with any particular yeast strains. The amount of added copper that is required for Ctr1p degradation is at least 10 μM, since little effect is apparent at lower concentrations (not shown).

To confirm that the loss of Ctr1p was due solely to degradation of pre-existing protein and not in part due to effects on the copper-responsive *CTR1* promoter, we examined expression of Ctr1p under the control of a *GAL1* promoter. As shown in Figure 1B, addition of 10 μM copper sulfate in the presence of glucose resulted in loss of Ctr1p at about the same rate as seen for Ctr1p expressed under the control of its native promoter.

The loss of Ctr1p in response to copper exposure could also be visualized by immunofluorescence microscopy using an anti-myc antibody. After growth in low copper medium, cells stained for Ctr1p showed bright rim structures, consistent with a plasma membrane localization (Figure 3A). Upon exposure of cells to 10 μM copper for 90 min, the staining was greatly diminished in intensity, reflecting loss of the protein (not shown). Quantitation by densitometric analysis of scanned cell images from a film negative indicated a loss of at least 75% of Ctr1p after 90 min exposure of cells to copper.

In order to ascertain if the effect of copper was specific for Ctr1p or a more general effect on plasma membrane proteins, we examined the yeast plasma membrane H^+-ATPase (Pma1p). Pma1p is an abundant plasma membrane protein comprising as much as 15% of the total plasma membrane protein (Serrano, 1991; Na *et al.*, 1995), and thus constitutes an appropriate marker. When strain 66 cells were exposed to 20 μM copper sulfate in the presence of cycloheximide, Pma1p remained stable for up to 3 h. In contrast, Ctr1p was substantially degraded in this time period (Figure 2). Thus, the degradation of Ctr1p that is triggered by copper is not a consequence of a general effect of copper on plasma membrane proteins.

Copper-dependent internalization of Ctr1p from the cell surface

Studies on plasma membrane proteins in yeast, for example the pheromone receptors and the uracil and inositol permeases, indicate that degradation of these proteins in response to various stimuli proceeds by endocytosis and delivery to the vacuole (Jenness and Spatrick, 1986; Davis *et al.*, 1993; Volland *et al.*, 1994; Lai *et al.*, 1995). Cytoplasmic membrane-bound endocytic intermediates can be trapped by incubation of cells at 15°C, a temperature at which internalization can still occur but progress to the vacuole is greatly impeded. These endosome-like intermediates have been detected biochemically in the case of internalized α-factor (Singer and Riezman, 1990; Singer-Kruger *et al.*, 1993) and by microscopy using a lipophilic dye as a marker (Vida and Emr, 1995). To see if Ctr1p is internalized from the cell surface upon exposure to copper, we looked by immunofluorescence microscopy to see if Ctr1p accumulated in cytoplasmic endosome-like intermediates upon incubation of cells at 15°C in the presence of copper.

When strain 66 cells were starved for copper and harvested, Ctr1p was found exclusively on the cell surface (Figure 3A). Incubation of these cells at 15°C in low copper medium (in the presence of 10 μM BCS) for up to 1 h did not affect this localization (Figure 3B). However, incubation at 15°C in the presence of 10 μM copper

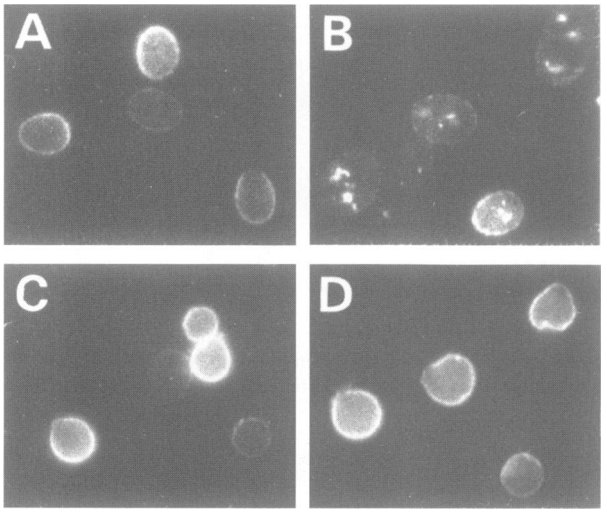

Fig. 4. Copper-induced internalization of Ctr1p is not observed in the endocytosis mutant *end3*. Strain RH266-1D (*end3*) and its parental wild-type strain RH144-3D were transformed with plasmid 352-CTR-myc. Cells were grown and harvested as described in Materials and methods. For each strain, the experiment was performed as described in the legend for Figure 3, except that cells were fixed 30 min after incubation at 15°C in either 10 μM BCS (**A** and **C**) or added 10 μM copper sulfate (**B** and **D**). (A) and (B) wild-type cells; (C) and (D) *end3* cells.

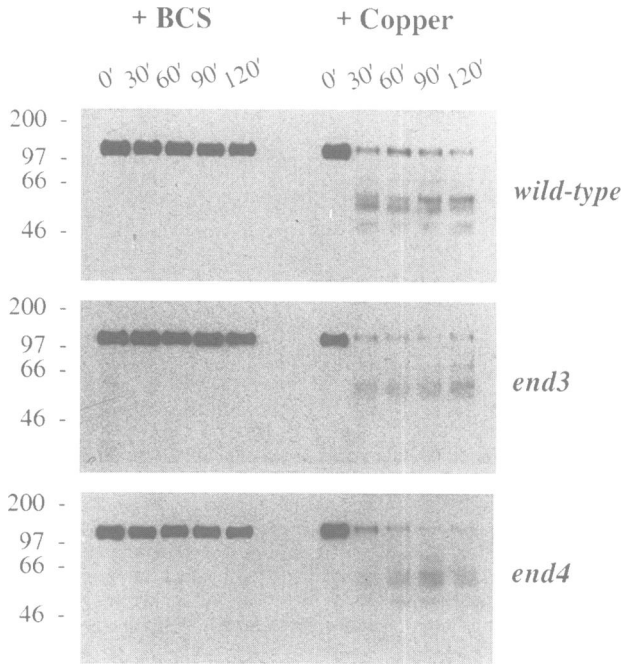

Fig. 5. Copper-induced degradation of Ctr1p in the *end3* and *end4* endocytosis mutant strains. Strains RH144-3D (wild-type parent), RH266-1D (*end3*) and RH268-1C (*end4*) were transformed with plasmid GAL-CTR-myc. Cells were grown and harvested as described in Materials and methods. Cells in media with or without 10 μM BCS were pre-incubated at 37°C for 10 min before addition of 10 μM copper sulfate to the sample without BCS. After addition of copper, cells were incubated at 37°C, and aliquots removed at 0, 30, 60, 90 and 120 min for immunoblot analysis. Left panels, incubation in BCS; right panels, incubation in copper. Top panels, wild-type strain; middle panels, *end3*; bottom panels, *end4*.

resulted in the appearance of Ctr1p in cytoplasmic punctate structures (Figure 3C). These structures were apparent by 30 min (Figure 4B).

We next asked if these punctate structures could be observed in a strain defective in endocytosis of α-factor, as a consequence of the *end3* mutation (Raths *et al.*, 1993; Benedetti *et al.*, 1994). The *end3* and its parental wild-type strains were transformed with the 352-CTR-myc plasmid. Transformants were starved for copper then incubated for 30 min at 15°C in the presence of 10 μM BCS or 10 μM copper sulfate. The wild-type cells showed similar results to those observed for strain 66 cells, i.e. incubation at 15°C in the presence of 10 μM copper resulted in the appearance of Ctr1p in cytoplasmic punctate structures (Figure 4A and B). In contrast, Ctr1p remained exclusively on the cell surface in the *end3* cells upon similar treatment (Figure 4C and D). The effect of the *end3* mutation confirms that the cytoplasmic punctate structures result from endocytosis of Ctr1p, and indicates that Ctr1p utilizes the same internalization pathway as for α-factor.

Copper-induced degradation of Ctr1p is unaffected in endocytosis mutant strains

The observation that copper triggers endocytosis of Ctr1p led us to ask if the copper-induced degradation of Ctr1p might proceed by internalization and delivery to the vacuole. We therefore tested to see if the endocytosis mutants *end3* and *end4* (Raths *et al.*, 1993) were defective in degradation of Ctr1p. We found that degradation of Ctr1p upon exposure to copper occurred in these mutant strains, in a manner similar to the wild-type parent (Figure 5). These experiments were performed at 37°C, a temperature that was non-permissive for growth and endocytic function in these mutant strains (Raths *et al.*, 1993). Similar results were observed whether Ctr1p was expressed from its native promoter (not shown) or from the *GAL1* promoter (Figure 5). These findings indicate that *end3/end4*-mediated endocytosis of Ctr1p is not required for its copper-induced degradation.

To confirm that copper-dependent degradation of Ctr1p was occurring in the absence of endocytosis in the mutant *end3* and *end4* strains, we performed similar experiments at 25°C. Under these conditions, copper-dependent degradation of Ctr1p occurred, but at a much slower rate, in both mutant and wild-type cells (not shown). The slower rate of degradation enabled us simultaneously to monitor internalization of Ctr1p by immunofluorescence. We observed, as for experiments carried out at 15°C (Figure 4), that internalization of Ctr1p occurred in wild-type cells but was blocked in the mutant *end3* and *end4* strains (not shown). Thus, copper-dependent degradation of Ctr1p can be observed under conditions where endocytosis of Ctr1p is blocked.

To obtain further evidence in support of these observations, we examined a mutant yeast strain with a temperature-sensitive allele of the clathrin heavy chain gene (*chc1-ts*), in which internalization of α-factor is retarded at 37°C (Tan *et al.*, 1993). As seen in Figure 6, the *chc1-ts* mutant showed copper-dependent degradation of Ctr1p at 37°C, and was indistinguishable from its wild-type parent. In addition, *act1-1* and *act1-2* mutants, which show an aberrant cytoskeleton and which do not internalize α-factor at the non-permissive temperature (37°C) (Kubler and Riezman, 1993), exhibited copper-dependent degradation of Ctr1p at 37°C (not shown).

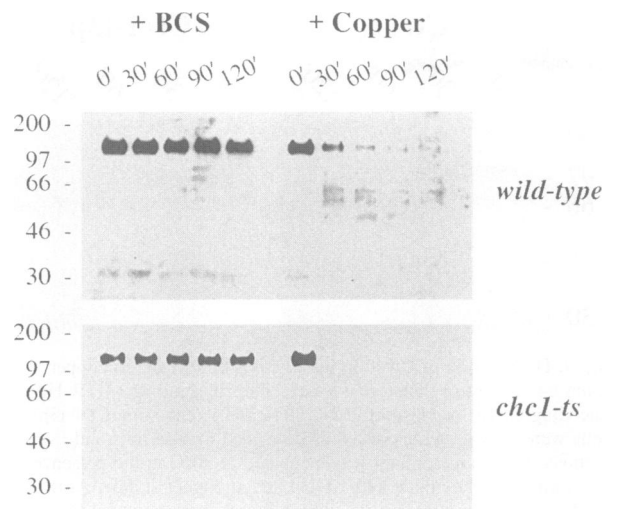

Fig. 6. Copper-induced degradation of Ctr1p in the clathrin mutant strain *chc1-ts*. Strain GPY418 (*chc1-ts*) and its parental wild-type strain GPY1100α were transformed with plasmid GAL-CTR-myc. Cells were grown and harvested as described in Materials and methods. Cells in media with or without 10 μM BCS were pre-incubated at 37°C for 10 min before addition of 10 μM copper sulfate to the sample without BCS. After addition of copper, cells were incubated at 37°C, and aliquots removed at 0, 30, 60, 90 and 120 min for immunoblot analysis. Left panels, incubation in BCS; right panels, incubation in copper. Top panels, wild-type cells; bottom panels, *chc1-ts* cells.

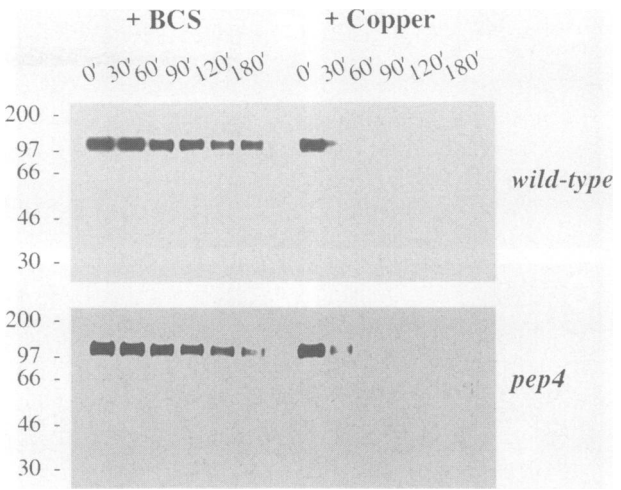

Fig. 7. Copper-dependent degradation of Ctr1p in *pep4* cells defective in vacuolar hydrolysis. Strain GPY449 (*pep4*) and its parental wild-type strain GPY1100α were transformed with plasmid 352-CTR-myc. Cells were grown and harvested as described in Materials and methods. Cells were incubated at 30°C in the presence of 100 μg/ml cycloheximide and either 10 μM BCS or 10 μM added copper sulfate. At 0, 30, 60, 90, 120 and 180 min after incubation, aliquots were removed for immunoblot analysis. Left panels, incubation in BCS; right panels, incubation in copper. Top panels, wild-type cells; bottom panels, *pep4* cells.

Copper-dependent degradation of Ctr1p occurs in cells defective in vacuolar hydrolysis

The lack of effect of the *end* mutants on copper-induced degradation of Ctr1p suggests that degradation does not occur in the lysosome-like vacuole, a process which requires internalization from the cell surface. To confirm this, we tested for copper-induced degradation of Ctr1p in *pep4* cells, which are defective in vacuolar degradation (Ammerer *et al.*, 1986; Woolford *et al.*, 1986). Degradation of internalized pheromone, pheromone receptors and the uracil and inositol permeases is inhibited in this mutant (Davis *et al.*, 1993; Schandel and Jenness, 1994; Volland *et al.*, 1994; Lai *et al.*, 1995). However, *pep4* cells exhibited copper-induced degradation of Ctr1p in a manner that was indistinguishable from its wild-type parent (Figure 7). Thus, vacuolar proteases do not appear to mediate degradation of Ctr1p.

A Ctr1p variant shows copper-dependent degradation despite an inability to be internalized

The above observations with yeast mutant strains suggest that endocytosis of Ctr1p is not required for its copper-dependent degradation. To confirm these findings further, we used another approach involving mutation of the Ctr1p molecule. It is well documented in mammalian systems that the amino acid motifs required for internalization from the plasma membrane are contained within the cytosolic tail portion of membrane proteins (Davis *et al.*, 1986; Trowbridge *et al.*, 1993; Voorhees *et al.*, 1995). In yeast, the only available information on internalization signals is on the α-factor receptor, where it has been shown that a five amino acid sequence within the cytosolic tail mediates ligand-dependent internalization of the receptor (Rohrer *et al.*, 1993). We thus reasoned that copper-dependent internalization of Ctr1p might also require amino acid sequences present in its cytosolic tail. A *CTR1* variant, *CTR1-t3*, encoding Ctr1p with a deletion of 78 residues from the C-terminus and an insertion of a hemagglutinin (HA) epitope tag at the new C-terminus, was created. The resulting truncated molecule, Ctr1-t3p, contained only 47 of the 125 amino acids in the cytosolic tail of Ctr1p. *CTR1-t3* functionally complemented strain 64 cells carrying a disruption in the *CTR1* gene (not shown). In these cells, Ctr1-t3p was localized to the plasma membrane, as assessed by immunofluorescence microscopy (Figure 8A).

We first assessed whether Ctr1-t3p was internalized upon addition of copper to the medium, using the 15°C treatment described above. Full-length Ctr1p bearing an HA epitope tag in strain 64 cells accumulated in cytoplasmic punctate structures upon incubation of cells in the presence of 10 μM copper at 15°C for 30 min (Figure 8A and B). In contrast, Ctr1-t3p bearing the deletion in the cytosolic tail did not accumulate in such structures under similar conditions and remained on the cell surface (Figure 8C and D). This result indicates that the C-terminal 78 residues of Ctr1p are required for the protein to be internalized in response to copper.

We next looked at the stability of Ctr1-t3p when copper is added to the extracellular medium. Strain 64 cells expressing either full-length HA-tagged Ctr1p or HA-tagged Ctr1-t3p were starved for copper and harvested as described for Figure 1A. Addition of 10 μM copper to the medium resulted in degradation of both full-length Ctr1p and the truncated Ctr1-t3p, at similar rates (Figure 9). Thus, the truncated variant of Ctr1p is able to be degraded in response to copper even though it cannot be internalized.

Discussion

In this study, we have investigated the regulation of surface expression of the plasma membrane copper trans-

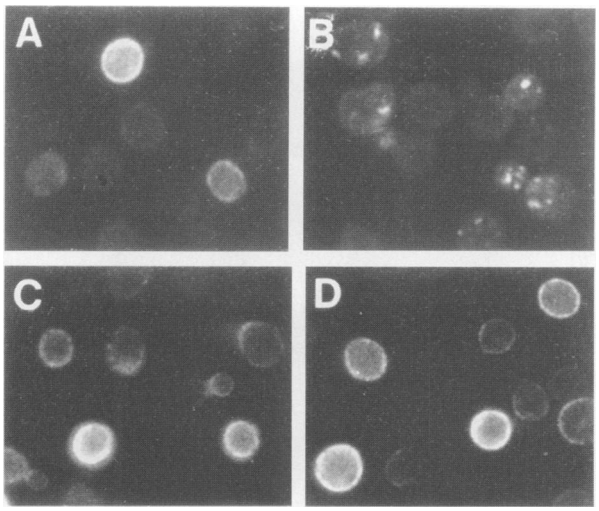

Fig. 8. Ctr1-t3p is not internalized in response to added copper. Strain 64 cells were transformed with either plasmid 352-CTR-HA (encoding full-length Ctr1p) or 352-CTR1-t3-HA (encoding Ctr1-t3p, which is Ctr1p with a deletion of 78 amino acids from the C-terminus). Cells were grown and harvested as described in Materials and methods. For each transformant, the experiment was performed as described in the legends for Figures 3 and 4. After incubation in either 10 μM BCS or 10 μM copper sulfate at 15°C for 30 min, cells were prepared for immunofluorescence microscopy. The proteins visualized and the incubation conditions are as follows: (**A**) Ctr1p, BCS; (**B**) Ctr1p, added copper; (**C**) Ctr1-t3p, BCS; (**D**) Ctr1-t3p, added copper.

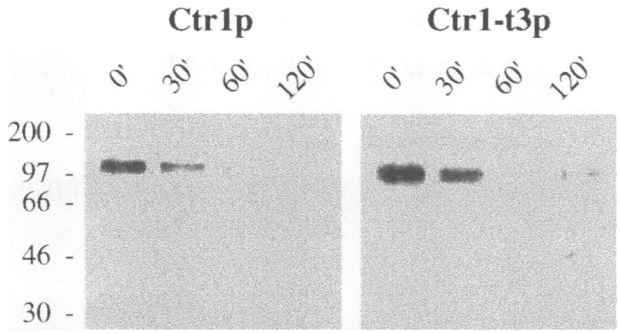

Fig. 9. Degradation of Ctr1-t3p upon exposure of cells to copper. Strain 64 cells were transformed with either plasmid 352-CTR-HA (encoding Ctr1p) or plasmid 352-CTR1-t3-HA (encoding Ctr1-t3p). Cells were grown and harvested as described in Materials and methods. Each transformant was incubated at 30°C in the presence of 100 μg/ml cycloheximide and 10 μM added copper sulfate. At 0, 30, 60, 90 and 120 min after incubation, aliquots were removed for immunoblot analysis. Left panel, Ctr1p; right panel, Ctr1-t3p.

porter, Ctr1p. We first determined whether homeostatic control of intracellular copper concentrations might involve an effect of copper on the turnover of the protein. We find that Ctr1p is a stable protein under conditions of low copper in the growth medium, but that exposure of cells to high copper triggers degradation of the protein. This copper-induced degradation appears to be specific for Ctr1p and not a general effect of copper on plasma membrane proteins.

Degradation of the copper transporter in response to increased extracellular copper is consistent with homeostatic control of the copper uptake system. Stable surface expression of the Ctr1p transporter in copper-starved cells allows for efficient uptake of limiting amounts of copper in the environment. On the other hand, rapid degradation of Ctr1p upon addition of high copper to the growth medium ensures cessation of excessive metal uptake that might lead to cell damage. Previous work from our laboratory has shown that copper regulates synthesis of Ctr1p via effects on transcription from the *CTR1* promoter; 10 nM added copper suffices to cause repression of transcription from the *CTR1* promoter (Yuan *et al.*, 1995). The concentration of copper at which degradation of Ctr1p is evident (10 μM) corresponds to a toxic level of copper at which the protective protein Cup1p is maximally expressed (not shown). It is possible that control of biosynthesis regulates the steady-state amount of Ctr1p, whereas degradation of Ctr1p may be used to reduce acutely the number of surface Ctr1p molecules under conditions of micromolar concentrations of copper when continued copper uptake could be deleterious.

What proteolytic pathway is involved in the copper-induced degradation of Ctr1p? In the case of the pheromone receptors and the inositol and uracil permeases, vacuolar degradation is responsible for protein turnover (Davis *et al.*, 1993; Schandel and Jenness, 1994; Volland *et al.*, 1994; Lai *et al.*, 1995). This mechanism requires internalization of the protein from the cell surface and transport to the vacuole. However, our findings indicate that for Ctr1p the principal mechanism responsible for copper-induced degradation of Ctr1p does not involve internalization for vacuolar hydrolysis. By immunofluorescence microscopy, we have observed that copper triggers the accumulation of Ctr1p in endocytic intermediates when cells are incubated at 15°C. This copper-dependent internalization of Ctr1p is not observed in the endocytosis mutant *end3*, which is defective in endocytosis of a number of other plasma membrane proteins. However, *end3* and other endocytosis mutants are able to exhibit copper-induced degradation of Ctr1p that is indistinguishable from that of wild-type strains. Furthermore, we have analyzed a Ctr1p variant lacking a portion of its cytosolic tail and which cannot be internalized in response to added copper as analyzed by immunofluorescence microscopy. This variant, Ctr1-t3p, is nevertheless degraded upon exposure of cells to copper. Finally, copper-induced Ctr1p degradation is unaffected in the *pep4* mutant which is defective for activation of most known vacuolar proteases (Jones, 1991). Together, these results provide compelling evidence that degradation of Ctr1p is occurring at the plasma membrane.

If the copper-induced degradation of Ctr1p is occurring at the plasma membrane, why do we observe internalization of Ctr1p into endosome-like structures upon addition of copper? One possibility is that internalization of Ctr1p is the mode by which copper is delivered into the cell, analogous to the uptake of iron-bound transferrin via internalization of the transferrin receptor (Klausner *et al.*, 1984). Another possibility is that vacuolar degradation represents an alternate minor route for copper-dependent turnover of Ctr1p. This alternate pathway could be responsible for constitutive turnover under conditions when acute response is not needed, e.g. at moderate copper levels. Indeed, we have observed that internalization of Ctr1p can be triggered by much lower concentrations of copper (0.1–1 μM) than that required for degradation (not shown). Whatever the role of Ctr1p internalization, interpretation of this phenomenon is limited by the fact that we cannot judge what proportion of surface Ctr1p is internalized in

response to copper under our experimental conditions. The internalized protein may represent only a small subpopulation of the total pool of Ctr1p, and can be recycled back to the plasma membrane where it is degraded.

The cell surface degradation of Ctr1p is a novel mechanism for plasma membrane protein turnover in yeast. Proteolysis at the plasma membrane has been described in bacterial and eukaryotic systems, and serves diverse purposes. Down-regulation of a number of leukocyte surface membrane proteins like L-selectin and CD43 is mediated by proteolytic cleavage at the cell surface (Bazil and Strominger, 1993; Kahn *et al.*, 1994). The extracellular domains of mammalian growth factor and cytokine receptors such as colony-stimulating factor 1, Met and Axl can be cleaved from the intact protein at the cell surface where they may perform inhibitory roles with respect to signaling, or enhance down-modulation of the receptor (Downing *et al.*, 1989; Prat *et al.*, 1991; O'Bryan *et al.*, 1995). Growth factors and cytokines themselves (like tumor necrosis factor and transforming growth factor-α) have transmembrane forms that are cleaved to release soluble forms (Kriegler *et al.*, 1988; Pandiella *et al.*, 1992). It is possible that cleavage of Ctr1p at the surface serves multiple purposes. In addition to providing a means of removing active copper transport activity under conditions of high copper, the cleavage may release the extracellular domain of Ctr1p that has the capacity to bind and sequester copper, thus further inhibiting copper uptake. Interestingly, the methionine/serine-rich repeats in the extracellular domain are structurally homologous to the copA protein of *Pseudomonas syringae* which apparently mediates sequestration of copper in the periplasm of this organism (Cha and Cooksey, 1991). Sequestration of ligand by an extracellular ligand binding protein domain has been described as a mechanism for inhibition of signaling via epidermal growth factor receptor (Basu *et al.*, 1989; Flickinger *et al.*, 1992). Detection of the N-terminal extracellular domain of Ctr1p by placing an epitope tag in this region would be evidence for such a model.

The protease(s) responsible for copper-dependent degadation of Ctr1p have yet to be identified. They could be plasma membrane resident proteins like the matrix metalloproteinases (Kaushal *et al.*, 1994; Cao *et al.*, 1995; Wolfsberg *et al.*, 1995), or could be recruited from the periplasmic space in a manner analogous to recruitment of urokinase to the cell surface for proteolysis (Ellis *et al.*, 1989; Barnathan *et al.*, 1990). To date, no plasma membrane or periplasmic protease has been identified in yeast.

How does copper cause degradation of Ctr1p? We have observed that copper-dependent degradation of Ctr1p does not occur in isolated membrane fractions (our unpublished results). This finding indicates that copper is not causing direct cleavage of the protein, and shows a requirement for integrity of cellular organization for the process. One possible scenario is that copper acts by causing some physical change in the protein that targets it for proteolysis. Indeed, precedent exists for an effect of copper binding on the proteolytic susceptibility of a protein in the case of the copper binding protein plastocyanin from *Chlamydomonas reinhardtii* (Li and Merchant, 1995). High extracellular copper may also promote degradation indirectly by causing an elevated cytosolic concentration of copper which would itself be the signal that sets in motion a cascade of events leading to degradation of Ctr1p. We have found that exposure of cells to copper changes the solubility of Ctr1p in Triton X-100 (our unpublished results). This is consistent with the idea of copper causing a change in the physical state of the Ctr1p protein, either by changing the conformation of the protein itself or by triggering an association with Triton-insoluble structures (e.g. the cytoskeleton or cell wall).

Our finding that a metal ligand, copper, can trigger proteolysis of its transporter illustrates a novel mode for homeostatic control of metal uptake. Initial characterization of the mechanism involved in this process has uncovered a pathway of protein turnover occurring at the yeast cell surface. Identification of the proteolytic machinery involved will help determine whether this is a general mechanism that extends to other plasma membrane proteins and whether it also exists in mammalian cells.

Materials and methods

Strains, media and growth conditions

Yeast strains used in this study are listed in Table I. DNA transformations were performed using the lithium acetate procedure (Ito *et al.*, 1983). Cells were propagated on agar plates with the appropriate SC dropout medium and grown overnight at 30°C (24–26°C for temperature-sensitive strains and their parental wild-types) in liquid medium to $OD_{600\ nm}$ ~1.0 ($0.5<OD<1.5$) for experiments. Cells transformed with plasmid bearing *CTR1* under the control of its native promoter were grown in low copper medium SUCFM [(0.67% yeast nitrogen base minus copper and iron (Bio101, Inc., La Jolla, CA), 2% dextrose, 0.08% CSM-ura, 50 mM MES buffer pH 6.1] supplemented with 10 μM of the copper chelator BCS. For experiments these cells were harvested the following way: (i) cells intended for incubation without addition of copper were washed once in SUCFM + 10 μM BCS and then resuspended in SUCFM + 10 μM BCS; (ii) cells intended for incubation with added copper sulfate were washed once in SUCFM without BCS, resuspended in SUCFM without BCS; copper sulfate was added from a freshly prepared 1 mM stock solution. Where indicated, cycloheximide was added from a 10 mg/ml stock solution. Cells transformed with plasmid bearing *CTR1* under the control of the *GAL1* promoter were grown overnight in SUCFM–raffinose medium containing 2% raffinose in place of dextrose. *CTR1* expression was induced by growth for 4 h (to $OD_{600\ nm}$ ~1.0) in SUCFM–raffinose supplemented with 0.5% galactose. For experiments, cells were centrifuged and resuspended in SUCFM (containing 2% glucose) with or without 10 μM BCS for subsequent incubation without or with copper sulfate, respectively. In all cases, cells were resuspended to 1.0 $OD_{600\ nm}$ at the start of the experiment, and incubations were at 30°C unless otherwise stated.

Plasmids

Plasmid 352-CTR-myc carries the *CTR1-myc* gene under the control of its native promoter in the 2μ vector YEp352, as previously described (Dancis *et al.*, 1994a). Plasmid 352-CTR-HA is similar to 352-CTR-myc except for the presence of the HA epitope (Wilson *et al.*, 1984) in place of the myc epitope. Plasmid GAL-CTR-myc contains the coding region of *CTR1-myc* inserted downstream of the *CYC-GAL* promoter in the 2μ vector pEMBLyex4 (kind gift of A.Hinnebusch). The open reading frame of *CTR1-myc* flanked by *Bam*HI (5′) and *Pst*I (3′) sites was generated by polymerase chain reaction (PCR), and the product subcloned into pEMBLyex4 using these restriction sites. Plasmid 352-CTR1-t3-HA contains *CTR1-t3* with an HA epitope at the C-terminus, in YEp352. *CTR1-t3-HA*, encoding *CTR1* with 78 residues deleted from the C-terminus was generated by PCR mutagenesis. The *Sph*I–*Eco*RI fragment in *CTR1* (in pRS414, Dancis *et al.*, 1994a) was replaced with the corresponding PCR product containing the deletion. The *Eag*I–*Xho*I fragment of this pRS414-CTR1-t3-HA construct was then subcloned in place of the corresponding fragment in 352-CTR-myc.

Immunoblot analysis

Total cell lysates were prepared by lysis of cells with base treatment followed by precipitation of proteins with trichloroacetic acid (TCA).

Cells (1 ml) from experimental samples were centrifuged at 14 000 *g* for 2 min. The cell pellet was resuspended in 150 µl of 1.85 M NaOH/1% β-mercaptoethanol and incubated on ice for 10 min. An equal volume of 50% TCA was added, followed by incubation on ice for at least 30 min. TCA precipitates were collected by centrifugation at 14 000 *g* for 5 min and resuspended in 50–100 µl of 2× SDS buffer (Laemmli, 1970) supplemented with 0.1 M Tris base to neutralize the TCA. The sample was heated at 95°C for 5 min and subjected to SDS–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and the blots incubated with either 9E10 (anti-myc, 30× dilution of culture supernate), 12CA5 (anti-HA, 1 µg/ml) or rabbit anti-Pma1p (500× dilution) in blocking buffer [5% non-fat dry milk, phosphate-buffered saline (PBS), 0.1% Triton X-100]. Detection by enhanced chemiluminescence (ECL) was performed after incubation with horseradish peroxidase-conjugated second antibody according to the instructions of the manufacturer (Amersham Corp., Arlington Heights, IL).

Immunofluorescence microscopy

Preparation of cells for immunofluorescence microscopy was performed essentially as described by Roberts *et al.* (1991). A 2.5 ml aliquot of cells (~2.5 $OD_{600\ nm}$) from the experiment was fixed by direct addition of 0.3 ml of formaldehyde (final concentraion 4% formaldehyde) and incubation with gentle agitation at 30°C for 1–2 h. Cells were centrifuged at 800 *g* for 5 min, resuspended in 1 ml of buffered formaldehyde (4% formaldehyde, 50 mM potassium phosphate pH 6.5, 0.5 mM $MgCl_2$) and incubated overnight at room temperature or 30°C with gentle shaking. Fixed cells were centrifuged and resuspended in 0.5 ml of TEB (200 mM Tris–HCl pH 8, 20 mM EDTA, 1% β-mercaptoethanol), incubated for 10 min at 30°C, then centrifuged. Cells were resuspended in 0.5 ml of SPM (1.2 M sorbitol, 50 mM potassium phosphate pH 7.3, 1 mM $MgCl_2$) and cell wall digestion was carried out with 800 U of oxalyticase (Enzogenetics, Corvallis, OR) for 1 h at 30°C. Spheroplasts were centrifuged (4000 *g*, 30 s), washed once with 1.2 M sorbitol, and treated with 1–2% SDS/1.2 M sorbitol for 1–2 min. They were then washed twice with 1.2 M sorbitol, resuspended in 1.2 M sorbitol, 50 mM potassium phosphate pH 6.5, 0.5 mM $MgCl_2$, and then applied to polylysine-coated coverslips. Cells were permeabilized during antibody incubations [antibodies diluted in 0.1% bovine serum albumin (BSA) in PBS, 0.05% saponin]. First antibody was either 9E10 (anti-myc) (5× dilution of 5-fold concentrated culture supernate) or 12CA5 (anti-HA) (5 µg/ml), and second antibody was rhodamine-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Lab., Inc., West Grove, PA). Antibody incubations were for 1 h at room temperature, with three washes of 0.1% BSA/PBS after each incubation. After a final rinse with PBS, cells were mounted with Fluoromount (Fisher Scientific, Pittsburgh, PA) and visualized by microscopy.

Acknowledgements

We thank David Haile for carrying out preliminary studies that gave rise to this project. We also thank Julie Donaldson, Mike Jeffers, Kazuhiro Iwai, Frans Hochstensbach and Dario Finazzi for helpful and critical discussions. We are grateful to D.Botstein, A.Hinnebusch, S.Jentsch, G.Payne and H.Riezman for generously providing yeast strains and plasmid, and to A.Chang for the kind gift of the yeast Pma1p antibody. C.E.O. was supported by a National Research Service Award post-doctoral fellowship.

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Received on February 9, 1996; revised on March 27, 1996