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A novel role for thioredoxin reductase in the iron metabolism of *S. cerevisiae*

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ABSTRACT

Intracellular levels of iron are tightly regulated. *Saccharomyces cerevisiae* uses well-defined pathways to extract iron molecules from the environment. Once inside the cell, the iron molecules must be transferred to target sites via an intracellular iron transporter. Although analogous carriers have been described for other metals, such as copper, an iron transporter has yet to be identified. We used two-dimensional gel electrophoresis and mass spectrometry techniques to attempt to identify the iron transporter from cytosolic fraction of *S. cerevisiae*. In this study, we identified the iron-binding activity of thioredoxin reductase, and our data suggest a potential role for this enzyme in intracellular iron transport.

Iron is an essential element for all organisms, because it plays an important role in many cellular processes, such as enzyme catalysis and electron transfer, and serves as a cofactor in cytochromes and iron–sulfur clusters. However, excess iron can generate deleterious reactive oxygen species, such as the hydroxyl radical, via the Fenton reaction. Thus, intracellular levels of iron are tightly regulated by conserved metabolic pathways [1–3].

Iron exists as an insoluble salt in nature and is difficult for living organism to obtain. The budding yeast, *Saccharomyces cerevisiae,* use two well-characterized iron uptake pathways: the reductive iron uptake pathway (*FET3/FTR1*) and the siderophore-mediated uptake pathway [4–6]. Metabolites obtained from the extracellular environment must be transported intracellularly to specific sites of use. For example, intracellular copper is delivered to specific target proteins by the copper carrier proteins, Atx1, Ccs, and Cox17 [5]. The intracellular delivery pathway for copper has been well-identified. In contrast, the intracellular delivery pathway for iron remains unknown. Recently, *Escherichia coli* thioredoxin reductase was shown to transport iron to the iron–sulfur cluster, but an analogous mechanism has not been found in eukaryotes [7–9].

To identify an intracellular iron carrier in *S. cerevisiae*, we screened for iron-binding proteins using iron-charged immobilized metal affinity chromatography (IMAC) and two-dimensional gel electrophoresis of cytosolic proteins. We identified cytosolic thioredoxin reductase protein (Trr1p), a functional homolog of bacterial thioredoxin reductase. In *S. cerevisiae,* two similar systems maintain intracellular redox homeostasis: the glutathione (GSH)–glutaredoxin and the thioredoxin systems. The thioredoxin system is divided into the cytoplasmic (*TRX1*, *TRX2*, and *TRR1*) and mitochondrial (*TRX3* and *TRR2*) systems. The oxidized disulfide form of thioredoxin (*TRX*) is reduced directly by NADPH and thioredoxin reductase (*TRR*). Although the cytoplasmic thioredoxin reductase (Trr1p) has been identified in genetic studies, little is known about its role in regulating the cellular redox environment [10–12]. Here, we report that Trr1p binds iron and may function in iron metabolism.

Materials and methods

Strains, media, and growth conditions. The *S. cerevisiae* strains used in this study were BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and its derivatives. Media with defined iron concentrations were prepared using a modified SD minimal medium containing 0.67% yeast nitrogen base without iron and copper, 2% glucose, and 25 mM MES buffer (pH 6.1), and amino acids with auxotrophic requirements. The iron-limited conditions were generated by the addition of 100 μM bathophenanthroline disulfonate (BPS) and various concentrations of ferrous ammonium sulfate (FAS). The *E. coli* strains used for expression and purification of Trr1 were grown at 37 °C in LB medium supplemented with ampicillin (100 μg/ml).

Protein extraction. To screen cytosolic iron-binding proteins of *S. cerevisiae*, yeast cells (BY4741) were cultured until mid-exponential phase ($A_{600} \sim 0.7$) at 30 °C in defined iron medium at three different iron concentrations, 0, 2, and 10 μM. Protein extracts were obtained by homogenization in lysis buffer [1.2 M sorbitol, 0.1 M potassium phosphate buffer (pH 7.5), and protease inhibitor cocktail] after zymolyase treatment for 60 min at 30 °C. The lysate was cleared at 10,000 g at 4 °C for 20 min and the protein concentration of the supernatant was determined using the Bio-Rad protein assay.

IMAC (Immobilized metal affinity chromatography). IMAC was performed with Chelating Sepharose Fast Flow resin (GE Healthcare, USA) according to the manufacturer's instructions. Fe(III) was coupled to the resin by applying a solution of 20 mM sodium citrate (pH 3.0), 50 mM $FeCl_3$, and 0.5 M NaCl to the column. The column was washed with a solution of 20 mM sodium acetate (pH 4.0) and 0.5 M NaCl and equilibrated with a solution containing 20 mM Tris–HCl (pH 7.4) and 0.5 M NaCl. Cytosolic proteins in binding buffer were allowed to bind to the column for 2 h at 4 °C with gentle shaking. The column was washed with 10 volumes of binding buffer, and bound proteins were eluted in a solution of 20 mM Tris–HCl

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(pH 7.4), 0.5 M NaCl, and 50 mM EDTA [13–15,24]. Protein concentrations were determined by the Bio-Rad protein assay, and fractions were monitored by SDS–PAGE.

Two-dimensional gel electrophoresis (2-DE) analysis. The proteins eluted from IMAC were precipitated in Trichloroacetic acid (TCA), lyophilized, and rehydrated in solution of 7 M urea, 2 M thiourea, 0.5% Immobilised pH buffer (IPG) buffer (pH 4–7, GE Healthcare, USA), 4% (w/v) of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 60 mM of dithiothreitol (DTT) before 2-DE separation [16]. Isoelectric focusing (IEF) was performed using IPG strips (pH 4–7, 24 cm, linear; GE Healthcare, USA) on IPGphor (GE Healthcare, USA) [17,18]. SDS–PAGE was then carried out on a 12% polyacrylamide gel. Gels were silver stained and scanned using a Power-Look III image scanner (UMAX data system, Hsinchu, Taiwan). Spot patterns of visualized gels were analyzed using ImageMaster 2 D Elite software (GE Healthcare, USA). Selected protein spots were excised from the gels, digested with trypsin, and analyzed by tandem mass spectrometry (MS/MS). The MS/MS results were searched in the NCBInr database with MASCOT (Matrix Science, London, UK; www.matrixscience.com).

Northern and Western blot analysis. Cells were cultured in defined iron medium at 30 °C until mid-log phase ($A_{600} \sim 0.7$). Total RNA was isolated with TRIzol Reagent according to the manufacturer's instructions (Gibco BRL, Grand Island, NY) and Northern blot was performed. DNA probes were synthesized by PCR with oligonucleotides, Trr1-nF (5′-AGTTGATCTGTCTTCCAAACC-3′) and Trr1-nR (5′-TCTTT TTAGTGTTCTTAATCC-3′). The probes were radioactively labeled by a Random Priming Kit (GE Healthcare, USA), and hybridization was performed overnight at 60 °C. To construct HA-tagged strains of *TRR1*, a PCR-amplified HA tagging cassette was used [26]. The HA-tagged strain of the *TRR1* gene was grown in YNB at various iron concentrations. Western blots were developed using the Amersham ECL detection kit (GE Healthcare, USA) and exposed to X-ray film.

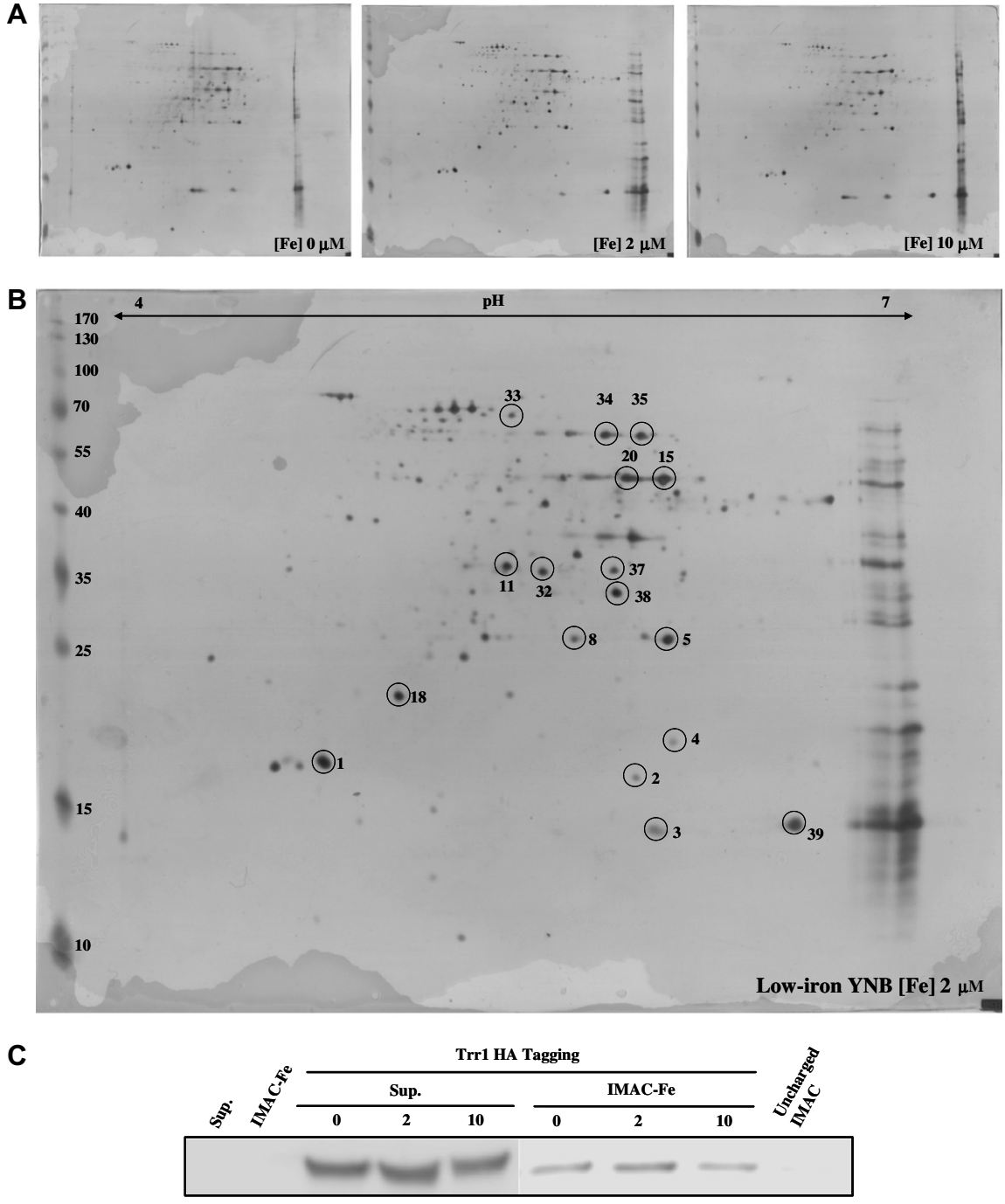

Fig. 1. 2-DE analysis of iron-binding proteins. (A) 2-DE analysis of iron-binding proteins extracted from cells grown at 0, 2, and 10 μM of FAS. (B) Protein spots selected for identification by MS/MS. (C) anti-HA Western blot. Lanes 1 and 2 are the untagged strains; Lane 9 is uncharged IMAC elution of Trr1-HA-tagged strain. The specific binding activities were identified from lanes 6, 7, and 8.

Protein expression and purification. *TRR1* was amplified from genomic DNA by PCR using the *TRR1* primers, inserted into the pQE30 expression vector (QIAGEN, USA) and transformed into *E. coli* JM109. Expression of the recombinant protein was induced with 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were disrupted by sonication in lysis buffer [50 mM Tris–HCl (pH 8.0), 100 mM NaCl, and protease inhibitor cocktail], and the lysate was pelleted by centrifugation at 10,000 g for 20 min at 4 °C. Recombinant Trr1-HA was affinity purified using Ni–NTA (Ni^{2+}–nitrilotriacetate)–agarose resin (QIAGEN, USA). The protein extracts and purified Trr1-HA were confirmed by SDS–PAGE. The protein concentration was determined by the Bradford method using bovine albumin as a standard.

Spectroscopic analysis. UV–vis absorption analysis was performed on a UV-1610PC double-beam spectrophotometer (Shimadzu, Japan) using 1-cm path-length quartz cuvettes [19–21]. Circular dichroism (CD) spectra were recorded with a Jasco J-810 spectropolarimeter (Jasco, Easton, MD) using a stoppered 0.2-cm path-length quartz cuvette at room temperature [22,23]. Each reported spectrum was obtained by averaging sixteen scans collected at 0.1-nm intervals at a bandwidth of 1 nm.

Results

IMAC analysis identified Trr1p as an iron-binding protein

To identify putative iron-binding proteins, we performed an IMAC assay on the cytosolic fraction of *S. cerevisiae* proteins. The eluted proteins from iron-IMAC were then separated by 2-DE and identified by MS/MS analysis. Fig. 1 shows the 2-DE gels derived from cultures grown on various iron concentrations (0, 2, and 10 μM of ammonium ferrous sulfate). We did not observe any protein spots that varied with iron concentration (Fig. 1A). To identify iron-binding proteins, we selected 40 spots on the 2-DE gel (Fig. 1B), which represented 20 individual proteins as identified by MS/MS (Table 1). Most of the identified proteins have known

Table 1
Proteins identified by MS/MS from iron-IMAC

Spot No.	Gene name	Description	Mw	pI	Matched peptides
1	AHP1/YLR109W	Thiol-specific peroxiredoxin	19274	5.01	SIGFELAVGDGVYWSGR
2	SOD1/YJR104C	Cytosolic superoxide dismutase	15828	5.63	TGNAGPRPACGVIGLTN
3, 39	RPL22A/YLR061W	Protein component of the large (60S) ribosomal subunit	13685	5.91	VEGAVGNLGNAVTVTEDGTVVTVVSTAKLAFY QVTPEEDEEEDEE
4	CDC33/YOL139C	Cytoplasmic mRNA cap binding protein	24239	5.29	TLLAVIGETIDEDDSQINGVVLSIR
5, 8	TPI1/YDR050C	Triose phosphate isomerase	26762	5.75	ILYGGSANGSNAVTFK
11	IPP1/YBR011C	Cytoplasmic inorganic pyrophosphatase (PPase)	32353	5.36	AVGDNDPIDVLEIGETIAYTGQVK
15	ERR3/YMR323W	Protein of unknown function has similarity to enolases	47312	5.19	RSGETEDTFIADLVVGLR
18	TSA1/YML028W	Ubiquitous housekeeping thioredoxin peroxidase	21690	5.03	DYGVLIEEEGVALR
20	ENO2/YHR174W	Enolase II	46830	6.16	VNQIGTLSESIK
32	RHR2/YIL053W	DL-glycerol-3-phosphatases, isoform of DL-glycerol-3-phosphatase	30591	5.31	VVVFEDAPAGIAAGK
33	EFT2/YDR385W	Elongation factor 2 (EF-2), also encoded by EFT1	93686	5.92	AYLPVNESFGFTGELR
34	PDC6/YGR087C	Minor isoform of pyruvate decarboxylase	61580	6.13	WAGNANELNAAYAADGYARIK
35	PDC1/YLR044C	Major of three pyruvate decarboxylase isozymes	60378	5.83	WAGNANELNAAYAADGYAR
37	TRR1/YDR353W	Cytoplasmic thioredoxin reductase	34474	5.46	IVAGQVDTDEAGYIK
38	ADH1/YOL086	Alcohol dehydrogenase	31954	6.38	ANGTTVLVGMPAGAKCCSDVFNQVVKSISIVGSYV

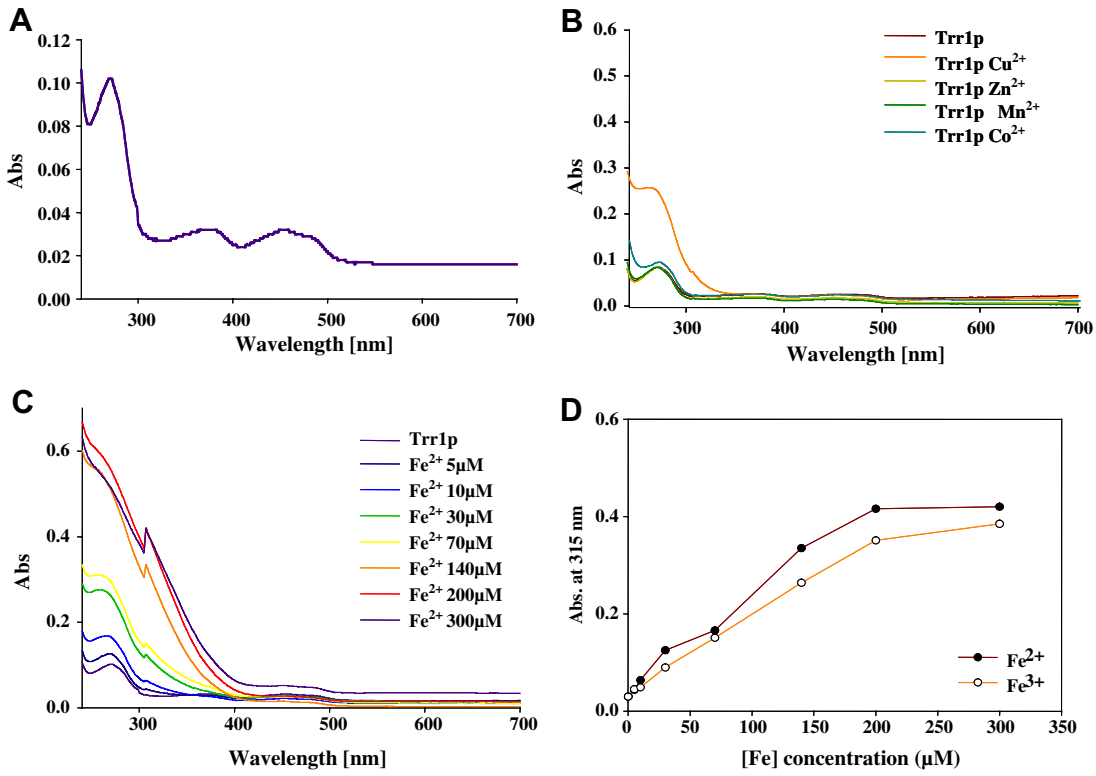

Fig. 2. UV–vis spectroscopy of recombinant Trr1p. The protein concentration was 0.1 μg/μl. (A) Recombinant Trr1p shows absorption peaks at 280, 359 and 452 nm in the absence of metal ions. (B) UV–vis absorption spectra of Trr1p with various metal ions. Each metal ion (100 μM $CuSO_4$, $ZnSO_4$, $MnSO_4$, or $CoCl_2$) was added to Trr1p and incubated for 30 min at 30 °C. (C,D) UV–vis spectra of Trr1p with increasing iron ($FeSO_4{\cdot}(NH_4)_2{\cdot}SO_4$ and $FeCl_3$) concentrations.

involvement in cellular redox, phosphorylation, or translation machinery (Table 1). Thioredoxin reductase (Trr1p), a NADPH-dependent oxidoreductase, is a known homolog of the bacterial Trx protein, a cellular iron transporter that transfers iron to the iron–sulfur cluster (ISC) [23]. To confirm that Trr1p binds to iron, we repeated the IMAC assay on lysates from a strain expressing HA-tagged Trr1p. Western blot analysis revealed a Trr1p-HA specific band in the IMAC-Fe resin-purified sample but not in the control sample (Fig. 1C) confirming that Trr1p has the ability to bind iron.

UV–vis and CD spectroscopic analysis of Trr1p

To further analyze the iron-binding ability of Trr1p, UV–vis and CD spectroscopic analyses were performed on recombinant Trr1p. As shown in Fig. 2A, UV–vis spectra of the recombinant Trr1p showed broad absorption peaks around 359 and 452 nm and an absorption peak at 280 nm [25]. However, when Trr1p was incubated with iron, the spectra of Trr1p showed an absorption peak at 315 nm and a broad absorption peak around 480 nm, which indicated iron binding (Fig. 2B and C) [19,21]. Recombinant Trr1p was then incubated with various concentrations of iron (from 5 to 300 μM of FAS). As the iron concentration was increased, the height of the absorption peak at 315 nm of Trr1p was increased with concentration dependent manner of iron until saturation at 200 μM (Fig. 2D) [23]. We did not observe significant changes in the spectrum with zinc, copper, manganese, or cobalt, indicating that Trr1p binds specifically to iron.

To investigate conformational changes in Trr1p upon metal ion binding, circular dichroism (CD) spectrum analysis was performed in the absence or presence of iron and other metal ions (Fig. 3). The spectrum of Trr1p in the absence of iron differed from that of Trr1p incubated with iron. The difference was a small change in the α-helical content of Trr1p upon iron binding [21–23]. The CD spectra of Trr1p in the absence or presence of copper, zinc, manganese, and cobalt were all similar, indicating that these metals have no effect on the protein structure. These results further confirm that Trr1p binds specifically to iron.

Expression level of TRR1 is not regulated by iron

To determine whether iron concentration influences the expression of *TRR1* at the transcriptional or protein level, Northern blot and Western blot analyses were performed. The cells were cultured in defined iron media at 30 °C, and total RNAs were extracted and subjected to Northern blot analysis. As shown in Fig. 4A, when cells were cultured in different iron concentrations, no difference in the mRNA level of *TRR1* was observed. To examine the expression levels of Trr1 protein, the *TRR1-HA*-tagged strain was used. Again, no change in the level of Trr1p-HA was observed with varying iron concentration. These results suggest that iron does not regulate *TRR1* expression.

trr1 has growth defects on SD medium

To investigate whether iron affects the growth of a *trr1* deletion strain, plate assays were performed on SD media containing 100 μM BPS supplemented with iron. On the 20 μM of iron, *trr1* had a severe growth defect, which was suppressed by high levels of exogenous iron (500 μM) (Fig. 4B). This is the expected phenotype of a strain with defects in the high affinity iron transport system, such as *fet3* or *ftr1*. The phenotype of the *trr1/trr2* double

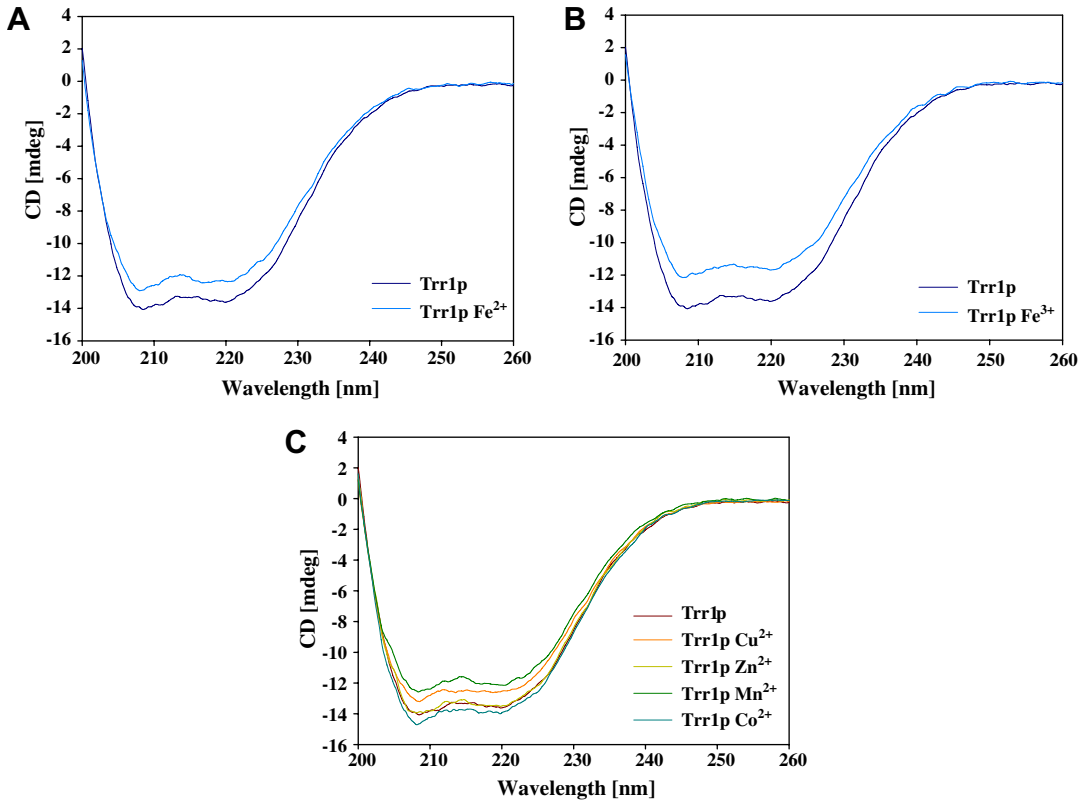

Fig. 3. CD spectroscopic analysis of recombinant Trr1p. All spectra shown represent the average of six recorded spectra.(A,B) CD spectra of Trr1p in the absence or presence of iron [$FeSO_4{\cdot}(NH_4)_2{\cdot}SO_4$ or $FeCl_3$]. Purified recombinant Trr1p was added to 100 μM Fe^{2+} or Fe^{3+} ions, incubated for 30 min at 30 °C and dialyzed. (C) The CD spectra of Trr1p with various metal ions. Each metal ion (100 μM $CuSO_4$, $ZnSO_4$, $MnSO_4$, or $CoCl_2$) color was added to Trr1p individually, and the corresponding spectra are indicated by different colors.

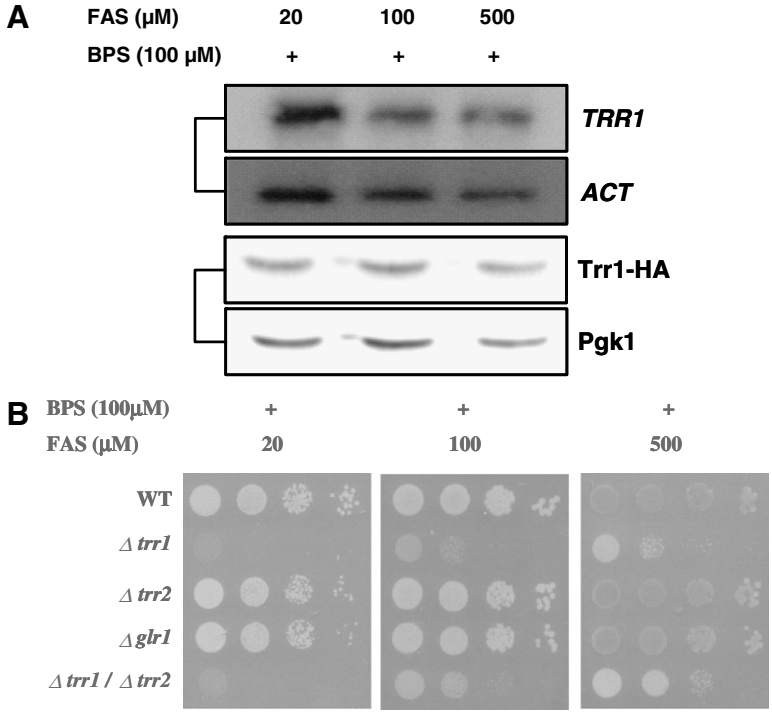

Fig. 4. Expression analysis and deletion phenotype of *TRR1* in low iron conditions. (A, top half) Northern blot analysis of *TRR1* from cells grown at various concentrations of iron (20, 100, and 500 μM; FAS); (lower half) corresponding anti-HA Western blot for Trr1p-HA. (B) Viability of deletion mutants at various concentrations of iron (20, 100, and 500 μM; FAS).

mutant was similar to that of *trr1* mutant. In contrast, the *trr2* and the *glr1* (glutathione reductase) mutants were not sensitive to low iron concentrations.

Discussion

The iron-uptake pathway is well-conserved from microorganisms to mammals. Iron taken up from the environment must be transferred to target molecules, where it is used as a cofactor in many enzymatic processes. Unlike the pathway for copper transport, the intracellular delivery pathway for iron in *S. cerevisiae* has yet to be identified. In this study, we sought to identify cytosolic iron-binding proteins. Out of 20 potential iron-binding proteins, we selected Trr1p as a candidate iron carrier, because this protein is functionally related to bacterial Trx1, which is a known iron carrier in *E. coli* [27].

Connections between redox proteins and iron metabolism exist in the literature. For example, inhibition of the reducing activity of Trr1p by heavy metals has been reported [28]. Furthermore, double deletion of *GRX3* and *GRX5* resulted in constitutive activation of Aft1p, a transcription factor for the iron transporters *FET3*, *FTR1*, and those in the *ARN* family [29]. In addition, GSH depletion induces defective cytoplasmic iron–sulfur cluster maturation [30,31]. In this study, we confirmed that Trr1p specifically binds iron molecules.

Taken together, current evidence suggests a role for Trr1p in iron metabolism, possibly as the elusive eukaryotic intracellular transporter. To characterize the exact mechanism of Trr1p in iron metabolism, the involvement of Trr1p in iron uptake and iron–sulfur cluster formation should be explored.

Acknowledgments

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