Post-transcriptional Regulation of the Yeast High Affinity Iron Transport System*

Received for publication, December 29, 2004, and in revised form, March 21, 2005
Published, JBC Papers in Press, April 7, 2005, DOI 10.1074/jbc.M414663200

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Saccharomyces cerevisiae transcriptionally regulates the expression of the plasma membrane high affinity iron transport system in response to iron need. This transport system is comprised of the products of the FET3 and FTR1 genes. We show that Fet3p and Ftr1p are post-translationally regulated by iron. Incubation of cells in high iron leads to the internalization and degradation of both Fet3p and Ftr1p. Yeast strains defective in endocytosis (aend4) show a reduced iron-induced loss of Fet3p-Ftr1p. In cells with a deletion in the vacuolar protease PEP4, high iron medium leads to the accumulation of Fet3p and Ftr1p in the vacuole. Iron-induced degradation of Fet3p-Ftr1p is significantly reduced in strains containing a deletion of a gene, VTA1, which is involved in multivesicular body (MVB) sorting in yeast. Sorting through the MVB can involve ubiquitination. We demonstrate that Ftr1p is ubiquitinated, whereas Fet3p is not ubiquitinated. Iron-induced internalization and degradation of Fet3p-Ftr1p occurs in a mutant strain of the E3 ubiquitin ligase RSP5 (rsp5-1), suggesting that Rsp5p is not required. Internalization of Fet3p-Ftr1p is specific for iron and requires both an active Fet3p and Ftr1p, indicating that it is the transport of iron through the iron permease Ftr1p that is responsible for the internalization and degradation of the Fet3p-Ftr1p complex.

Transition metals are essential for life, yet transition metals in high concentrations can be toxic. Both eukaryotes and prokaryotes tightly regulate the concentration of free intracellular metals by either regulating metal uptake or sequestration. High affinity iron transport in the budding yeast Saccharomyces cerevisiae requires the expression of two cell surface proteins, the multicopper oxidase Fet3p and the transmembrane permease Ftr1p (1, 2). Transcription of these genes, as well as genes that encode proteins required for the processing of Fet3p, is regulated by the iron sensing transcription factor Aft1p (3). In Saccharomyces cerevisiae transporters for the transition metals copper and zinc are regulated post-translationally. High levels of zinc induce the internalization and vacuolar degradation of Zrt1p, the high affinity zinc transporter (4). High levels of copper induce the degradation of Ctr1p, the high affinity copper transporter, whereas Ctr3p, another high affinity copper transporter, is not affected (5). A previous study from our laboratory suggested that regulation of the high affinity iron transport system was predominantly transcriptional (6), although there is evidence that the activity of the iron transport system may be regulated by cAMP (7). Studies in Schizosaccharomyces pombe, however, suggested that the multicopper oxidase-based high affinity iron transport system might be regulated post-translationally. Incubation of S. pombe, expressing the high affinity transport system, with high concentrations of iron led to a rapid inhibition of iron transport (8). High levels of iron transport activity are seen when FET3/FTR1 are expressed using the iron-independent GAL10 promoter. There is a 50% reduction in transport activity when such cells are incubated in high iron as opposed to low iron medium (8). Based on these observations, we re-examined whether the Fet3p-Ftr1p transport system is post-translationally regulated. We demonstrate that high levels of iron induce the internalization and degradation of the Fet3p-Ftr1p transport system.

MATERIALS AND METHODS

Strains and Media—The S. cerevisiae strains used in this study are listed in Table I. The cells were grown in either medium containing yeast extract-peptone-dextrose (YPD) or yeast nitrogen base synthetic complete medium (CM) with supplements as needed (9). Low iron growth medium was made by adding 40 or 80 μM bathophenanthroline disulfonate (BPS), an iron chelator, to CM or YPD and then adding back varying amounts of FeSO4. Low iron medium used in this work is referred to as BPS (x), in which the media contains BPS and x equals the concentration in micromolar of added FeSO4.

61 Nuclease Protection Analysis—Total RNA was isolated and analyzed using standard techniques (10). All samples were isolated from mid-log phase cultures grown in either CM or CM BPS (5). The 32P-labeled FET3 and CMD1 probes were generated.

Preparation of Antisera against Fet3p—A secreted Fet3p (Fet3p lacking the transmembrane and cytoplasmic domains) was generated as described by Hasset et al. (11). Procedures for the isolation and deglycosylation of secreted-Fet3p have been described previously (12). The N-glycanase-treated Fet3p was injected into rabbits, and antisera were prepared. The soluble Fet3p was attached to an Amino-link gel using the manufacturer’s instructions (Pierce Inc.). The antiserum was applied to the column, and the column was extensively washed with phosphate-buffered saline and eluted with 0.1 M glycine (pH 2.5) and immediately neutralized with 1.0 M Tris-HCl (pH 9.0). The purified antibody was useful for both immunofluorescence and Western analysis.

Immunofluorescence—Cells were prepared for immunofluorescence as described previously (13). For visualization of Fet3p, the rabbit anti-Fet3p antibody was used (1:500) followed by either an Alexa 594- or Alexa 488-conjugated goat anti-rabbit antibody (1:500). All of the

* This work was supported by National Institutes of Health Grant DK30534. Support for DNA oligonucleotides and sequencing was provided by Cancer Center Support Grant CA49014. 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.

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1 The abbreviations used are: YPD, yeast extract-peptone-dextrose; BPS, bathophenanthroline disulfonate; CM, complete media; GFP, green fluorescent protein; CFP, cyan fluorescent protein.
fluorescent secondary antibodies were obtained from Molecular Probes. Western Analysis—Western blot analysis was performed on Fet3p-containing membrane fractions as described previously (14) using our purified rabbit anti-Fet3p (1:1000). The only variation in protocol was that membranes (15 μg) were treated with endoglycosidase Hf per the manufacturer's protocol (New England Biolabs) before being analyzed by SDS-PAGE using 10% gels followed by Western analysis. For Western analysis of Gap1p-GFP or Ftr1p-CFP, membranes were isolated using a procedure described previously (15) and analyzed on 10% SDS-PAGE, and proteins were transferred to nitrocellulose membranes. The membranes were blocked with milk and incubated with either a rabbit anti-Fet3p, mouse anti-GFP (1:10,000, Covance), or mouse anti-ubiquitin (1:1,000, Covance) as the primary antibody and peroxidase-conjugated goat anti-mouse or rabbit IgG as the secondary antibody (1:10,000, Jackson ImmunoResearch Laboratories, Inc.).

Atomic Absorption Assay—Cells were grown to log phase in low iron medium and then transferred to medium containing a range of FeSO₄ for 2 h. Log phase cells were collected and washed by centrifugation except that 100 μg/ml cycloheximide was added at the same time as cells were incubated in iron-containing medium. D, cells (Δfet3) were transformed with a plasmid containing a GAL10-regulated FET3. The cells were incubated in low iron galactose-containing medium overnight and then incubated for 2 h in glucose-containing medium supplemented with different concentrations of iron. The cells were harvested, and extracts were analyzed by Western analysis using antibodies against Fet3p and Gas1p.

**TABLE I**

| Strain     | Genotype   | Ref. |
|------------|------------|-----|
| DY150      | MATα ade2, can1, his3, leu2, trp1, ura3 | (9) |
| DY1457     | MATα ade6, can1, his3, leu2, trp1, ura3 | (9) |
| DY150 (FET3-GFP) | MATα ade2, can1, his3, leu2, trp1, ura3, FET3-GFP::KanMX | This study |
| DY1457 (FTR1-CFP) | MATα ade6, can1, his3, leu2, trp1, ura3, FTR1-CFP::KanMX | This study |
| Δfern       | MATα ade2, can1, his3, leu2, lys2, trp1, Δfern::TRP1 | (37) |
| Δfet1 42C (FET3-GFP) | MATα ade2, can1, his3, leu2, trp1, ura3, Δfet1::FET3-GFP::KanMX | This study |
| Δfet4       | MATα ade2, can1, his3, leu2, trp1, ura3, Δfet4::FET3::KanMX | This study |
| Δfet1       | MATα ade2, can1, his3, leu2, trp1, ura3, Δfet1::FET3::KanMX | This study |
| BY4742      | MATa his3 leu2 lys2 ura3 | (21) |
| Δvtl1–5a    | MATa his3 leu2 met 15 ura3, Δvtl1::KanMX | (21) |
| OCY 354     | MATa ade2, can1, his3, leu2, trp1, ura3, HO::FET3 LacZ | This study |
| 23344       | MATa, ura3 | (15) |
| 27038 (rsp5) | MATa, ura3, rop5 | (15) |
| 23344 (FET3-GFP) | MATa, ura3, FET3-GFP::KanMX | This study |
| 27038 (rop5) | MATa, rop5, FET3-GFP::KanMX | This study |
| 23344 (FTR1-CFP) | MATa, ura3, FTR1-CFP::KanMX | This study |
| 27038 (rop5) | MATa, rop5, FTR1-CFP::KanMX | This study |
| LHY291      | His3, trp1, ade2, ura3, leu2, bar1 | (24) |
| LHY23       | rop5–1, ura3, leu2, trp1, bar1 GAL | (24) |

**FIG. 1. Effect of iron on FET3 protein and mRNA levels.** Cells grown overnight in iron-limited medium BPS (5) were incubated with different concentrations of FeSO₄ for 2 h. A, samples were taken for Western analysis, and blots were probed with antibodies to Fet3p and to Gas1p. B, samples were harvested for S1 nuclease protection analysis using probes for FET3 and CMD1. C, cells were treated as in A except that 100 μg/ml cycloheximide was added at the same time as cells were incubated in iron-containing medium. D, cells (Δfet3) were transformed with a plasmid containing a GAL10-regulated FET3. The cells were incubated in low iron galactose-containing medium overnight and then incubated for 2 h in glucose-containing medium supplemented with different concentrations of iron. The cells were harvested, and extracts were analyzed by Western analysis using antibodies against Fet3p and Gas1p. 

**TABLE I**

| Strain     | Genotype   | Ref. |
|------------|------------|-----|
| DY150      | MATα ade2, can1, his3, leu2, trp1, ura3 | (9) |
| DY1457     | MATα ade6, can1, his3, leu2, trp1, ura3 | (9) |
| DY150 (FET3-GFP) | MATα ade2, can1, his3, leu2, trp1, ura3, FET3-GFP::KanMX | This study |
| DY1457 (FTR1-CFP) | MATα ade6, can1, his3, leu2, trp1, ura3, FTR1-CFP::KanMX | This study |
| Δfern       | MATα ade2, can1, his3, leu2, lys2, trp1, Δfern::TRP1 | (37) |
| Δfet1 42C (FET3-GFP) | MATα ade2, can1, his3, leu2, trp1, ura3, Δfet1::FET3-GFP::KanMX | This study |
| Δfet4       | MATα ade2, can1, his3, leu2, trp1, ura3, Δfet4::FET3::KanMX | This study |
| Δfet1       | MATα ade2, can1, his3, leu2, trp1, ura3, Δfet1::FET3::KanMX | This study |
| BY4742      | MATa his3 leu2 lys2 ura3 | (21) |
| Δvtl1–5a    | MATa his3 leu2 met 15 ura3, Δvtl1::KanMX | (21) |
| OCY 354     | MATa ade2, can1, his3, leu2, trp1, ura3, HO::FET3 LacZ | This study |
| 23344       | MATa, ura3 | (15) |
| 27038 (rsp5) | MATa, ura3, rop5 | (15) |
| 23344 (FET3-GFP) | MATa, ura3, FET3-GFP::KanMX | This study |
| 27038 (rop5) | MATa, rop5, FET3-GFP::KanMX | This study |
| 23344 (FTR1-CFP) | MATa, ura3, FTR1-CFP::KanMX | This study |
| 27038 (rop5) | MATa, rop5, FTR1-CFP::KanMX | This study |
| LHY291      | His3, trp1, ade2, ura3, leu2, bar1 | (24) |
| LHY23       | rop5–1, ura3, leu2, trp1, bar1 GAL | (24) |
RESULTS

To determine if the Fet3p-Ftr1p transport system is post-transcriptionally regulated by iron, we exposed wild type cells expressing the transport system to high iron medium and then examined Fet3p levels by Western analysis. When cells were exposed to high iron medium there was a concentration-dependent decrease in Fet3p. Relative to Gas1p, employed as a loading control, exposure of cells to 1 mM FeSO₄ resulted in the disappearance of 50% of Fet3p within 1 h (data not shown) and 80% within 2 h (Fig. 1A). It may be possible that the disappearance of surface Fet3p is the result of the steady-state turnover of Fet3p, as FET3 transcription is iron-sensitive (9).
We observed that FET3 mRNA levels were dramatically decreased when cells were incubated with as little as 10 μM iron (Fig. 1B). There was little further change in transcript level with increased medium iron. Examination of Fet3p levels revealed little decrement in Fet3p when cells were incubated with 10 μM iron for 2 h. Decreased protein levels were only seen at higher concentrations of iron (Fig. 1A).

These results suggest that Fet3p levels may be regulated independently of FET3 mRNA. We confirmed this result using two different approaches. First, we measured Fet3 protein in cells treated with the protein synthesis inhibitor cycloheximide. Cells were grown in low iron medium, and cycloheximide was added at the same time as high iron. In the presence of cycloheximide, there was an iron-dependent decrease in Fet3p when cells were incubated with 10 μM iron for 2 h. Decreased protein levels were only seen at higher concentrations of iron (Fig. 1A).

The post-translational regulation of the high affinity iron transport system was confirmed using immunofluorescence. Cells incubated in iron-depleted medium showed fluorescent staining of the cell surface, whereas Δfet3 cells stained with the same anti-Fet3p antibody showed no fluorescence (Fig. 2A). Addition of iron for 2 h resulted in the disappearance of Fet3p fluorescence. Expression of FET3 regulated by the GAL10 promoter leads to abundant Fet3p on the cell surface, and there was little change in the surface expression of Fet3p when galactose-grown cells were incubated in glucose-containing medium. Upon addition of iron, there was a dramatic decrease in fluorescence (data not shown). We generated strains containing an integrated FET3-GFP. As shown previously, addition of an epitope to either Fet3p or Ftr1p does not alter their ability to transport iron (17). Addition of iron also resulted in the loss of surface fluorescence in cells that had a chromosomal copy of FET3 with a carboxyl-terminal GFP (Fig. 2B). The loss of Fet3p was confirmed by Western analysis.

Both components of the high affinity iron transport system, Fet3p and Ftr1p, have to be synthesized simultaneously for appropriate cell surface targeting (2). In the absence of Fet3p, Ftr1p does not localize to the surface and is degraded, as is
Fet3p in the absence of Ftr1p. These results suggest that Fet3p and Ftr1p form a complex. Based on the observation that iron induced the loss of Fet3p, we asked whether iron also induced the loss of Ftr1p. We generated strains containing an integrated FTR1-CFP, because published studies show the utility of this fusion protein (17). Expression of Ftr1p-CFP permitted those cells to grow on low iron media, indicating that the protein was functional. When Ftr1p-CFP-expressing cells were incubated with high iron there was a loss of cell surface Ftr1p-CFP fluorescence (Fig. 2C). The predicted molecular mass of Ftr1p is 45.7 kDa, and addition of CFP would add 27 kDa. Our data show Ftr1p-CFP migrating on SDS-PAGE with a predicted molecular mass of 70 kDa, which is close to the predicted size of the fusion protein.

We took advantage of mutant cell lines to show that the loss of surface Fet3p was due to internalization and vacuolar degradation. A deletion of END4 attenuates but does not completely inhibit endocytosis, as shown by decreased uptake of the fluorescent dye FM4–64 (18). In Δend4 cells, the iron-induced loss of surface Fet3p was reduced (Fig. 3A). In cells that lack the vacuolar protease Pep4p, the iron-induced loss of cell surface fluorescence correlated with the appearance of fluorescence in the vacuole (Fig. 3B). The targeting of many cell surface proteins to the vacuoles requires their sorting in the

**Fig. 4. Internalization of Fet3p is specific for iron.** Cells (Δpep4) were grown overnight in iron-limited medium and then incubated in medium containing 50 μM of the specified metals for 2 h. The cells were then processed for immunofluorescence using an antibody to Fet3p.

**Fig. 5. Ftr1p is ubiquitinated.** A, wild type cells containing an integrated copy of FET3-GFP or FTR1-CFP were grown in low iron medium overnight and then incubated in either low iron or high iron containing medium for 2 h. Cells were harvested; membranes were detergent-extracted and immunoprecipitated with antibodies against GFP/CFP. The immunoprecipitates were analyzed by Western blot using antibodies to ubiquitin or GFP/CFP. B, cells with a chromosomal copy of FET3-GFP or FTR1-CFP were transformed with either a control plasmid or a plasmid containing a CUP1 regulated ubiquitin c-myc construct. The cells were incubated in low iron medium overnight and then transferred to copper containing low or high iron containing medium for 2 h. Cells were harvested; membranes were detergent-extracted and immunoprecipitated with antibodies against GFP/CFP. The immunoprecipitates were analyzed by Western blots using antibodies to c-myc. C, cells treated as in B were incubated in low iron medium overnight then incubated in either low iron or high iron medium in the presence or absence of copper for 45 min and then examined for fluorescence. Note that, in the absence of ubiquitin expression, fluorescence was seen in vacuoles only in cells incubated with iron. In contrast, when ubiquitin was expressed both Fet3p-GFP and Ftr1p-CFP were found in the vacuole even in low iron medium.
FIG. 6. The iron-induced degradation of Fet3p is independent of Rsp5p. A, internalization and degradation of the ammonia transporter Gap1p is reduced in r-sp5 cells. Wild type and r-sp5 cells, transformed with a GAP1-GFP-containing plasmid, were grown in ammonia-containing medium overnight. The cells were examined for Gap1-GFP by both fluorescence and Western analysis in which an anti-GFP antibody was used to probe Western blots. B, wild type and r-sp5 cells were transformed with a plasmid containing ZRT1 with a hemagglutinin epitope. The cells were grown overnight in low Zn\(^{2+}\)/H\(_{11001}\) medium and then incubated with Zn\(^{2+}\)/H\(_{11001}\) for 1 h. The cells were then examined by Western analysis, and the blots were probed with an antibody directed against hemagglutinin. C, wild type and r-sp5 cells, containing an integrated copy of FET3-GFP or FTR1-CFP, were incubated overnight in low iron medium. The cultures were divided into aliquots, and iron was added to one of the aliquots for 2 h. Samples were taken for fluorescence or for Western analysis in which the blots were probed with either Gas1p or an antibody to GFP. D, wild type cells and r-sp5-1 cells were transformed with an STE2-GFP plasmid. Cells were incubated at the restrictive temperature for 15 min, and then α-factor was added.
multivesicular body (19). Vta1p is a class E protein involved in multivesicular body sorting (20, 21). In iron-exposed Δvta1 cells, Fet3p was not degraded and was found at the plasma membrane as well as in a class E prevacuolar compartment (Fig. 3C).

The Fet3p-Ftr1p transport system is highly specific for iron and will not transport other transition metals. Cells with a deletion of PEP4 (Δpep4) were incubated overnight in low iron medium and then exposed to different transition metals. At a concentration of 50 μM, only iron led to the vacuolar accumulation of Fet3p. Incubation of cells in metals such as Cu²⁺, Mn²⁺, and Zn²⁺ in concentrations as high as 50 μM did not lead to internalization of Fet3p (Fig. 4). These results show that internalization of surface Fet3p is specific for iron.

Entry of most plasma membrane proteins into the vacuole through the multivesicular body is a consequence of their being ubiquitinated. We therefore examined whether either Fet3p or Ftr1p was ubiquitinated. Cells expressing either Fet3p-GFP or Ftr1p-CFP were incubated in the presence of iron for 2 h and harvested, and detergent extracts were immunoprecipitated with anti-GFP antibodies. Western blots of the immunoprecipitates were probed with an anti-ubiquitin antibody. No ubiquitin was seen in the immunoprecipitate from Fet3p-GFP-expressing cells, but ubiquitin was found in extracts from Ftr1p-CFP-expressing cells (Fig. 5A). Ubiquitin could be seen on Ftr1p-CFP from cells grown in low iron medium; however, the addition of iron resulted in a significant increase in ubiquitin levels. Immunoprecipitated Ftr1p (detected by Western analysis) had an apparent molecular mass of 45 kDa, much lower than that of Ftr1p-CFP seen in extracts of cells probed by Western analysis. We think that it is likely that the lower molecular mass results from proteolytic cleavage occurring during immunoprecipitation, because we did not observe this change in molecular mass prior to immunoprecipitation (compare Figs. 2B and 5A).

We confirmed the presence of ubiquitin on Ftr1p by taking advantage of cells transformed with a plasmid containing a copper regulated (CUP1) ubiquitin with a carboxyl-terminal c-myc epitope. Cells were grown in high copper-containing medium to induce the expression of ubiquitin-c-myc, and detergent extracts were immunoprecipitated using antibodies to GFP. Again, no ubiquitin was seen in immunoprecipitates from Fet3p-GFP cells, but ubiquitin was seen in immunoprecipitates from Ftr1p-CFP cells (Fig. 5B). We observed that Ftr1p was ubiquitinated in both low and high iron medium with multiple ubiquitin-containing bands. Other plasma membrane proteins have been found to be hyper-ubiquitinated in cells overexpressing ubiquitin (15). If ubiquitination of Ftr1p was responsible for the internalization of the Fet3p-Ftr1p complex, then we might expect increased internalization of hyper-ubiquitinated Ftr1p-CFP in low iron medium. In Fet3p-GFP- or Ftr1p-CFP-expressing cells incubated in low iron medium, fluorescence was found predominately on the cell surface. Under the same conditions, in cells expressing ubiquitin, fluorescence was now found in the vacuole. Addition of iron to cells overexpressing ubiquitin resulted in an increase in the rate of vacuolar accumulation of Fet3p-Ftr1p. These results suggest that ubiquitination of Ftr1p leads to internalization of the Fet3p-Ftr1p complex.

Most plasma membrane transporters are ubiquitinated by the ubiquitin ligase Rsp5p (for reviews see Refs. 19 and 22). To determine if Rsp5p is required for the iron-induced internalization of Fet3p-Ftr1p transport system, we utilized a yeast strain with a mutation in RSP5. We first confirmed that rsp5Δ cells showed a defect in ubiquitination by following the degradation of the high affinity amino acid transporter Gap1p. Wild type and rsp5Δ cells were transformed with a GAP1-GFP plasmid. In cells grown in ammonia-free (nitrogen-poor) media Gap1p is localized to the plasma membrane (15, 23). Addition of ammonia results in the internalization of Gap1p-GFP and its localization in the vacuole, as seen by fluorescence or by the presence of cleaved GFP on Western blots. In the rsp5Δ cells, Gap1p-GFP is found at the plasma membrane and in a prevacuolar compartment (Fig. 6A). Western analysis of rsp5Δ extracts showed full-length Gap1p-GFP, whereas in wild type cells only GFP is seen, indicating that Gap1p-GFP is degraded. Gitan and Eide (4) reported a decrease in the Zn²⁺-mediated internalization and degradation of the high affinity zinc transporter Zrt1p in rsp5Δ cells. We confirmed that result, as shown in Fig. 6B. To determine if Rsp5 was necessary for the iron-induced degradation of Fet3p-Ftr1p, we generated an rsp5Δ strain with an integrated FET3-GFP or FTR1-CFP. We were surprised to find no reduction in iron-induced internalization or degradation of Fet3p or Ftr1p in rsp5Δ cells (Fig. 6C). It is possible that the rsp5Δ mutant retains sufficient activity to ubiquitinate Ftr1p. We therefore took advantage of a temperature-sensitive allele of RSP5 (rsp5-1), which shows a severe defect in ubiquitination (24). We confirmed that rsp5-1Δ has a temperature-sensitive defect in ubiquitination by showing a severe alteration in α-factor-mediated internalization of Ste2p-GFP (Fig. 6D). Fet3p-GFP showed no of iron-dependent loss in rsp5-1Δ cells at the restrictive temperature (Fig. 6E). These results suggest that Rsp5p is not required for the iron-induced loss of the high affinity iron transport system.

We considered three mechanisms to explain how iron signals the internalization and degradation of Fet3p-Ftr1p: a signal generated by iron at the cell surface, a signal generated by iron inside the cell, or a signal generated as a consequence of movement of iron through the transport system. To test these possibilities, we transformed a Δfet3Δpep4 strain with a GAL10-regulated allele of FET3 that was unable to transport iron due to a mutation in one of the amino acids that ligate the Type 1 copper (25). Cells were grown in galactose to induce the expression of the mutant Fet3p. Although the inactive Fet3p is still translocated to the cell surface, incubation of cells with high levels of iron did not result in the degradation of the mutant Fet3p, as assessed by either Western blot analysis (data not shown) or by immunofluorescence (Fig. 7A). We then examined the effect of mutations in Ftr1p by expressing a form of Ftr1p that had defective iron transport. A mutation in the putative iron binding REXLE domain of Ftr1p does not prevent Ftr1p and Fet3p from being localized to the cell surface but reduces iron transport by ~80% (17). When exposed to high iron, the degradation of the transport system was reduced compared with wild type cells (Fig. 7B). The concentration of media iron in these experiments was high, because it was sufficient to support the growth of Δfet3 cells but was also sufficient to inhibit transcription of a FET3lacZ reporter construct in Δfet3 cells (data not shown).

To further show that a Fet3p-Ftr1p transport system is required for iron-induced internalization, we took advantage of cells with a deletion in the GEF1 gene. Gef1p is a voltage-regulated chloride channel present in the post-Golgi compart-
ment in which apo-Fet3p is copper-loaded (14, 26). In the absence of Gef1p, apo-Fet3p is not copper-loaded but is still targeted to the cell surface. Cell surface apo-Fet3p, lacks multicopper oxidase activity and is unable to transport iron. Incubation of Δgef1 cells with iron did not lead to the degradation of apo-Fet3p (Fig. 8). Apo-Fet3p on the cell surface can be copper-loaded by incubation of cells at 0 °C in the presence of Cl⁻, Cu²⁺, and reduced pH (14). Copper loading of apo-Fet3p resulted in increased iron transport activity and increased multicopper oxidase activity. Once copper-loaded, addition of iron leads to the internalization and degradation of Fet3p. These results demonstrate that an active iron transport system is required for iron to induce the internalization of Fet3p-Ftr1p.

Extracellular iron is not the signal for the internalization and degradation of the high affinity iron transport system. S. cerevisiae can acquire iron through the low affinity iron transport system, Fet4p, as well as through siderophore-iron transporters. There are two separate routes by which S. cerevisiae can acquire iron provided by iron-siderophore complexes (27, 28). The first route involves reduction of siderophore-iron complexes at the cell surface followed by the uptake of iron by the Fet3p-Ftr1p transport system. The impermeable Fe(II) chelator BPS can inhibit uptake of iron by this route. The second route of uptake of siderophore iron involves transport of the siderophore iron complex through a siderophore transporter. This route of iron acquisition cannot be inhibited by BPS. In the presence of high concentrations of BPS, yeast can grow on siderophore-iron complexes (28, 29). Addition of high concentrations of ferrioxamine-iron to cells grown in BPS did not lead to the internalization of Fet3p-Ftr1p (Fig. 9A). The same concentration of ferrioxamine-iron, however, can provide enough iron to support the growth of cells and to prevent the expression of a FET3lacZ reporter construct (Fig. 9B). It may be possible that the amount of siderophore-iron accumulated...
within cells may be enough to suppress the transcription of the iron-regulon but is insufficient to induce the internalization of Fet3p-Ftr1p. To examine the effect of intracellular iron on the degradation of Fet3p-Ftr1p, we took advantage of the observation that, in the absence of high affinity, iron transport system cells increase the expression of the low affinity transition metal transporter Fet4p (30). As shown above, \( \Delta gef1 \) cells do not internalize Fet3p-Ftr1p. At low concentrations of media iron wild type cells accumulated more iron than \( \Delta gef1 \) cells (Fig. 9C). This is expected, because \( \Delta gef1 \) cells do not have a functional high affinity iron transport system. As media iron increases, \( \Delta gef1 \) cells showed a greater accumulation of iron than wild type cells. Increased iron accumulation reflects the increased expression of the Fet4p low affinity iron transporter on \( \Delta gef1 \) cells and low levels of Fet4p on wild type cells. Even in the face of greater than wild type levels of cellular iron, no degradation of Fet3p-Ftr1p was observed (see Fig. 8). These results suggest that intracellular iron does not provide the signal for the internalization of Fet3p-Ftr1p.

**DISCUSSION**

Transcriptional regulation of the high affinity iron transport system, comprising the products of the FET3 and FTR1 genes, by the transcription factor Aft1p has been well described (3, 31–33). We now demonstrate that Fet3p and Ftr1p are regulated post-translationally, as iron induces the internalization and degradation of both Fet3p and Ftr1p. The simultaneous synthesis of Fet3p and Ftr1p is required for their appropriate targeting to the cell surface suggesting that these molecules are in a complex (2, 8). The observation that iron induces the simultaneous internalization of both Fet3p and Ftr1p provides further support for the view that these two proteins exist in an obligate complex. Iron-induced internalization of the iron transport system is consistent with studies showing post-translational regulation of copper (5), zinc (4, 34), and manganese (35) transport systems. For both iron and zinc transport systems, transcriptional regulation is more sensitive than post-translational regulation. Iron, at concentrations as low as 10 \( \mu M \), can reduce transcription of FET3 by \( >90\% \), whereas the same concentration only had minimal effects on protein levels.

Ubiquitination is the signal that targets most membrane proteins for degradation (19, 22). Our data indicate that Ftr1p can be ubiquitinlated and that ubiquitination is required for degradation, because deletions of genes required for sorting into the multivesicular body pathway prevent the vacuolar localization of Fet3p-Ftr1p. Most plasma membrane proteins are ubiquitinlated by Rsp5p (22). Rsp5p, in combination with Bsd2p, is responsible for ubiquitination of the Mn\(^{2+}\) transporter Smf1p in the biosynthetic pathway (36). The rate of degradation of the amino acid transporter Gap1p and the zinc transporter Zrt1p was severely reduced in \( \text{rpsp5} \) cells. It was surprising to find that the iron-induced degradation of Fet3p-Ftr1p was not affected in \( \text{rpsp5} \) cells. Furthermore, no iron-induced change in surface Fet3p was seen at the restrictive temperature in cells that had temperature-sensitive allele of RSP5, although effects were seen on the internalization of Ste2p. These results suggest that a ubiquitin ligase other than
Rsp5p is required for the ubiquitination of Ftr1p.

Given that metals can induce the internalization of surface transporters via ubiquitination leading to transporter degradation, what is the signal that leads to ubiquitination? The metal-induced event that leads to Rsp5p-mediated ubiquitin addition in Smf1p may be a conformational change in the transporter resulting from transport of the metal (36). Mutations that abolish transport activity for Smf1p abolish metal-induced internalization. These studies suggest that Rsp5p (in combination with Bsd2p) recognizes alterations in the hydrophobic domain of membrane proteins. Transport of substrate might be expected to lead to perturbation in the lipid bilayer resulting from movements in the transporter as substrate is passed through the bilayer. This is an attractive model for post-translational regulation of the iron transport system, because it would correlate transport activity with both cellular metal requirement and transcriptional regulation of the high affinity iron transporter. In conditions of iron sufficiency, iron transport will lead to degradation of the transporter. In the face of iron insufficiency, even though transport of iron increases the rate of degradation of the transporter, the increased degradation rate will be offset by increased transcription of the transporter. Linking transport activity to degradation rate provides a simple feedback mechanism that ensures tight control of cytosolic metal levels, as well as assuring the specificity of membrane targeting to the specific transporter.

The demonstration that the Fet3p-Ftr1p transport system must be active to effect iron-induced internalization indicates that cell surface iron is not the signal for internalization/ubiquitination. There are two issues that must be resolved before accepting a model for iron-induced internalization in which the movement of iron through the channel is responsible for ubiquitination. First, why are high concentrations of iron required for post-translational regulation? The $K_m$ for the Fet3p-Ftr1p transport system is in the sub-micromolar (0.15–0.2 μM) range (9), yet much higher concentrations of iron (>50 μM) are required for substantial rates of transporter degradation. Second, what is the ubiquitin ligase responsible for “marking” Ftr1p? We have ruled out Rsp5p, although it is formally possible that, even in the rsp5 mutant strain or the temperature-sensitive rsp5-I strain, residual enzyme activity is sufficient to ubiquitinate Ftr1p. The best way to show that Rsp5p is not required is to identify the ubiquitin ligase that is required. Those studies are in progress.

Acknowledgments—We thank Drs. David Eide (University of Wisconsin), Chris Kaiser (Massachusetts Institute of Technology), Linda Hicke (Northwestern), and Howard Riezman (University of Basel) for their generous gifts of plasmids, cells, and antibodies.

REFERENCES

1. Askwith, C. C., de Silva, D., and Kaplan, J. (1996) Mol. Microbiol. 20, 27–34
2. Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D., and Dancis, A. (1996) Science 271, 1552–1557
3. Yamaguchi-Iwai, Y., Dancis, A., and Klausner, R. D. (1995) EMBO J. 14, 1231–1239
4. Gitan, R. S., Luo, H., Rodgers, J., Broderius, M., and Ede, D. (1998) J. Biol. Chem. 273, 28617–28624
5. Osi, C. E., Rabinovich, E., Dancis, A., Bonifacino, J. S., and Klausner, R. D. (1996) EMBO J. 15, 3515–3523
6. Ede, D., Davis-Kaplan, S., Jordan, I., Sipe, D., and Kaplan, J. (1992) J. Biol. Chem. 267, 20774–20781
7. Lesuisse, E., Horion, B., Labbe, P., and Hilger, F. (1991) Biochem. J. 280, 543–549
8. Askwith, C., and Kaplan, J. (1997) J. Biol. Chem. 272, 401–405
9. Askwith, C., Ede, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., and Kaplan, J. (1994) Cell 76, 403–410
10. Chen, O. S., Hemenway, S., and Kaplan, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16922–16927
11. Hassett, R. F., Yuan, D. S., and Kosman, D. J. (1998) J. Biol. Chem. 273, 23274–23282
12. Harris, Z. L., Davis-Kaplan, S. R., Gittin, J. D., and Kaplan, J. (2004) Blood 103, 4672–4673
13. Davis-Kaplan, S. R., Ward, D. M., Shiflett, S. L., and Kaplan, J. (2004) J. Biol. Chem. 279, 4322–4329
14. Davis-Kaplan, S. R., Askwith, C. C., Bengtzen, A. C., Radisky, D., and Kaplan, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13641–13645
15. Springael, J. Y., and Andre, B. (1998) Mol. Biol. Cell. 9, 1253–1263
16. Mason, P. B., and Struhl, K. (2003) Mol. Cell. Biol. 23, 8325–8333
17. Severance, S., Chakrabrotty, S., and Kosman, D. J. (2004) Biochem. J. 380, 487–496
18. Rathe, S., Rohrer, J., Craussaz, F., and Riezman, H. (1993) J. Cell Biol. 120, 55–65
19. Katzmann, D. J., Odorizzi, G., and Emr, S. D. (2002) Nat. Rev. Mol. Cell. Biol. 3, 891–905
20. Yes, S. C., Xu, L., Ren, J., Beulon, V. J., Wagle, M. D., Liu, C., Ren, G., Wang, P., Zahn, R., Sasajala, P., Yang, H., Piper, R. C., and Munn, A. L. (2003) J. Cell Sci. 116, 3957–3970
21. Shiflett, S. L., Ward, D. M., Hoynh, D., Vaughan, M. B., Simmons, J. C., and Kaplan, J. (2004) J. Biol. Chem. 279, 10982–10990
22. Hicke, L., and Dunn, R. (2003) Annu. Rev. Cell Dev. Biol. 19, 141–172
23. Soetens, O., De Craene, J., and Andre, B. (2001) J. Biol. Chem. 276, 43949–43957
24. Dunn, R., and Hicke, L. (2001) Mol. Biol. Cell 12, 421–435
25. Askwith, C. C., and Kaplan, J. (1999) J. Biol. Chem. 274, 22415–22419
26. Gaxiola, R. A., Yuan, D. S., Klausner, R. D., and Fink, G. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6494–6500
27. Lesuisse, E., Simon-Casteras, M., and Labbe, P. (1998) Microbiology 144, 3455–3462
28. Yun, C. W., Pereira, T., Rashford, J., Ardon, O., Brown, P. O., Botstein, D., Kaplan, J., and Philpott, C. C. (2000) J. Biol. Chem. 275, 10797–10799
29. Kosman, D. J. (2003) Mol. Microbiol. 47, 1185–1197
30. Li, L., and Kaplan, J. (1998) J. Biol. Chem. 273, 22181–22187
31. Casas, C., Aldea, M., Espinet, C., Gallego, C., Gil, R., and Herrero, E. (1997) Yeast 13, 621–637
32. Rutherford, J. C., Jaron, S., and Winge, D. R. (2003) J. Biol. Chem. 278, 27636–27643
33. Yamaguchi-Iwai, Y., Stearman, R., Dancis, A., and Klausner, R. D. (1996) EMBO J. 15, 3377–3384
34. Gitan, R. S., and Ede, D. J. (2000) Biochem. J. 346, 329–336
35. Liu, X. F., and Culotta, V. C. (1999) J. Biol. Chem. 274, 4863–4868
36. Hettema, E. H., Valdez-Taubas, J., and Pelham, H. R. (2004) EMBO J. 23, 1279–1288
37. Li, L., Chen, O. S., McVey Ward, D., and Kaplan, J. (2001) J. Biol. Chem. 276, 25615–25621
38. Spizzo, T., Byersdorfer, C., Duesterhoeft, S., and Ede, D. (1997) Mol. Gen. Genet. 256, 547–556
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J. Biol. Chem. 2005, 280:22181-22190. doi: 10.1074/jbc.M414663200 originally published online April 7, 2005

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