Characterization of iron-binding motifs in *Candida albicans* high-affinity iron permease CaFtr1p by site-directed mutagenesis

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A peptide motif Glu-Xaa-Xaa-Glu has been implicated in direct binding of ferric iron in several proteins involved in iron transport, sensing or storage. However, it is not known whether the motif alone is sufficient for iron binding and whether functional replacement of the conserved residues by other amino acids with similar properties is possible. We previously identified a *Candida albicans* iron permease, CaFtr1p, which contains five Glu-Xaa-Xaa-Glu motifs [Ramanan and Wang (2000) *Science* **288**, 1062–1065]. In this study, we investigated the role of each of these motifs in iron uptake by site-directed mutagenesis. Substitution of Ala for any one of the two Glu residues in Glu-Gly-Leu-Glu^{158–161} abolished iron-uptake activity, while the same substitution in any of the other four motifs had little effect, indicating that only the motif at position 158–161 is required for iron transport. We then evaluated the importance of each of the

residues within and immediately adjacent to this motif in iron uptake. The permease remained active when any one of the Glu residues was replaced by Asp, while it became inactive when both were replaced. We also found that the amino acid immediately in front of Glu-Gly-Leu-Glu^{158–161} must be either Arg or Lys. In addition, substitution of any of the two residues in the middle with several structurally distinct amino acids had no detectable effect on iron uptake. Here we propose to extend the iron-binding motif to Arg/Lys-Glu/Asp-Xaa-Xaa-Glu or Arg/Lys-Glu-Xaa-Xaa-Glu/Asp, which may serve as a guide for the identification of potential iron-binding sites in proteins.

Key words: ferrichrome, ferrioxamine B, iron transport, iron-uptake assay, siderophore.

INTRODUCTION

Nearly all organisms need iron for growth, because it serves as an obligate cofactor of many essential proteins [1,2]. In Nature iron is largely present in ferric form with extremely low solubility. Micro-organisms play an important role in solubilizing the ferric iron by secreting ferric reductases and iron chelators [3–10]. Iron uptake is then carried out by specific iron-transport apparatus residing on the cell surface [11–16]. Iron is toxic to cells when present in excess, because it causes the production of hydroxyl free radicals that damage DNA, proteins and lipids [1,2]. Thus the level of intracellular free iron must be tightly regulated via controlled uptake and storage. Proteins play pivotal roles in virtually every step of iron metabolism and some of them make direct contact with elemental iron. To understand the mechanisms by which these proteins perform iron binding, transport and storage, it is necessary to define the peptide motifs or amino acid residues required for interaction with iron. A peptide motif Glu-Xaa-Xaa-Glu has been proposed as a ferric iron-binding site in several proteins involved in different aspects of iron metabolism [15,17,18]. It was first implicated in iron binding in a study of the crystal structures of ferritin light chain [19–21]. Trikha et al. [19] found evidence suggesting direct interaction of the Glu residues in a motif Glu-His-Ala-Glu with iron, forming the nucleation site for iron core formation. Later, such motifs were found to be present in and essential for the activities of high-affinity iron permeases in fungi [15,17]. Wosten et al. [18] recently reported the presence of Glu-Xaa-Xaa-Glu motifs in bacterial iron-sensing protein PmrB of *Salmonella* and provided evidence for iron

binding at these sites. Despite this evidence, it is not known whether Glu-Xaa-Xaa-Glu alone is sufficient for iron binding and whether other amino acid residues immediately flanking the motif are also required. It appears that many of the known or proposed Glu-Xaa-Xaa-Glu motifs have a positively charged amino acid, Arg, Lys or His, immediately in front of them. However, there have been no experimental data demonstrating that these positively charged amino acids are essential for iron binding and are functionally interchangeable. It is also not clear whether the Glu residues can be replaced by Asp, which is similar to Glu in both structure and charge. In other words, can Asp-Xaa-Xaa-Glu, Glu-Xaa-Xaa-Asp or Asp-Xaa-Xaa-Asp motifs potentially serve as iron-binding sites?

The mechanism of high-affinity iron transport has been intensively studied in *Saccharomyces cerevisiae*. This system consists of two proteins, a permease Ftr1p and an oxidase Fet3p [4,5]. Recently iron-transport systems consisting of homologous components have also been found in *Schizosaccharomyces pombe* and *Candida albicans* [17,22]. All these proteins contain multiple Glu-Xaa-Xaa-Glu motifs, although the positions of some of the motifs differ among these proteins [15,17,23]. Currently, only one of the motifs in Ftr1p is known to be essential for the permease activity [17]. However, it is not known whether all the other motifs are real iron-binding sites and required for iron-uptake activity.

In this study, we used systematic site-directed mutagenesis to evaluate the role of each Glu-Xaa-Xaa-Glu motif in the iron permease CaFtr1p from *C. albicans*. We found that only one motif, Glu-Gly-Leu-Glu^{158–161}, was essential for the iron-uptake

Abbreviations used: BPS, bathophenanthroline disulphonate; GFP, green fluorescent protein; LIM0, limited-iron medium; SDF, SD medium containing iron.

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activity. We then mutated the residues within and immediately adjacent to this motif to investigate the role of each amino acid. Although mutation of any one of the Glu residues to Ala abolished the iron-uptake activity, the enzyme remained active when one of them was replaced by Asp. We also found that the nature of the amino acid immediately in front of Glu¹⁵⁸ is equally essential for the iron-transport activity, and must be Arg or Lys. The residues between the two glutamates can be replaced by a range of structurally distinct residues without detectable deteriorating effects.

EXPERIMENTAL

Strains, media and culture conditions

C. albicans strains used were SC5314 [24], CAI4 (*ura3::λimm434/ura3::λimm434* [25]), CaWYNR1 (*Cafr1::hisG/Cafr1::hisG/Cafr2::hisG/Cafr2::hisG-URA3-hisG* [15]) and CaWYNR2 (the same as CaWYNR1 except *ura*). *C. albicans* cells were normally grown in YPD medium containing 2% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) glucose or SD medium. A litre of SD medium contains 6.7 g of yeast nitrogen base without amino acids (Difco), 0.8 g of complete supplement mixture (CSM) or CSM – ura (complete supplement mixture without uracil; Bio101), 20 g of glucose and 50 mM Mes (pH 6.1). To prepare iron-limiting and iron-sufficient solid medium we followed procedures described previously [15,17]. Briefly, 1 mM ascorbic acid and 1 mM ferrozine were added to chelate the iron in the SD medium (SDF). FeCl₃ was then added to the SDF to final concentrations of 50 and 350 μM to generate the iron-limiting SDF50 and the iron-sufficient SDF350 media, respectively. Liquid medium with undetectable amounts of iron called limited-iron medium (LIM0) was prepared as described previously [15,26,27]. FeCl₃ was added to LIM0 to a final concentration of 2 or 200 μM to prepare the iron-limiting LIM2 and iron-sufficient LIM200 media.

S. cerevisiae FTR1 deletion mutant (*frt1Δ::HIS3*) was constructed by replacing the complete coding sequence with *HIS3* in the haploid strain CRY2α (*can1-100 ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3*). The deletion was verified by Southern blotting analysis and its inability to grow in iron-limiting medium.

Site-directed mutagenesis

The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate different *CaFTR1* mutants. The primers used for mutagenesis are listed in Table 1. A DNA fragment containing the *CaFTR1* promoter and coding sequence followed by an in-frame insertion of green fluorescent protein (GFP) and the 400 bp 3'-untranslated region was first constructed. Then this DNA fragment was cloned into a *C. albicans* autonomous-replicating plasmid pABSK1. The *CaFTR1-GFP* fusion gene was also cloned under the control of the GAL1/10 promoter in an *S. cerevisiae* CEN plasmid containing *URA3* as selection marker. These two plasmids were used as templates for mutagenesis of *CaFTR1*. Mutagenesis was performed by following the manufacturer's protocol (Stratagene). The resulting plasmids were isolated and sequenced to verify the desired mutation. The mutated *CaFTR1* in pABSK1 was transformed into *C. albicans* *Cafr1* *Cafr2* cells and transformants selected on GMM-ura (glucose minimal medium containing 6.7 g/l yeast nitrogen base, without amino acids, and 2% glucose, without uracil) plates. The mutated gene in the *S. cerevisiae* CEN plasmid was transformed into *S. cerevisiae* *frt1Δ* and transformants selected on a SDF350 – ura (SDF350 without uracil) plate containing 2% (w/v) galactose instead of glucose as a carbon source.

Table 1 Oligonucleotide primers used for site-directed mutagenesis

Sequences in bold indicate mutated codons.

Mutation	Primer
E16A	TTCATCGTTTTCAGAGCATCTTTGGAAGCTAT
E158A	ATTACTTGTTCGCTGCAGGTTTAGAAGCTGT
E347A	GAATTAACAAAGCTGCAAGGATGAATTGTT
E365A	TTGGTCAAGCTGCAGCAGTTGATGAACTTC
E376A	CAAAACAAATTGATCGCATCCAAAGAAAACA
E16/19A	CGTTTTTCAGAGCATCTTTGCGAGCTATCATTGT
E158/161A	ACTTGTTCGCTGCAGGTTTAGCAAGCTGTCTCTTC
E347/350A	TTAAACAAAGCTGCAAGGATGCATTGTTCACTAA
E365/368A	GGTCAAGCTGCAGCAGTTGATGCAACTTCATCAAA
E376/379A	AACAAATTGATCGCATCCAAAGCAACAAAGGATCC
E161A	CGGTGAAGGTTTAGCAGCTGTCTCTCGT
E158D	CATTACTTGTTCGCTGACGGTTTAGAAGCT
E161D	CGGTGAAGGTTTAGCAGCTGTCTCTCGT
E158/161D	ACTTGTTCGCTGCAGGTTTAGCAGCTGTCTCTTC
R157A	ACCATTACTTGTTCGCTGAGGTTTAGAAGCT
R157K	ACCATTACTTGTTCGCTGAGGTTTAGAAGC
R157H	ACCATTACTTGTTCGCTGAGGTTTAGAAGCT
G159A	CTTGTTTCGCTGAAGCTTTAGAAAGCTGTTGT
G159D	CTTGTTTCGCTGAAGGATTAGAAAGCTGTTGT
G159H	CTTGTTTCGCTGAAGCATTAGAAAGCTGTTGT
G159K	CTTGTTTCGCTGAAGATTAGAAAGCTGTTGT
G159W	CTTGTTTCGCTGAAGTTAGAAAGCTGTTGT
L160A	GTTTGCCTGAAGGTCGCAAGGCTGTTGTCTTC
L160C	GTTTGCCTGAAGGTTGTGAAGCTGTTGTCTTC
L160D	GTTTGCCTGAAGGTCATGAAGCTGTTGTCTTC
L160H	GTTTGCCTGAAGGTCATGAAGCTGTTGTCTTC
L160W	GTTTGCCTGAAGGTTGGGAAGCTGTTGTCTTC
A162W	GTGAAGGTTAGAAATGGGTTGTCTCTGTTGG

To randomly mutate Arg¹⁵⁷ of CaFtr1p two mutagenic primers targeting different strands of the same regions were used: 5'-ACCATTACTTGTTCGCTGAGGTTTAGAAGC-3' and 5'-GCTTCTAAACCTTCNNNCAACAAGTAATGGT-3'. The CEN plasmid containing *CaFTR1* described above was used as a template. The mutagenesis product was transformed into *Escherichia coli* XL-Blue Supercompetent Cells (Stratagene) and plated on Luria–Bertani (LB)/ampicillin plates. All the colonies (about 800) were harvested as a mixture and plasmids isolated as a library of mutant genes. The library plasmids were transformed into *S. cerevisiae* *frt1Δ* cells and the transformants selected on the iron-limiting SDF50-ura plate containing galactose as a carbon source. The colonies were picked and grown separately. Total DNA from each clone was isolated and re-transformed into *Escherichia coli* DH5α cells by electroporation. The plasmids were isolated and sequenced to verify the mutations.

Iron-dependent growth assay of *C. albicans* and *S. cerevisiae* strains carrying various *CaFTR1* mutants

The *C. albicans* strains to be tested were grown in LIM200 to saturation. Then the cells were spun down and washed once with 10 mM EDTA, thrice with a solution containing 10 mM EDTA and 100 μM bathophenanthroline disulphonate (BPS) and thrice with LIM0. The cells were resuspended in LIM0 and grown at 30 °C for 8 h with shaking at 200 rev./min to exhaust the intracellular iron. Then the cells were spun down, washed twice with LIM0 and resuspended in LIM0 to a density of 1 × 10⁶ cells/ml. For growth on solid medium, 10-fold serial dilutions were spotted on to SDF350 and SDF50 plates. The plates were incubated at 30 °C for 36 h. For liquid culture, cells were diluted to 1 × 10⁴ cells/ml in LIM2 or LIM200 and incubated

at 30 °C for 48 h with shaking at 200 rev./min. The D_{600} value of the cultures was measured every 2 h.

To examine the growth of *S. cerevisiae* transformants, cells were first grown in SDF200-ura overnight at 30 °C. Then the cells were pelleted, and washed twice with and resuspended in SDF medium. Serial dilutions of cells were spotted on to SDF50–ura or SDF350–ura agar with glucose replaced by galactose as a carbon source. The plates were incubated at 30 °C for 4 days.

Iron-uptake assays

The iron-uptake assay was carried out as described previously with some modifications [27]. *C. albicans* cells were grown in LIM200 at 30 °C for 36 h to the stationary phase. The cells were pelleted and washed once with 10 mM EDTA, twice with 10 mM EDTA/100 μ M BPS and twice with LIM0. The cells were resuspended in LIM0 at a density of 1×10^7 cells/ml and grown at 30 °C for 8 h with shaking at 200 rev./min. Then the cells were pelleted, washed twice with LIM0 and resuspended in LIM0 at a density of 2×10^8 cells/ml. The cell suspension (50 μ l; $\approx 10^7$ cells) was used for iron-uptake assay. ^{59}Fe was added to a final concentration of 2 μ M and the cells were incubated at 30 °C for 15 min. An identical aliquot of the cell suspension with ^{59}Fe was kept on ice for estimating the level of background binding of ^{59}Fe to the cell surface. Radioactivity was counted by using an LKB Compu-gamma counter (model 1282). After subtracting the background the iron uptake is expressed in fmol of ^{59}Fe /min per 10^6 cells.

Western blotting analysis

We used anti-GFP antibody in Western blotting analysis to examine the expression of the mutated *CaFtr1p*-GFP. Each strain tested was grown in 30 ml of LIM200 to a D_{600} of 0.6 and then the cells were transferred to LIM2 for another 3 h of growth. Cells were then spun down and washed in ice-cold 0.9% (w/v) NaCl containing 1 mM NaN_3 , 10 mM EDTA and 50 mM NaF. Then, cells were resuspended in 500 μ l of lysis buffer containing 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris/HCl (pH 7.2), 1 mM PMSF, 20 μ g/ml leupeptin, 40 μ g/ml aprotinin, 0.1 mM sodium orthovanate and 15 mM *p*-nitrophenylphosphate. An equal volume of glass beads was added and cells were disrupted by two rounds (2 min each) of bead beating at maximum power using a Mini-Beadbeater (Biospec Products). Cells were kept on ice for 1 min between the beatings. Cell extracts were cleared by centrifugation at 15 700 *g* for 10 min. A total of 40 μ l of each cell extract (about 30 μ g of protein) was resolved by 8% (w/v) SDS/PAGE. Then the proteins were transferred to nitrocellulose membranes by electroblotting. A mouse anti-GFP monoclonal antibody (dilution 1:500; Clontech) was used as the primary antibody followed by peroxidase-conjugated anti-mouse IgG (1:2000; Clontech). The GFP-tagged proteins were detected using the Super-Signal chemiluminescent assay (Pierce Chemicals).

RESULTS

Expression of *CaFTR1* mutants and testing their ability to support cell growth in iron-limiting medium

There are five Glu-Xaa-Xaa-Glu motifs in *CaFtr1p*: Glu-Ser-Leu-GLU^{16–19}, Glu-Gly-Leu-Glu^{158–161}, Glu-Lys-Asp-Glu^{347–350}, Glu-Val-Asp-Glu^{365–368} and Glu-Ser-Lys-Glu^{376–379}. To deter-

Figure 1 Expression of Glu-Xaa-Xaa-Glu mutants in *Cafr1 Cafr2* cells

(A) All the *Cafr1p* mutants are correctly localized to the plasma membrane. Cells of each strain tested were first grown in LIM200 overnight. Then the cells were inoculated into LIM200 and LIM2 at a density of 10^5 cells/ml and grown at 30 °C for 3 h before visualization by fluorescence microscopy. The photographs only show the cells grown in LIM2. The cells grown in LIM200 did not exhibit detectable fluorescence at the same exposure. *Cafr1 Cafr2* cells transformed with the wild type (WT) or any one of the five Glu-Xaa-Xaa-Glu mutants were included in this experiment. (B) Western blotting analysis detected a protein of expected size in *Cafr1 Cafr2* cells transformed with various *CaFTR1* mutants. The cells inoculated into LIM2 described above were grown to a D_{600} of ≈ 0.6 before Western blotting analysis. Protein extracts from *Cafr1 Cafr2* cells and *Cafr1 Cafr2* cells transformed with the vector (Vector) alone were included as negative controls.

mine which motif is required for iron transport, we used site-directed mutagenesis to generate five mutants, each having the first Glu replaced by Ala in one of the motifs. We then cloned these five mutated genes in the autonomous-replicating vector pABS1 driven by the *CaFTR1* promoter and transformed them into *C. albicans Cafr1 Cafr2* cells that are completely defective in oxidase/permease-mediated high-affinity iron uptake [15,27]. Each of these mutants was also C-terminally tagged by GFP, which allowed us to determine whether each mutant protein can correctly localize to the plasma membrane by fluorescence microscopy and to quantify the amount of the protein by anti-GFP Western blotting analysis. We had previously demonstrated that the C-terminal GFP tag had no damaging effect on permease function [15]. Each transformant was grown in both the iron-limiting LIM2 and iron-sufficient LIM200. Fluorescence microscope examination showed that all the transformants carrying different mutant genes exhibited a bright periphery in cells grown in LIM2 but not in LIM200 (Figure 1A), indicating that all the mutated permeases were expressed in the right growth conditions and correctly localized in the cells. Western blotting analysis confirmed the expression in each transformant of a protein of expected size (Figure 1B). All the new mutants described below were similarly examined for their correct expression and cellular localization (results not shown).

The transformants were then tested for their ability to correct the growth defect of the *Cafr1 Cafr2* strain under iron-limiting conditions. Since it is difficult to remove contaminating iron from agar, to prepare iron-limiting and sufficient solid medium we first added 1 mM ascorbic acid and 1 mM ferrozine to chelate iron in the SD medium containing dissolved agar. FeCl_3 was then added to the medium to final concentrations of 50 and 350 μ M to generate the iron-limiting SDF50 and the iron-sufficient SDF350 media. It has been demonstrated previously that SDF50 represented iron-limiting conditions for both *S. cerevisiae* and *C. albicans*, because strains lacking the permease/oxidase-mediated high-affinity iron-uptake system were unable to grow in this medium [15,17]. In contrast, these mutants grew as well as the

Figure 2 Iron-dependent growth assay of Glu-Xaa-Xaa-Glu mutants on solid media

Cells were first grown in LIM200 (LIM contains uridine) overnight and then serially diluted. Two inocula from each strain, containing approx. 1000 and 100 cells, were spotted on to SDF50 and SDF350 agar plates and grown at 30 °C for 36 h. The wild-type strain SC5314, *Caftr1 Caftr2* (*ura⁻* strain) and *Caftr1 Caftr2* cells transformed with the vector alone, *CaFTR1* or various Glu-Xaa-Xaa-Glu mutants were included in this assay.

wild type in SDF350, because under this condition the high-affinity iron-uptake system is normally shut down and the low-affinity system is responsible for iron uptake [15,17]. In the experiment shown in Figure 2, the wild-type strain SC5314 was included as a positive control, which should grow on both iron-limiting and iron-sufficient media. The *ura⁻ Caftr1 Caftr2* was included as a negative control, which was not expected to grow on either medium because of the lack of uridine in the SD medium. Since each transforming plasmid carries a mutated *Caftr1* and a copy of the *URA3* gene, only the transformants expressing a functional *CaFtr1p* mutant would be able to grow on both iron-limiting and iron-sufficient media, while transformants expressing an inactive form of *CaFtr1p* or containing a vector plasmid alone would only grow on the iron-sufficient plates. Figure 2 shows that, on the iron-limiting SDF50 medium, the transformants expressing the wild-type *CaFtr1p* or any of the mutants E16A, E347A, E365A and E376A grew equally well. In contrast, the *Caftr1 Caftr2* cells transformed with either the vector pABSK1 alone or with the mutant E158A did not grow. On the iron-sufficient SDF350 medium, except for the *ura⁻ Caftr1 Caftr2* mutant all the strains tested grew equally well. To evaluate the iron-dependent growth of each strain in a more quantitative manner, we determined growth curves for all the strains cultured in liquid media. Differing from the SDF solid medium, contaminating iron was first nearly completely removed from liquid media by iron-chelating chromatography and then a known amount of iron was added back. For example, LIM2 and LIM200 contain 2 and 200 μM FeCl_3 , representing iron-limiting and iron-sufficient conditions, respectively. It was reported previously that mutants lacking the high-affinity system could only grow in LIM200 but not in LIM2 [15]. Figure 3 shows that in LIM200 all strains examined exhibited similar growth curves with some differences largely within experimental variations. In contrast, like the *Caftr1 Caftr2* mutant the transformants containing the vector or expressing E158A did not show any growth in the iron-limiting LIM2 after 36 h, whereas the transformants

Figure 3 Iron-dependent growth assay of Glu-Xaa-Xaa-Glu mutants

Cells of each strain were first grown in LIM200 to saturation before dilution into LIM2 (bottom panel) and LIM200 (top panel) at a density of 10^4 cells/ml. The cultures were then incubated at 30 °C with vigorous shaking for 36 h and the D_{600} was measured at 2 h intervals.

expressing the wild-type and the other four mutated permeases grew nearly as well as the wild-type strain SC5314.

We also generated a second set of *CaFTR1* mutants by replacing the second Glu residue with Ala in each of the five Glu-Xaa-Xaa-Glu motifs and examined the effect on the permease activity by repeating the growth assays described above. Again, only E161A failed to rescue the iron-uptake defect of *Caftr1 Caftr2* under iron-limiting conditions, while the permeases carrying mutations in the other four motifs were functional (results not shown). Taken together, the results indicate that only one of the five motifs, Glu-Gly-Leu-Glu¹⁵⁸⁻¹⁶¹, is essential for permease activity. The result is consistent with previous reports on the indispensability of this motif in *Ftr1p* for iron-uptake activity [17].

The role of Arg¹⁵⁷ in *CaFtr1p* activity

When aligning a number of short amino acid sequences containing known iron-binding motifs, we and others [18] noticed that the Glu-Xaa-Xaa-Glu motifs were often preceded by a positively charged amino acid: Arg, Lys or His (Figure 4). In fungi, in all the members of the *Ftrp* family of permeases the motif corresponding to Glu-Gly-Leu-Glu¹⁵⁸⁻¹⁶¹ has an Arg immediately in front of it. The Glu-Xaa-Xaa-Glu motif in ferritin light chain has either Arg or Lys at this position. In bacteria, a group of proteins related to the *Salmonella* PmrB iron sensor contain Glu-Xaa-Xaa-Glu motifs that often have a His

Figure 4 Amino acid sequence alignment of protein regions containing iron-binding motifs

(A) The iron-interacting region in ferritins from *Rana catesbeiana* (Ra), *Homo sapiens* (Hu), *Mus musculus* (Mu) and *Glycine max* (Gl). The region shown corresponds to amino acids 53–65 of the protein from *R. catesbeiana*. (B) Iron transporters of *S. cerevisiae* and *C. albicans*. The region aligned corresponds to amino acids 154–163 of CaFtr1p. (C) PmrB-related iron-sensing proteins from *Salmonella enterica*, *E. coli*, *Yersinia pestis* and *Klebsiella pneumoniae*. The region corresponds to amino acids 35–64 of PmrB from *S. enterica* [18].

Figure 5 Iron-dependent growth assay of CaFtr1p Arg-Glu-Gly-Leu-Glu^{157–161} mutants on solid media

The assay was performed as described in Figure 2. (A) Growth of Arg¹⁵⁷ mutants. (B) Growth of Glu¹⁵⁸ and Glu¹⁶¹ mutants.

residue immediately in front of the first Glu. This observation prompted us to determine whether Arg¹⁵⁷ of CaFtr1p is required for iron uptake and, if so, whether it can be functionally substituted by Lys or His. We constructed R157A, R157K and R157H mutants and examined the ability of each to rescue the iron-dependent growth defect of *Cafr1 Cafr2* (Figure 5A). We found that cells expressing R157A exhibited no growth on the iron-limiting plate, indicating that Arg¹⁵⁷ is essential for the function of the permease. Interestingly, the R157K mutant could fully rescue the growth defect of *Cafr1 Cafr2*, while the cells expressing R157H did not exhibit any growth. The result suggests that the Arg residue can only be functionally replaced by Lys but not His.

To further investigate whether any other amino acids besides Lys might be able to substitute Arg¹⁵⁷, we randomly mutated the codon encoding Arg¹⁵⁷. In order to quickly identify the mutations

Figure 6 ⁵⁹Fe-uptake assay of CaFtr1p Arg-Glu-Gly-Leu-Glu^{157–161} mutants

Cells were grown in LIM200 overnight and then inoculated into LIM0 to exhaust intracellular iron. The assay was then carried out in LIM0 supplemented with 2 μ M ⁵⁹Fe. The uptake is expressed in fmol of ⁵⁹Fe/min/10⁶ cells.

that did not result in a loss of the permease activity, we transformed the mixture of mutated *CaFTR1* genes into *S. cerevisiae fir1Δ* under the control of the GAL1/10 promoter to select clones that would grow on iron-limiting plates in the presence of galactose. We had demonstrated previously that *CaFTR1* could rescue the growth defect of *fir1Δ* [15]. From over 200 colonies of *S. cerevisiae fir1Δ* transformed with the library of mutated *CaFTR1* genes, the plasmid from each clone was recovered by transforming *E. coli* and the mutated gene analysed by sequencing. About half of the mutants were found to contain the various degenerate codons for Arg at amino acid position 157 and the other half contained codons for Lys. No other substitutions were found. This result is consistent with that of expressing the Arg¹⁵⁷ mutants in *C. albicans* described above, indicating that the amino acid residue immediately in front of Glu-Gly-Leu-Glu^{158–161} must be either Arg or Lys for the permease activity.

One of the two glutamates in Glu-Gly-Leu-Glu^{158–161} can be functionally replaced by Asp

The Glu residue carries a negative charge in its side chain. Next we asked whether the Glu residues in Glu-Gly-Leu-Glu^{158–161} could be substituted by another negatively charged amino acid, Asp. We generated three mutants, E158D, E161D and E158,161D to transform *Cafr1 Cafr2*. Figure 5(B) shows that *Cafr1 Cafr2* cells expressing E158D or E161D grew as well as the cells expressing the wild-type CaFtr1p on iron-limiting plates, whereas the cells expressing E158,161D or E161A did not grow. The result demonstrates that the permease remains functional when one of the Glu residues is replaced by Asp, but loses function when both Glu residues are replaced.

⁵⁹Fe-uptake assay of Arg-Glu-Gly-Leu-Glu^{157–161} mutants

To better quantify the effect of amino acid substitutions of the Arg and Glu residues in Arg-Glu-Gly-Leu-Glu^{157–161}, we determined ⁵⁹Fe-uptake rate in *Cafr1 Cafr2* cells expressing different mutants. The assay was carried out in LIM0 supplemented with 2 μ M ⁵⁹Fe and the results are summarized in Figure 6. The wild-type strain SC5314 showed 140 fmol of ⁵⁹Fe uptake/min in every 1 $\times$ 10⁶ cells, whereas *Cafr1 Cafr2* showed no ⁵⁹Fe

uptake. The transformant expressing *CaFTR1* restored iron uptake to the level of SC5314, while the transformants expressing R157A and E158A exhibited no iron uptake, demonstrating again the essentiality of Arg¹⁵⁷ and Glu¹⁵⁸ in the iron-uptake activity of the permease. The cells expressing E158D or R157K restored the iron uptake to about 50–60 % of the level of SC5314, indicating that the permeases carrying these mutations remain active, albeit at a reduced level. In the growth assay performed on agar plates described above the same two transformants exhibited similar growth rates as the wild-type cells, suggesting that the 40–50 % permease activity was sufficient to support cell growth under the iron-limiting condition used. Since there is an Arg immediately in front of Glu-Ser-Leu-Glu^{16–19}, it appears to have all the residues required for iron binding. To explore the possibility that the E16A mutation may have some effect on iron transport that was not detected by the growth assays described above, we determined the ⁵⁹Fe uptake of the *Cafr1* *Cafr2* cells expressing E16A and found that this mutant was nearly as active as the wild-type permease in iron uptake.

Examination of potential roles of other amino acids within or immediately adjacent to Arg-Glu-Gly-Leu-Glu^{157–161}

Although the two residues in the middle of Glu-Xaa-Xaa-Glu motifs are variable among the iron-binding proteins from different organisms, most members of the Ftrp family of permeases in *S. cerevisiae*, *C. albicans* and *S. pombe* have Arg-Glu-Gly-Leu-Glu at the position corresponding to position 157–161 of CaFtr1p (see Figure 4B), suggesting that the residues between the Glu residues may also be conserved to a certain extent, perhaps within each family of iron-binding proteins. Furthermore, Ala¹⁶² and Val¹⁶³ also appear to be conserved among these permeases. To test the importance of these residues, we mutated Gly¹⁵⁹ and Leu¹⁶⁰ to a range of amino acids distinct in structure and charge, including G159A, G159D, G159H, G159K, G159W, L160A, L160C, L160D, L160H and L160W. Ala¹⁶² and Val¹⁶³ were mutated to Trp and Ala, respectively. The *CaFTR1* gene cloned in both pABSK1 and the *S. cerevisiae* CEN plasmid described above were used as templates for mutagenesis, and then the mutants were tested for activity in *C. albicans* *Cafr1* *Cafr2* and *S. cerevisiae* *ftr1Δ*, respectively. Results showed that all these mutants could fully rescue the growth defect of both *Cafr1* *Cafr2* and *Δftr1* on iron-limiting plates (results not shown), indicating that these residues are not essential for the activity of the permease. However, we did not determine whether there would be any subtle effects of these mutations on permease activity by the more sensitive ⁵⁹Fe-uptake assay.

DISCUSSION

There has been strong evidence suggesting that the peptide motif Glu-Xaa-Xaa-Glu participates in iron binding in several proteins involved in iron metabolism [15,17–19]. In this study we used the *C. albicans* high-affinity iron permease CaFtr1p as a model molecule to explore the amino acid residues required for the formation of a functional iron-binding motif. The members of the Ftrp family of permeases all contain multiple Glu-Xaa-Xaa-Glu motifs. Do they all participate in the interaction with iron? By mutating the conserved Glu residues to Ala, we found that only one motif, Glu-Gly-Leu-Glu^{158–161}, is indispensable for iron-transport activity. The position of this motif corresponds to the one in Ftr1p that has previously been shown to be essential for iron-uptake activity [17]. The same amino acid substitution in any of the other motifs had no detectable effect, suggesting that they are not critically important for the iron-transport activity of the permease.

Does the Glu-Xaa-Xaa-Glu motif contain all the elements required for iron binding? Can the presence of this motif serve as a good predictor for possible interactions with iron? By mutating the residues between the two glutamates and those flanking Glu-Gly-Leu-Glu^{158–161}, we found that the nature of the amino acid immediately in front of the first Glu is also critical. In CaFtr1p there is an Arg at this position, substitution of which by Ala abolished iron transport. By random mutagenesis of the codon for Arg¹⁵⁷ we found that this residue could only be functionally replaced by Lys, an amino acid highly similar to Arg in both side-chain structure and basicity. Another positively charged amino acid, histidine, failed to functionally replace Arg¹⁵⁷. This could be explained by the much weaker affinity for protons of the His side chain (pK_a , 6) and its significantly different structure in comparison with Arg (pK_a , 12.48) and Lys (pK_a , 10.53). Since all the culture media used in this study had nearly neutral pH, during cell growth the histidine residues would only be partially charged. In some of the ferritin light chains (Figure 4A), the region implicated in direct iron binding contains both Arg-Glu-Xaa-Xaa-Glu and Lys-Glu-Xaa-Xaa-Glu [19], suggesting that in nature Arg and Lys might also be functionally interchangeable as an essential component of iron-binding motifs in certain proteins. In the bacterial proteins related to the iron sensor PmrB of *Salmonella enterica* [18] all the three positively charged amino acids are found immediately in front of different Glu-Xaa-Xaa-Glu motifs, and it appears that His is more frequently present. Although it is not known whether all the three amino acids are functionally interchangeable in these proteins, perhaps the requirement for both the level of basicity of the side chain and its structure is less stringent for iron binding in these bacterial proteins. In Ftr1p and CaFtr1p none of the Glu-Xaa-Xaa-Glu motifs at the C-terminal end has an Arg or Lys immediately in front of the first Glu, and Glu → Ala mutation had no effect on iron transport, suggesting that they are unlikely to be iron-binding motifs. The motif at the N-terminal end appears to be reasonably well conserved, being Arg-Glu-Xaa-Xaa-Glu in several fungal iron permeases. However, mutation of this motif did not have any detectable effect on iron uptake either. It could be that a functional motif must be located within a certain structural context, such as within a loop, like the functional motifs in Ftr1p and PmrB [15,18].

Another interesting observation made in this study is the functional substitution of Asp for one of the two glutamates, the 'signature' of the iron-binding motif. Though this mutation reduced the rate of iron uptake by ≈ 50 %, this level of activity was found to be sufficient to support normal cell growth under our experimental conditions. However, we are not aware of any protein that uses Glu-Xaa-Xaa-Asp or Asp-Xaa-Xaa-Glu for iron binding.

Taken together, we propose to extend the iron-binding motif to Arg/Lys-Glu/Asp-Xaa-Xaa-Glu or Arg/Lys-Glu-Xaa-Xaa-Glu/Asp. The first position may also be a His in some proteins. This proposed motif might serve as a better guide for identifying potential iron-binding proteins.

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