Pse1p Mediates the Nuclear Import of the Iron-responsive Transcription Factor Aft1p in *Saccharomyces cerevisiae*  

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In *Saccharomyces cerevisiae*, the iron-responsive transcription factor Aft1p plays a critical role in maintaining iron homeostasis. The activity of Aft1p is induced in response to iron starvation and as a consequence the expression of the iron-regulon is increased. We have shown previously that Aft1p is localized to the cytoplasm under iron-replete conditions but that it is localized to the nucleus under iron-depleted conditions. In this study, we identified the transport receptor that mediates the import of Aft1p into the nucleus, the location of the nuclear localization signal (NLS) sequences of Aft1p, and examined whether the nuclear import of Aft1p is affected by iron status. In *pse1-1* cells, which bear a temperature-sensitive mutation of *PSE1*, Aft1p was misdirected to the cytoplasm during iron starvation at the restrictive temperature. Aft1p could also directly bind to Pse1p and was dissociated from the complex by Ran-GTP in vitro. These results indicate that Aft1p is imported into the nucleus by Pse1p. Supporting this is that the induction of an Aft1p target gene, *FTR1*, in response to iron starvation was greatly reduced in *pse1-1* cells. Furthermore, we demonstrated that the nuclear localization of a mutant Aft1 protein that contains an NLS derived from SV40 was regulated by iron status regardless of whether Pse1p could interact with Aft1p. This suggests that the interaction between Aft1p and Pse1p is not a critical step that controls the iron-regulated nucleocytoplasmic transport of Aft1p.

Regulation of the nucleo-cytoplasmic localization of transcription factors is a strategy by which eukaryotic cells respond to environmental changes. The nuclear translocation of transcription factors occurs commonly in response to extracellular signals and appears to be a key mechanism by which these factors alter the expression of their target genes (1, 2).

The nucleus and the cytoplasm are separated by the nuclear envelope (NE). Molecules are exchanged through the nuclear pore complex (NPC), which is embedded in the NE. Macromolecules, such as proteins with molecular masses exceeding 40–60 kDa, are imported to or exported from the nucleus via sequence-mediated processes. Transport receptors called karyopherins (also called importins or exportins) recognize specific nuclear import and export sequences (called nuclear localization signals (NLS)\(^3\) and nuclear export signals (NES), respectively) within their cargo proteins and mediate their translocation across the NPC. The small GTPase Ran plays an essential role in this transport process since it, along with its effector molecules, establishes the directionality of transport. A large family of transport receptors that contain the conserved region required for binding to Ran-GTP have been identified and shown to operate within the multiple pathways of nucleocytoplasmic transport (3–5). Fourteen transport receptors that are members of the karyopherin β family have been identified in yeast genome databases (6). To date, eight of these have been designated as import receptors, five as export receptors, and one as an import and export receptor. In metazoans, the existence of more than 25 karyopherin β members has been demonstrated (7, 8). These include importin β, the best studied karyopherin β, which often functions in association with an adaptor protein, importin α, to bind cargo proteins (9, 10). Importin α recognizes cargo proteins containing one or two stretches of basic amino acid residues termed the classical NLS (11). The sequences recognized by other receptors identified so far are not similar to the classical NLS (2), and the mechanisms by which these receptors recognize their cargo remain to be elucidated.

Fluctuation in environmental nutrient concentrations elicits signals that modulate gene expression. Iron is an essential nutrient, but it can be toxic in excess. Therefore, cells have acquired mechanisms through which they maintain iron homeostasis (12, 13). The major iron-responsive transcriptional activator Aft1p plays a critical role in iron homeostasis in budding yeast as its activity is induced in response to iron starvation and as a consequence the expression of the iron-regulon is up-regulated (14, 15). Aft2p, a paralog of Aft1p, shares 39% homology with Aft1p (16) and plays overlapping but non-redundant roles with Aft1p in the transcriptional regulation of the iron regulon (17). We have shown previously that Aft1p is localized to the cytoplasm in *Saccharomyces cerevisiae* under iron-replete conditions, but that it is localized to the nucleus under iron-depleted conditions (18).

Here we demonstrate that Aft1p is imported into the nucleus of *S. cerevisiae* by the nonclassical import receptor Pse1p/Kap121p. Consistent with the importance of *PSE1* in the nuclear localization of Aft1p it is the iron-regulon that was poorly expressed in a mutant strain deficient in the Pse1p activity and had been subjected to iron-impoverished conditions. We also identified two NLSs of Aft1p and demonstrate that each is sufficient for its *PSE1*-dependent nuclear import in vivo, which

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\(^3\) The abbreviations used are: NLS, nuclear localization signal; NES, nuclear export signal; GFP, green fluorescent protein.
suggests that these two regions are independently recognized by Pse1p. In addition, we demonstrate that a mutant Aft1p containing a classical NLS responds to iron status independently of Pse1p, which suggests that the recognition of Aft1p by Pse1p is not important for the iron-regulated nuclear localization of Aft1p.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The yeast strains used in this study are listed in Table I. Strains expressing hemagglutinin (HA)-tagged Aft1 (Aft1p-HA) or its derivatives were created by transforming the strain indicated with pRS416-AFT1-HA or its derivatives. Cells were grown routinely in YPD or synthetic dextrose (SD) medium supplemented with amino acids. To create iron-deprived conditions, cells were grown in SD-Fe media consisting of yeast nitrogen base lacking iron, 2% glucose, amino acid supplements, and 50 mM MES buffer (pH 6.1). To create iron-replete conditions, ferrous sulfate and ferrozine were added to final concentrations of 200 and 500 μM, respectively.

Plasmids—pRS416-AFT1-HA has been described previously (18). To create histidine-tagged Aft1 protein (Aft1p-His) synthesis in vitro, pEU-AFT1-His/S6 was constructed by inserting the SmaI-HindIII fragment encoding the region encompassing amino acids 1372 to 1473 of Aft1p and the HindIII-BamHI fragment encoding the histidine tag into the EcoRV and BamHI sites of pEU-NII (Invitrogen). pGEX-PSE1 (20) and pGEX-KAP95p (21) were used to express mutant histidine-tagged Gsp1 proteins. pET-GSP1(ADH) and pET-GSP1(T26N) were used to express mutant histidine-tagged Gsp1 proteins. pET-GSP1(ADH) was constructed by inserting the NdeI-XhoI fragment encoding Gsp1pT26N into NdeI and XhoI sites of pET15b (Novagen). Expression plasmids for the green fluorescent protein (GFP)-fused proteins were constructed as follows: Within pU1372, the ADH promoter was replaced with the MET25 promoter, and the NE5 derived from the protein kinase inhibitor (PKI) was mutated to create pMET25-SV40 NLS-GFP. The DNA fragments encoding various regions of Aft1p with a ClaI site and SmaI site at each end were then inserted into the NdeI-XhoI fragment encoding Aft1p-HA by indirect immunofluorescence microscopy. For indirect immunofluorescence and GFP microscopy—For indirect immunofluorescence microscopy, cells expressing HA-tagged proteins were fixed by permeabilization with 1% formaldehyde, 0.5 mM MgCl2, and 50 mM MES buffer (pH 6.1). To create iron-replete conditions, ferrous sulfate and ferrozine were added to final concentrations of 200 and 500 μM, respectively.

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Indirect Immunofluorescence and GFP Microscopy—For indirect immunofluorescence microscopy, cells expressing HA-tagged proteins were fixed by permeabilization with 1% formaldehyde to a final concentration of 4% for 30 min, followed by the addition of buffered formaldehyde (4% formaldehyde, 50 mM potassium phosphate buffer, pH 6.5, 0.5 mM MgCl2) for 90 min. Cell walls were digested with 300 units of zymolase (Seikagaku Kogyo) in SPM (1.2 mM sorbitol, 50 mM potassium phosphate buffer, pH 6.5, 0.5 mM MgCl2) at 1 h at 30 °C, followed by the addition of 2% SDS for 2 min. Sperms were fixed on polylysine-coated cover glass using phosphate-buffered saline (PBS) containing formaldehyde, permeabilized with 0.05% saponin for 10 min, and then incubated with an anti-HA antibody (Roche Diagnostics). Signals were amplified by Alexa Fluor 594 Signal-Amplification Kit (Molecular Probes) and visualized by fluorescent microscopy.

For GFP microscopy, cells were cultured in medium lacking methionine to induce the expression of the GFP-fused proteins. Shifting the temperature was performed before the proteins were induced. GFP-fused proteins were visualized directly by fluorescent microscopy. The expression of the tagged proteins was measured by Western blot and found to be similar in each of the strains under the different iron conditions employed.

Recombinant Protein Expression and Purification—The expression and purification of recombinant GST-Pse1p and GST-Kap95p were performed as follows: Escherichia coli BL21(DE3) cells transformed with pGEX-FSE1 or pGEX-KAP95p were grown in LB at 37 °C. At an OD600 of 0.5, the incubation temperature was shifted to 20 °C and then isopropyl-β-D-thiogalactoside (IPTG) containing formaldehyde, permeabilized with 0.05% saponin for 10 min, and then incubated with an anti-HA antibody (Roche Diagnostics). Signals were amplified by Alexa Fluor 594 Signal-Amplification Kit (Molecular Probes) and visualized by fluorescent microscopy.

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NMD5, and PDR6, no defect in the nuclear accumulation of Aft1p was observed (data not shown). However, we found that when the strain carrying a temperature-sensitive mutation in PSE1 (pse1-1) was cultured at the restrictive temperature (37 °C), Aft1p-HA does not accumulate in the nucleus during iron starvation (Fig. 1A, panel 7), which suggests that the nuclear localization of Aft1p requires Pse1p. However, the import of proteins with classical NLSs was not perturbed in
Pse1p Directly Binds to Aft1p and Is Dissociated from Aft1p by Ran-GTP—To confirm that Pse1p is the transport receptor for Aft1p, we examined the interaction between Aft1p and Pse1p in vitro. GST-fused Pse1 protein (GST-Pse1p) and histidine-tagged Aft1 protein (Aft1p-His) were synthesized and subjected to a GST pull-down assay. Thus, GST-Pse1p was immobilized on glutathione-Sepharose and incubated with Aft1p-His. GST-fused Rap1p/Kap95p (GST-Kap95p) was used as a negative control. After washing, the bound proteins were eluted and subjected to SDS-PAGE and analyzed by immunoblotting with an anti-His antibody and by protein staining. As shown in Fig. 2A, Aft1p-His was retained by GST-Pse1p but not by GST-Kap95p, which indicates that Pse1p directly interacts with Aft1p.

In general, stable complexes of the import receptors and cargos that are formed in the cytoplasm are dissociated in the nucleus by binding to the GTP-bound form of Ran (3, 4). To further investigate the specificity of the interaction between Pse1p and Aft1p, the disassembly of this interaction by the GTP-bound form of Gsp1p, a yeast homolog of Ran, was examined using histidine-tagged mutant Gsp1 proteins (His-Gsp1pG21V and His-Gsp1pT26N) that had been purified from E. coli. His-Gsp1pG21V harbors a mutation that causes the loss of the GTPase activity of Gsp1p (23), while Gsp1p T26N is known to bind only GDP (24). Thus, GTP-loaded His-Gsp1pG21V (His-Gsp1pG21V-GTP) or GDP-loaded His-Gsp1pT26N (His-Gsp1pT26N-GDP) was added to glutathione-Sepharose bead-immobilized GST-Pse1p to which Aft1p-His was bound. In the presence of His-Gsp1pG21V-GTP, Aft1p-His was dissociated from GST-Pse1p (Fig. 2B, lanes 1 and 2). However, unlike His-Gsp1pG21V-GTP, Aft1p was still associated with Pse1p in the presence of His-Gsp1pT26N-GDP (Fig. 2B, compare lanes 1 and 2 with lanes 3 and 4). Together, these results suggest that Pse1p mediates the nuclear import of Aft1p.

pse1-1 Cells Have a Defect in Aft1p-induced Transcription—As revealed above, Pse1p mediates the nuclear import of Aft1p. Consequently, we hypothesized that the impaired nuclear import of Aft1p may cause the pse1-1 cells to be defective in the induction of the Aft1p regulon, thereby leading to a defect in iron homeostasis. To address this, we analyzed the expression in wild-type and pse1-1 cells at 37 °C of an Aft1p target gene, FTR1, which encodes the iron permease that is required for high-affinity iron uptake in response to iron starvation. The mRNA levels of FTR1 in pse1-1 cells were indeed much lower than in wild-type cells in iron-starved conditions (Fig. 3, lanes 2 and 4), although the induction of FTR1 was not completely lost in pse1-1 cells (Fig. 3, lanes 3 and 4). This implies that pse1-1 cells have a defect in iron homeostasis.
Identify the region in Aft1p required for its nuclear import, we constructed a series of vectors that express various regions of Aft1p fused in-frame with GFP under the control of the MET25 promoter. The localization of each fusion protein under iron-replete (+Fe) or iron-depleted (−Fe) conditions is indicated on the right (N, nuclear; C, cytoplasmic). B, subcellular localization of Aft1p-(198–225)-GFP (panels 1 and 3) and Aft1p-(332–365)-GFP (panels 2 and 4) in wild-type cells (panels 1 and 2) or in pse1-1 cells (panels 3 and 4) under iron-depleted conditions.

Fig. 4. Two independent regions (amino acids 198–225 and 332–365) within Aft1p are sufficient for the nuclear import mediated by Pse1p. A, NLS activity of various regions of Aft1p. The various regions of Aft1p indicated on the left were expressed in wild-type cells as GFP fusion proteins. The localization of each fusion protein under iron-replete (+Fe) or iron-depleted (−Fe) conditions is indicated on the right (N, nuclear; C, cytoplasmic). B, subcellular localization of Aft1p-(198–225)-GFP (panels 1 and 3) and Aft1p-(332–365)-GFP (panels 2 and 4) in wild-type cells (panels 1 and 2) or in pse1-1 cells (panels 3 and 4) under iron-depleted conditions.

Each of these two regions contains a stretch of basic amino acid residues, namely, KPKKKK (residues 202–207) and RKPK (residues 352–355), respectively. This is similar to what is seen in classical NLSs and consequently we wondered if these regions could be recognized by the classical nuclear import receptor complex, even though whole Aft1p is imported into the nucleus by Pse1p. However, when Aft1p-(198–225)-GFP and Aft1p-(332–365)-GFP were expressed in the srp1-31 and rsl1-4 strains, both regions were able to localize to the nucleus at the restrictive temperature (data not shown). In contrast, the mislocalization of these fusion proteins was evident in pse1-1 cells cultured at the restrictive temperature (Fig. 4B, panels 3 and 4). This implies that each region in Aft1p (namely, amino acids 198–225 or 332–365) functions independently as an NLS for Pse1p. Further deletion of each region led to the loss of nuclear localization (data not shown), as has been previously reported for the NLSs in other cargo proteins that are imported by Pse1p (20, 25, 26).

Some of the sequences that have been reported to date as being NLSs recognized by Pse1p are not homologous to each other except in that they contain basic amino acids (20, 25–27). As described above, many basic amino acids are located in the Aft1p NLSs. Thus, we assessed the contribution of these residues to NLS function. The residues within or proximal to the basic amino acid stretch were mutated, and the subcellular localization of the resulting mutant Aft1p-GFPs in wild-type cells under iron-depleted conditions was then determined. Aft1p-(198–225)-GFP that contains six alanine substitutions in amino acids 202–207 (m(198–225)-GFP) was misdirected (Fig. 5, A and B, panel 1). Mutation of amino acids 332–335 (m1(332–365)-GFP) or 352–356 (m2(332–365)-GFP) to alanine residues also partially impaired the NLS function of the 332–365 region, and the double mutation (m3(332–365)-GFP) completely abolished the nuclear localization of Aft1p-(332–365)-GFP (Fig. 5, A and B, panel 2).

In addition, we tested whether the mutations introduced in
these two regions abolish the nuclear localization of Aft1p. Thus, we introduced the same alanine substitutions in m(198–225)-GFP and m3(332–365)-GFP into the full-length Aft1p fused with GFP (Aft1p(mut-nls)-GFP) (Fig. 5C), and observed its subcellular localization. As shown in Fig. 5D (panel 2), GFP-fused Aft1p(mut-nls) no longer accumulates in the nucleus, even under iron-starved conditions. However, when the alanine substitutions in either m(198–225)-GFP or m3(332–365)-GFP were mutated in m(198–225)-GFP and m3(332–365)-GFP in A were mutated to alanine residues, C, schematic depiction of wild-type (Aft1p(1–690)-GFP) and mutant Aft1p containing alanine substitutions in both NLSs (Aft1p(mut-nls)-GFP). In Aft1p(mut-nls)-GFP, the same amino acids residues that were mutated in m(198–225)-GFP and m3(332–365)-GFP in A were mutated to alanine residues. D, alanine substitutions of basic amino acid stretches within Aft1p-NLS eliminate the nuclear accumulation of Aft1p under iron-depleted conditions. Wild-type cells expressing Aft1p-GFP (panel 1) or Aft1p(mut-nls)-GFP (panel 2) were cultured in iron-depleted medium, and the subcellular localization of the GFP-fusion proteins was examined.
Aft1p NLSs are required for NLS function within these proteins are relatively short stretch of basic amino acids, including serine and proline. We identified these NLSs in the cargo proteins can be recognized by Pse1p. It has been reported that Pse1p preferentially binds to unphosphorylated Pho4p (25). As Aft1p has been reported to be a phosphoprotein (30), it would be of interest to determine if the phosphorylation of Aft1p affects its interaction with Pse1p.

The data reported here suggest that the interaction of Aft1p and Pse1p is not regulated by iron status, as we found that the iron concentration did not affect the nuclear localization of the two Aft1p NLS-GFP fusion proteins. This indicates that the binding of Aft1p NLSs to Pse1p occurs with similar affinity in iron-replete and iron-depleted conditions, although we did not extensively compare the rate of import. It has been reported that the nuclear localization of a protein can be regulated by the intra- or intermolecular masking of the NLS that inhibits the interaction between the NLS and its import receptor (31, 32). However, this is unlikely to be a mechanism affecting the recognition of Aft1p by Pse1p since SV40 NLS fused to the C terminus of Aft1p can substitute for the Aft1p NLSs. Nevertheless, these findings do not completely exclude the possibility that the import of Aft1p may play some role in regulating its activity. It may be that this protein is anchored in the cytoplasm, which overrides the presence of any NLS, even though the NLS of the cargo is accessible to the import receptor. Supporting this notion is a recent report that demonstrated that the transcription factor Nrf2, which is essential for the antioxidant responsive element (ARE)-mediated induction of phase II detoxifying and oxidative stress enzymes, is bound in the cytoplasm to Keap1. Keap1 is closely related to the Drosophila actin-binding protein Kelch, which is anchored to the actin cytoskeleton. Upon exposure to electrophiles, the Keap1-Nrf2 complex is disrupted and Nrf2 migrates to the nucleus (33). Thus, it is possible that Aft1p interacts with such an anchoring protein and is located to the cytoplasm in the presence of iron but that when iron is scarce, it is released and results in its translocation into the nucleus. It is also possible that the subcellular localization of Aft1p might be regulated solely at the export step, as is the case for Yap1p (20). The recognition of Yap1 by the export receptor, Crm1pXpo1p is regulated in a redox-sensitive manner (34, 35). Further investigation is required to determine which of these possibilities are true and to fully understand the mechanisms that regulate Aft1p localization in response to iron status.

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