The Monothiol Glutaredoxin Grx4 Exerts an Iron-Dependent Inhibitory Effect on Php4 Function

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When iron is scarce, Schizosaccharomyces pombe cells repress transcription of several genes that encode iron-using proteins. Php4 mediates this transcriptional control by specifically interacting with the CCAAT-binding core complex that is composed of Php2, Php3, and Php5. In contrast, when there is sufficient iron, Php4 is inactivated, thus allowing the transcription of many genes that encode iron-requiring proteins. Analysis by bimolecular fluorescence complementation and two-hybrid assays showed that Php4 and the monothiol glutaredoxin Grx4 physically interact with each other. Deletion mapping analysis revealed that the glutaredoxin (GRX) domain of Grx4 associates with Php4 in an iron-dependent manner. Site-directed mutagenesis identified the Cys172 of Grx4 as being required for this iron-dependent association. Subsequent analysis showed that, although the thioredoxin (TRX) domain of Grx4 interacts strongly and constitutively with the C-terminus of Fep1 (17). Further analysis has shown that the monothiol glutaredoxin Grx4 associates with Php4 through its N terminus. A po-


**Iron Inhibition of Php4 Function**

1. **Materials and Methods**

   **Yeast strains and growth media.** The *S. pombe* strains used in this study were all isogenic derivatives of FY435 (his7-366 leu1-32 ura4-Δ18 ade6-M210) (27) and included gdx4Δ (his7-366 leu1-32 ura4-Δ18 ade6-M210 gdx4Δ::KANMX) and php4Δ gdx4Δ (his7-366 leu1-32 ura4-Δ18 ade6-M210 php4Δ::loxP gdx4Δ::KANMX). *S. pombe* cultures were isolated in yeast extract plus supplements (YES) medium that contained 3% glucose and 225 mg/liter of adenine, histidine, leucine, uracil, and lysine. Strains used for plasmid integration were grown in synthetic Edinburgh minimal medium (EMM) in which specific amino acids were absent as required for plasmid selection and maintenance. *S. pombe* liquid cultures were seeded at an A660 of 0.5 and then grown to exponential phase. Once at log phase (A660 of ~0.9), the cells were treated with either 2.2'-dipiridyl (Dip) (250 μM) or FeCl3 (100 μM) or were left untreated for 90 min, unless otherwise indicated. gdx4Δ and php4Δ gdx4Δ mutant strains and control strains were grown in culture jars under microaerobic conditions using the BD GazPack EZ system (BD Diagnostic System, Sparks, MD). In the case of two-hybrid experiments, *S. cerevisiae* strain L40 (MATa his3Δ200 trp1-901 leu2-3,112 ade2 lys2::lexAop-HIS3 URA3::(lexAop)2-lacZ) (47) was grown in a synthetic minimal medium (pH 6.1) containing 83 mg/liter of histidine, adenine, uracil, and lysine, 2% dextrose, 50 mM MES [2-(N-morpholino)ethanesulfonic acid], and 0.67% yeast nitrogen base lacking both copper and iron (MP Biomedicals, Solon, OH).

   **Plasmids.** Thirteen previously described plasmids, PV16-1pHpa295, PV16-1pHpa254, PV16-1pHpa218, PV16-1pHpa254, PV16-1pHpa254, PV16-1pHpa254, PV16-1pHpa254, PV16-1pHpa254, LexA-GRx4-44, LexA-GRx4-42 (domain TRX), LexA-105Gx4-244 (domain GRX), LexA-GRx4-44 (domain GRX), LexA-GRx4-42 (domain GRX), LexA-GRx4-42 (domain GRX), LexA-GRx4-44 (domain GRX), LexA-GRx4-42 (domain GRX), and LexA-GRx4-44 (domain GRX), were used in this study. The prey plasmids PV16-54pHpa295, PV16-112pHpa295, PV16-152pHpa295, pVP16-188pHpa295, PV16-219pHpa295, and PV16-152pHpa295 (C221A; C272A) were created by cloning different truncated versions of the php4 Δ gene into pVP16 (47). The truncated versions of the php4 Δ gene were generated by PCR using primers which contained BamHI and NotI restriction sites at their ends. After amplification, the purified DNA fragments were digested with these two enzymes and then cloned into the corresponding sites of pVP16 (47) as described previously (27).

   The *S. pombe grx4 Δ* gene was obtained by PCR amplification using primers that contained Sall and Asp718 restriction sites and used genomic DNA from strain FY435 as the template. The purified DNA fragment was digested and then cloned into the *Sall* and *Asp718* sites of the *pJK-194* promphp4 Δ-TAP plasmid (prom stands for promoter), creating *pJK-194* promphp4 Δ−TAP-grx4 Δ. The *pJK-194* promphp4 Δ−GFP-grx4 Δ plasmid (GFP stands for green fluorescent protein) has been described previously (27). The gene encoding GFP was PCR amplified from the *pfGFP1* plasmid (32) and inserted into the BamHI and Sall restriction sites of *pGEM-7ZI* (Promega, Madison, WI). A *Sall-Asp718* grx4 Δ gene fragment was generated by PCR and cloned into the plasmid *pGEM-7ZI* in which the GFP gene had been previously introduced. The resulting *pGEM-7ZI*−GFP-grx4 Δ plasmid was subsequently digested with BamHI and XhoI, and the insert was cloned into the corresponding sites of the previously described pSP1-194*promphp4 Δ* plasmid (27). The resulting construct was named pSP1-194*promphp4 Δ−GFP-grx4 Δ. To create the *pSP1-194* promphp4 Δ−GFP, *Gx4 Δ* plasmid, pSP1-194*promphp4 Δ−GFP-grx4 Δ* was codigested with *Sall* and *Apal*. A copy of the C-terminal region of *Grx4* (the coding sequence corresponding to amino acid residues 105 to 244) was generated by PCR using primers that contained *Sall* and *Apal* sites and then exchanged with the *Sall*-*Apal* DNA fragment in plasmid pSP1-194*promphp4 Δ−GFP-grx4 Δ*.

   A DNA fragment encoding *Gx4-VC* was isolated from *pGEM-grx4 Δ-V* (27) using the BamHI and *Asp718* restriction enzymes. The purified DNA fragment was insert into the integrative vector *pJK-1200grx4 Δ* (17) from which the BamHI-*Asp718* grx4 Δ gene fragment had previously been removed, leaving only the *Sall*-BamHI grx4 Δ promoter segment at the 5′ terminal end of the polylinker region (*pJK-1200promgrx4 Δ*). The resulting plasmid was denoted *pJK-1200promgrx4 Δ* grx4 Δ-VC. To generate *pJK-1200promgrx4 Δ*-VC, *Gx4 Δ* strain, the first BamHI-Sall DNA restriction fragment containing the coding region of VC was produced by PCR amplification using *pF6a-VC*-kanMX6 (45) as the template. A second *Sall*-Asp718 DNA restriction fragment (fragment) display fluorescence signals in the nuclei of iron-deficient cells, whereas BiFC signals accumulate in the cytoplasm of cells exposed to high iron levels (27).

   *S. pombe* Grx4 is a multidomain monothiol glutaredoxin (4). The primary amino acid sequence of Grx4 contains two major regions that are denoted as the thioredoxin (TRX)-like and glutaredoxin (GRX)-like domains, respectively. The N-terminal TRX-like domain of Grx4 contains a WAAPC34H sequence that is reminiscent of the thioredoxin active site motif WCGrPK (4, 11). Mutational analysis of the Cys35 residue located within the TRX domain of Grx4 revealed that it is required for the establishment of a strong, iron-independent association with Php4 (17). Consistently, a recent study of *S. cerevisiae* suggested that the TRX domain may serve as a docking site for the interacting partners of the multidomain monothiol glutaredoxins Grx3 and Grx4 (12). In baker’s yeast, other functions for the TRX domain have been proposed, including a role in the targeting of the monothiol glutaredoxin Grx3 to the nucleus (31), as well as a regulatory role in active cytoskeleton remodeling and a role in cellular defense against oxidative stress (40). However, whether the TRX domain’s participation in the repolarization of the actin cytoskeleton involves any protein–protein interactions remains to be determined.

   Based on biochemical studies with other multidomain glutaredoxins orthologous to *S. pombe* Grx4, it has been shown that the C-terminal GRX-like domain contains a CGFS active site motif (16, 42). Indeed, the combination of two glutaredoxin molecules (containing one CGFS motif each) provides two Cys ligands with which they can hold a 2Fe-2S cluster (1, 16, 38). Within the complex, the addition of two glutathione molecules provides the other two cluster ligands, resulting in a glutathione–ligated [2Fe-2S] bridged homodimers (1, 16, 38).

   Several studies have pointed out important roles for multidomain monothiol glutaredoxins. Of note, these proteins participate in mitochondrial and cytosolic Fe–S protein biogenesis (41), they specifically deliver and transfer Fe–S clusters into proteins and subcellular compartments (32) and they relay cellular iron status to several iron-responsive transcription factors (17, 21, 23, 27, 33, 39, 43).

   Based on the previous finding that Grx4 is a Php4-binding partner that is required for its inactivation under conditions of high levels of iron (27), the mechanism by which Grx4 and Php4 physically interact with each other as a function of iron availability was investigated. Deletion mapping analysis revealed that the TRX domain interacts constitutively with Php4, whereas the GRX domain associates in an iron-dependent manner with a domain corresponding to amino acids 188 to 254 of Php4. Further analyses by BiFC assays revealed that the GRX domain is required for iron-mediated inhibition of Php4 activity, as well as for its nuclear export behavior. Taken together, the results reported here reveal that the presence of the GRX domain of Grx4 is critical to the communication of an excess of iron to the Grx4-Php4 complex.
fragment containing the C-terminal region of Grx4 (amino acid residues 105 to 244) was generated by PCR amplification from the pSP1-194promPhp4promGrx4-GFP-105Grx4 plasmid. The pJK-1200promGrx4VC-105Grx4 plasmid was constructed by a three-piece ligation that simultaneously introduced the BamHI-Sall PCR-amplified fragment containing VC and the Sall-Asp718 PCR-amplified fragment harboring 105Grx4 into the BamHI-Asp718-digested pJK-1200promGrx4 vector.

RNA isolation and analysis. Total RNA was extracted using a hot phenol method as described previously (3) and was quantified spectrophotometrically. In the case of the RNAse protection assays, 15 μg of RNA per reaction were used as described previously (28). Riboprobes derived from the plasmids pSKisa1, pSKphp4, and pSKgrx4 (27) were used to detect the isa1, php4, and grx4 transcripts, respectively. An act1 riboprobe derived from the linearized plasmid pSKact1 (28) was used to detect act1 mRNA as an internal control for normalization during quantification of the RNAse protection products. The riboprobes derived from the plasmids pSlsacZ, pSKSACT1 (25), and pSKVP16 (27) were used to determine the lacZ, ACT1, and VP16 mRNA levels, respectively.

Two-hybrid analysis. Precultures of each L40 cotransformed strain harboring the indicated bait and prey plasmids were grown to an A600 of 0.4 and were then either left untreated or were cultured in the presence of Dip (250 μM) or FeCl3 (100 μM) for 4 h. Aliquots were withdrawn, and the β-galactosidase activity was assayed using o-nitrophenyl-β-D-galactopyranoside as the substrate. β-Galactosidase activity levels were measured within the linear response range and were expressed in standard Miller units (30). The values reported here are the averages of triplicates of three independent cotransformants. In addition to β-galactosidase assays, a riboprobe derived from the plasmid pSlsacZ was used to monitor steady-state levels of the lacZ mRNA derived from the integrated (lexAsys)lacZ reporter construct in the L40 strain. Furthermore, a riboprobe detecting the act1 mRNA levels from S. cerevisiae was used as an internal control. For Western blotting experiments, total cell lysates were prepared as described previously (49). After electrophoresis on 9% sodium dodecyl sulfate-polyacrylamide gels, protein samples were analyzed by immunoblotting. Antibodies used for protein detection were monoclonal antibodies anti-LexA 2-12, which is directed against the LexA DNA-binding domain, and anti-VP16 1-21, which is directed against the VP16 activation domain (Santa Cruz Biotechnology, Santa Cruz, CA). A monoclonal anti-3-phosphoglycerate kinase (anti-PGK) antibody (Molecular Probes, Eugene, OR) was used to detect PGK and served as an internal control.

Bimolecular fluorescence complementation assay. Analysis by BiFC assays was performed as described previously (27). Fluorescence and differential interference contrast images of the cells were obtained using an Eclipse E800 epifluorescence microscope (Nikon, Melville, NY) equipped with an ORCA ER digital cooled camera (Hamamatsu, Bridgewater, NJ). BiFC signals were visualized using a magnification of \( \times 1,000 \) with a transmission window of 465 to 495 nm, whereas the chromosomal material (as marked by Hoechst 33342 staining) was detected with a window of 340 to 380 nm. The cell fields shown in this study represent a minimum of five independent experiments. The merged images were obtained using the Simple PCI software, version 5.3.0.1102 (Compix, Sewickly, PA).

RESULTS

Php4 interacts with both the N- and C-terminal regions of Grx4. Previously it has been shown that full-length Php4 and Grx4 physically interact with each other (27). More specifically, the minimal region of Php4 (residues 152 to 254) necessary for interaction with Grx4 was elucidated (27); however, the amino acid regions of Grx4 that are required for its association with Php4 remain unknown. To gain insight on the regions of Grx4 that interact with Php4, an N-terminal segment (residues 2 to 142) and a C-terminal segment (residues 105 to 244) of Grx4 were initially studied. Notably, the N-terminal 142-residue region of Grx4 includes a thioredoxin (TRX)-like domain, whereas its C-terminal region (amino acids 105 to 244) contains a glutaredoxin (GRX)-like domain. β-Galactosidase assays of LexA-2Grx4 and LexA-105Grx4 coexpressed with VP16-Php4 revealed high activity levels (1,072 ± 70 and 686 ± 118 Miller units, respectively). Although these levels of β-galactosidase activity were lower by 32% and 57% compared to that of the full-length LexA-2Grx4 protein (1,578 ± 119 Miller units), a clear and elevated transactivation of the reporter gene expression was observed, revealing that both N- and C-terminal regions of Grx4 interact with Php4 (Fig. 1A). For negative controls, the VP16 transactivation domain without the Php4 protein or the LexA DNA-binding domain was assayed without the Grx4 protein. In both cases, β-galactosidase activity was absent, resulting in background values typical of pairs of noninteracting proteins (Fig. 1A). Immunoblot analyses of protein extracts using anti-LexA and anti-VP16 antibodies clearly indicated that the fusion proteins were expressed in the cotransformed cells (Fig. 1B). Although we consistently detected LexA polypeptide alone, full-length VP16-Php4 protein, LexA-Grx4 protein, and its truncated derivatives, we were unable to detect the VP16 polypeptide alone. This result may be due to its low predicted molecular mass (~8 kDa). On the basis of these data, we concluded that the TRX- and GRX-containing regions of Grx4 both interact with Php4.

Whereas the association between the TRX domain and Php4 is constitutive, the GRX domain interacts in an iron-dependent manner with Php4. As we have previously shown using a two-hybrid approach, the interaction between the full-length LexA-Grx4 and VP16-Php4 fusion proteins is not modulated by the cellular iron status (27). Indeed, in the present study, the full-length LexA-Grx4 and VP16-Php4 chimeric proteins interacted with each other, producing a constitutive steady-state level of lacZ mRNA as assayed by RNase protection experiments under both low and high iron concentrations (Fig. 2A and B). Similarly, when the GRX domain of Grx4 was deleted (LexA-2Grx4142), leaving only the TRX-containing region, and the resulting construct was then tested for interaction with the full-length VP16-1Php4295 protein, the levels of lacZ mRNA were constitutive and unresponsive to cellular iron status (Fig. 2A and B). Surprisingly, when the TRX domain was removed (LexA-105Grx4444, leaving only the GRX-containing region) and the resulting construct was then tested for association with VP16-1Php4295, the lacZ mRNA was exclusively detected in the presence of iron and not under iron-limiting conditions (Fig. 2A and B). In this study, iron was limited by the addition of the permeant iron chelator 2,2′-dipyridyl (Dip) at a concentration of 250 μM. In contrast, iron-treated cultures were supplemented with 100 μM FeCl3. Based on these data and the fact that we had previously determined that the Php4 domain corresponding to amino acids 152 to 254 constituted the minimal module sufficient for interaction with Grx4 (27), the region on Grx4 that was required for interaction with the Php4 domain corresponding to amino acids 152 to 254 was investigated. Initially, the possibility of interaction between VP16-152Php4 and the N-terminal 2 to 142 residues of Grx4 (including the TRX domain) was examined. This first combination showed an absence of lacZ transcript, irrespective of iron status (Fig. 2A and B). We then tested whether the C-terminal 105 to 244 residues of Grx4 (including the GRX domain) was involved in the interaction with amino acids 152 to 254 of Php4. In these experiments, LexA-105Grx4444 clearly activated lacZ mRNA expression under iron-replete conditions, but not under iron-limit-
ing conditions. These results revealed an interaction between the GRX domain of Grx4 and the amino acid region consisting of residues 152 to 254 of Php4 that was iron dependent. We consistently found that the interaction between the full-length LexA-Grx4 and VP16-152Php4254 occurred only in response to excess iron and not under conditions of iron deficiency (Fig. 2A and B). These results suggested that the iron-dependent interaction between these two proteins took place through the GRX domain of Grx4. With the exception of the VP16 polypeptide alone and the short VP16-152Php4254 protein, all of the fusion proteins tested for two-hybrid interactions were expressed as confirmed by immunoblot analyses (Fig. 2C). Given this situation, the mRNA levels of VP16 alone, VP16-152Php4254, and VP16-152Php4254 were assayed by RNase protection experiments. The results showed that constructs containing VP16 alone or fused with full-length or truncated php4, were all expressed, with transcripts being detected in the case of each prey construct (Fig. 2D). We therefore concluded, on the basis of this data that iron fosters the interaction between the GRX domain of Grx4 and the C-terminal 152 to 254 amino acid residues of Php4, whereas the TRX domain establishes a constitutive and iron-independent association with Php4.

Cys35 of Grx4 is required for interaction between Php4 and the TRX domain, whereas Cys172 is necessary for the iron-dependent association between the GRX domain and the C-terminal region of Php4. To further investigate the interaction between both the TRX and GRX domains of Grx4 with Php4, two-hybrid experiments using LexA-grx4 fusion alleles that contained point mutations in two highly conserved cysteine residues were performed. The N-terminal TRX domain of Grx4 contains a WAAP C35K sequence that is reminiscent of the thioredoxin active site motif WCGPCK (11). The C-terminal GRX-like domain of Grx4 contains the highly conserved residues C172GFS that are required for monothiol glutaredoxin cellular functions (41). Initially, the LexA-Grx4(C35A) mutant construct was coexpressed with either VP16-1Php4295 or VP16-152Php4254. Under low-iron conditions, no significant levels of lacZ mRNA were detected, indicating that the interactions between LexA-Grx4(C35A) and VP16-1Php4295 or LexA-Grx4(C35A) and VP16-152Php4254 were either not significant or were absent (Fig. 3A and B). In contrast, when these two cotransformants were incubated in the presence of iron, lacZ mRNA was readily detected, revealing the presence of iron-dependent interactions between LexA-Grx4(C35A) and VP16-1Php4295 or LexA-Grx4(C35A) and VP16-152Php4254 (Fig. 3A and B).
Two domains of Grx4 are involved in the association with Php4, but only the GRX domain interacts in an iron-dependent manner. (A) Schematic illustrations of chimeric VP16-Php4 and LexA-Grx4 molecules that were used as prey and bait, respectively. The N-terminal 142 amino acid residues of Grx4 encompass its TRX domain, whereas residues 105 to 244 of Grx4 contain the GRX domain. The amino acid sequence numbers refer to the positions relative to the first amino acid of each protein. (B) Each set of constructs was coexpressed in the *S. cerevisiae* strain L40 grown to an *A*₆₀₀ of 0.4 and then treated with Dip (250 μM) or FeCl₃ (100 μM) for 3 h. After total RNA extraction, the *lacZ* and *ACT1* steady-state mRNA levels were analyzed by RNase protection assays. Results shown are representative of three independent experiments. (C) Cell lysates from aliquots of the cultures containing the constructs shown in panel B were analyzed by immunoblotting using anti-VP16, anti-LexA, or anti-PGK (as an internal control) antibodies. The positions of the molecular mass standards are indicated on the left. (D) Aliquots of the cultures containing the constructs in panel B were also examined by RNase protection assays for the steady-state levels of the VP16 transcript. Actin (*ACT1*) mRNA levels were probed as an internal control.
When the GRX domain was mutated [LexA-Grx4(C172A)] and then tested for interaction with the full-length VP16-1Php4 fusion protein, high levels of lacZ mRNA were detected under both iron-limiting and iron-replete conditions (Fig. 3A and B). In contrast, two-hybrid assays of LexA-Grx4(C35A) coexpressed with VP16-152Php4 showed no lacZ mRNA under either iron-limiting or iron-replete conditions (Fig. 3A and B). On the basis of
these data, we concluded that Cys35 of Grx4 was necessary for interaction between Php4 and the TRX domain of Grx4, whereas Cys172 of Grx4 was required for Grx4 interaction with the C-terminal 152 to 254 residues of Php4. Furthermore, the results showed that interaction was modulated by iron in the absence of the Cys35 of Grx4. To further investigate the requirement for Cys172, a truncated version of the N-terminal end of Grx4 containing only residues 105 to 244 of the GRX domain was constructed. When the Cys172 → Ala mutation [LexA-105Grx4C172A(C172A)] was tested for interaction with either VP16-1Php4295 or VP16-152Php4254 by two-hybrid analysis, no LacZ mRNA was detected by RNase protection assays (Fig. 3A and B). Given these results and those obtained above (Fig. 2), we concluded that the Cys172 residue located within the GRX domain of Grx4 is absolutely required because its exchange abrogates the interaction between the LexA-Grx4 and VP16-1Php4295 or LexA-152Php4254 and VP16-152Php4254 in response to iron. Western blot analyses of protein extracts using anti-LexA and anti-VP16 antibodies showed that the fusion proteins were expressed in the cotransfected cells, independently of the iron levels (Figs. 3C and D). Since we were unable to detect the VP16-152Php4254 fusion protein (presumably due to its low molecular weight), we ascertained the mRNA levels of the VP16-php4 fusion alleles by RNase protection assays (Fig. 3E). Results showed that VP16-1Php4295 and VP16-152Php4254 fusion alleles were clearly expressed, with transcripts detected in the case of each prey construct (Fig. 3E).

Minimal C-terminal region of Php4 required for interaction with the GRX domain of Grx4. The full-length Grx4 protein has previously been shown to interact with the C-terminal 152 to 254 residues of Php4 (27). To gain additional insight into the Php4 domain that is responsible for interaction with the GRX domain of Grx4, seven chimeric proteins were generated using different segments of the Php4 protein. These segments comprised amino acid residues 1 to 295 (VP16-1Php4295), 1 to 254 (VP16-1Php4254), 1 to 218 (VP16-1Php4218), 152 to 254 (VP16-152Php4254), 188 to 254 (VP16-188Php4254), 219 to 254 (VP16-219Php4254), and 152 to 254 (VP16-152Php4254(C221A; C227A)), in which both Cys221 and Cys227 were mutated to alanine residues. To gain additional insight into the Php4 regions that are required for optimal interaction between Php4 and the GRX domain of Grx4, these experiments were in keeping with the fact of the importance of the Php4-dependent regulation of iron deficiency, isa1 gene expression was repressed (74%). Further deletion to amino acid 219 drastically abolished almost all of the β-galactosidase activity (by 97%) compared to the level observed with the full-length VP16-1Php4295. Further deletion to amino acid 219 drastically abolished almost all of the β-galactosidase activity (by 97%) compared to the level observed with the full-length VP16-1Php4295 (Fig. 5A). The LexA protein alone and LexA-2Grx4 fusion proteins were detected by immunoblotting using an anti-LexA antibody (Fig. 5B). Although the long VP16-Php4 fusions (VP16-1Php4295, VP16-152Php4254, and VP16-24Php4254) were consistently detected by immunoblotting (Fig. 5B), the short fusion products (VP16-152Php4254, VP16-188Php4254, VP16-152Php4254, and VP16-219Php4254) were not consistently detected by immunoblotting (Fig. 5B). To ensure that these fusion proteins were expressed in the cotransfected cells, RNase protection analyses were performed so as to verify the expression levels of these VP16-Php4 constructs. Results showed that the transcripts were detected in each case (Fig. 5C). Taken together, the two-hybrid interaction assays revealed that the last 41 amino acid residues of the C terminus of Php4 and the first 218 N-terminal amino acids of Php4 constitute two important regions that are required for optimal interaction between Php4 and the TRX domain of Grx4.

The GRX domain is sufficient for the iron-mediated inhibition of Php4 function. Given the fact that two-hybrid assays showed that the GRX domain strongly associates with Php4 in an iron-dependent manner, we further investigated the effect of this domain on Php4 function. These experiments were in keeping with the fact of the importance of the Php4-dependent regulation of two target genes, isa1 and sdh4. The isa1 gene encodes an iron-using protein implicated in the iron-sulfur cluster assembly paths (48), whereas sdh4 encodes an iron-dependent mitochondrial membrane anchor subunit of the succinate dehydrogenase (28). Integrative plasmids harboring the grx4, TAP-grx4, and GFP-grx4 alleles were expressed under the control of the grx4+ promoter. Similarly, the GFP-GRX and GFP-GRX C172A alleles, which expressed only the GRX domain (wild type and mutated, respectively), were integrated into a grx4 Δ mutant strain and were also expressed under the grx4+ promoter. To assess the effects of the expression of the different Grx4 fusion proteins on Php4 function, we carried out RNase protection experiments examining the relative transcriptional profiles of two Php4-regulated target genes, isa1 and sdh4+ (Fig. 6). When grx4 Δ cells expressing the GFP-GRX (GRX domain alone) allele were grown under conditions of iron deficiency, isa1+ and sdh4+ gene expression was repressed (~5.5- and ~6.2-fold, respectively) compared to the transcript levels detected from either control (untreated) or iron-treated cells. As shown in Fig. 6, grx4 Δ cells in which wild-type grx4+ or functional TAP-grx4+ and GFP-grx4+ alleles were reintegrated regained their capacity to downregulate isa1+ or sdh4+.
gene expression in response to iron starvation. In the case of these transformed grx4Δ cells, isa1+ and sdh4+ transcript levels were readily detected under both standard (untreated) and iron-replete conditions. In fact, isa1+ and sdh4+ transcript levels were more abundant (∼9- to 16-fold) than those of mRNAs observed under iron-limiting conditions. Under iron-replete conditions, grx4Δ cells expressing GFP-GRX exhibited a less pronounced derepression of the isa1+ and sdh4+ transcript levels than grx4Δ cells expressing the grx4C, TAP-grx4 or GFP-grx4+ alleles (Fig. 6 and data not shown). This result may be due to the fact that the GRX domain alone (without the TRX domain) is less competent in inhibiting Php4 in response to iron. When an integrative plasmid expressing the GFP-GRX C172A allele was transformed in grx4Δ cells, no sdh4+ transcript was observed, revealing that Cys172 was required for the GRX domain-mediated inhibition of Php4 function in response to iron (Fig. 6C and D). Taken together, the results revealed that the GRX domain of Grx4 appears to be sufficient to inactivate a large proportion of the Php4 proteins in response to iron.

The GRX domain and Php4 associate with each other in the presence of iron. The ability of the GRX domain to interact with Php4 in an S. cerevisiae two-hybrid system led us to investigate whether such interaction could be detected in S. pombe. To address this point, we used a BiFC approach in fission yeast in which case Venus amino-terminal fragment (VN) and Venus carboxyl-terminal fragment (VC) were fused to the N-terminal portions of...
Php4 and the GRX domain, respectively. Integrative plasmids expressing the tagged (VN-php4\(^{+}\) and VC-GRX) coding sequences were cotransformed into grx4\(^{Δ}\) php4\(^{Δ}\) mutant cells which were grown to mid-logarithmic phase. Protein-protein interactions were detected by \(β\)-galactosidase assays, and the results are indicated in Miller units. The values are means plus standard deviations (error bars) of triplicate experiments. Amino acids are numbered relative to the first amino acid of either the Grx4 or Php4 protein. (B) Total cell extract preparations from aliquots of the cultures used in the assays described above for panel A were analyzed by immunoblotting with anti-VP16, anti-LexA, or anti-PGK antibodies. (C) Aliquots of the cultures used in the assays described above for panel A were also examined by RNase protection assays for steady-state VP16 and ACT1 mRNA levels.

**FIG 5** The TRX domain of Grx4 interacts with two distinct regions of Php4. (A) The TRX domain of Grx4 was coexpressed with a series of VP16-Php4 truncations in the *S. cerevisiae* L40 strain. Protein-protein interactions were detected by \(β\)-galactosidase assays, and the results are indicated in Miller units. The values are means plus standard deviations (error bars) of triplicate experiments. Amino acids are numbered relative to the first amino acid of either the Grx4 or Php4 protein. (B) Total cell extract preparations from aliquots of the cultures used in the assays described above for panel A were analyzed by immunoblotting with anti-VP16, anti-LexA, or anti-PGK antibodies. (C) Aliquots of the cultures used in the assays described above for panel A were also examined by RNase protection assays for steady-state VP16 and ACT1 mRNA levels.

The interaction between the GRX domain and Php4 occurred in an iron-dependent manner when assayed by BiFC in *S. pombe* and was dependent on the presence of the Cys172 residue. As previously reported (27), the VN-tagged Php4 and VC-tagged Grx4 proteins used in this study (as positive controls) produced a BiFC signal in the nucleus under low-iron conditions, whereas the BiFC signal was mainly detected in the cytoplasm of the cells under iron-replete conditions (Fig. 7A). To ensure that the VN- and VC-tagged versions of the Php4 and Grx4 protein derivatives were expressed, total RNA was prepared from the cultures analyzed by BiFC under both iron starvation and iron-replete conditions and was analyzed by RNase protection assays (Fig. 7B and C). Although the transcripts for each construct were not found at the same steady-state levels, the php4\(^{+}\) and grx4\(^{+}\) mRNAs were clearly detected under both iron starvation and excess iron. Together, these results indicated that both the GRX domain and iron are required for relocalization of the Grx4-Php4 complex from the nucleus to the cytoplasm.
FIG 6 The GRX domain inhibits Php4 activity in response to iron. (A) Cells harboring a grx4Δ deletion were transformed with an empty integrative vector or with the grx4Δ, TAP-grx4Δ, GFP-grx4Δ, or GFP-GRX allele. For the GFP-GRX allele, results obtained with two independent transformants are shown. Mid-logarithmic-phase cultures were left untreated (−) or were treated with Dip (250 μM) or FeCl3 (Fe) (100 μM) for 90 min. Total RNA was prepared from each sample and analyzed by RNase protection assays. The steady-state levels of the isa1+ and act1+ mRNAs are indicated by the black arrowheads. (B) Graphic representation of the quantification of the results of three independent RNase protection assays, including the experiment shown in panel A. The histogram values represent the averages plus standard deviations (error bars) of triplicate determinations. (C) Strain AMY34 (grx4Δ) was transformed with an empty integrative vector or with the grx4Δ, GFP-GRX, or GFP-GRX C172A allele. Mid-logarithmic-phase cultures of isogenic strains FY435 (WT) and AMY34 (grx4Δ) were left untreated (−) or were treated with Dip (250 μM) or FeCl3 (Fe) (100 μM) for 90 min. Total RNA was prepared from each sample and analyzed by RNase protection assays. The steady-state levels of the sdh4+ and act1+ mRNAs are indicated by the black arrowheads. (D) The histogram values represent the averages plus standard deviations of triplicate determinations.
**DISCUSSION**

In *S. pombe*, the *php4* gene encodes a subunit of the CCAAT-binding protein complex which includes three other subunits that are denoted Php2, Php3, and Php5 (26). The genes encoding Php2, Php3, and Php5 are constitutively expressed, whereas transcripts of *php4* are induced under conditions of iron starvation and repressed under iron-replete conditions (28). Under low-iron conditions, Php4 acts as a negative regulatory subunit of the CCAAT-binding factor and fosters the repression of several genes encoding iron-using proteins. It has previously been shown that the deletion of the *grx4* gene makes Php4 constitutively active, suggesting that Grx4 plays a critical role in inhibiting Php4 function (27). Further analysis by BiFC assays showed that Grx4 is a binding partner of Php4 (27). On the basis of these findings, we sought to determine the mechanism by which Grx4 and Php4 interact with each other as a function of iron availability. The results presented here indicate that both the TRX and GRX domains of Grx4 interact with Php4. Although the TRX domain interacted strongly and constitutively with Php4, the GRX domain associated in an iron-dependent manner with Php4. These results were different than those reported in the case of the *S. cerevisiae* monothiol glutaredoxins Grx3 and Grx4 with respect to their associations with Aft1 (39). In this case, two-hybrid experiments showed that both the GRX and TRX domains of Grx3 and Grx4

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**FIG 7** Php4 and the GRX domain interact in an iron-dependent manner in living *S. pombe* cells. (A) A mutant strain disrupted in the *grx4* and *php4* genes (*grx4Δ php4Δ*) was transformed with a vector alone (VN alone) and with *grx4*-VC or VN-php4 and *grx4*-VC or VN-php4 and VC-GRX or VN-php4 and VC-GRX C172A. Cells expressing the indicated alleles were grown to mid-logarithmic phase and then treated with 250 μM Dip or 100 μM FeCl₃ (Fe) for 2 h. After treatment, the cells were visualized by fluorescence microscopy using BiFC and Hoechst stain. The merged images are shown in the last row of color panels. Nomarski optics was used to monitor cell morphology. For simplicity, the images of cells cotransformed with VN alone and *grx4*-VC or VN-php4 and VC-GRX C172A were taken from iron-replete cells because the fluorescent images from the iron-deficient cells were identical. Aliquots of the cultures described above for panel A were analyzed by RNase protection assays. Steady-state mRNA levels of *php4* and *act1* were probed in panel B, while those of *grx4* and *act1* were analyzed in panel C. Each RNase protection experiment in panels B and C was assayed three independent times.

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interacted with Aft1, exhibiting similar levels of β-galactosidase activity, with no specification with respect to the interactions (between these polypeptides) as a function of iron availability. Although the nature of the differences between these respective observations is unclear, it is possible that the composition and length of the GRX and TRX domains may be contributing factors that would explain the differences between the results reported here and those published by others (39). Alternatively, the differences between the results of the two-hybrid studies may be due to the fact that Php4 (S. pombe) and Aft1 (S. cerevisiae) do not share significant amino acid sequence identity with each other (only 4.3%). It is possible that these two proteins use distinct mechanisms or partners in their interactions with monothiol glutaredoxins. Recently, it has been determined that the last 16 amino acid residues of S. cerevisiae Grx4 serve as a specific binding region for the transcription factor Aft1 (12). When this region was substituted for the last 14 amino acid residues of S. pombe Grx4 (which represents the C-terminal end of the protein), the interaction between the chimeric Grx4 and Aft1 was lost (12). This lack of interaction was consistent with the fact that there is only limited identity (~31%) between the C-terminal ends of the Grx4 proteins from S. pombe and S. cerevisiae.

In S. cerevisiae, the monothiol glutaredoxins Grx3 and Grx4 can transport Fe/S clusters to diverse locations and then subsequently sort them to different enzymes (32). It has been demonstrated that both the TRX and GRX domains of Grx3 and Grx4 are required for this function (12). Although a similar function for S. pombe Grx4 has not yet been ascertained, this protein may also participate in intracellular Fe/S cluster delivery. A second role for S. cerevisiae Grx3 and Grx4 is to communicate the presence of iron to Aft1. This condition leads to Aft1 inactivation and its subsequent export from the nucleus to the cytoplasm (5, 21, 37, 46). Deletion mapping analysis has revealed that neither the TRX domain alone nor the GRX domain alone can mediate the iron-dependent inhibition of Aft1 (12). S. cerevisiae Grx4 must contain the two domains, TRX and GRX, to inactivate Aft1 function. The results presented here are different from those reported for S. cerevisiae Grx4. In the case of S. pombe Grx4, the expression of the GRX domain alone was sufficient to mediate a significant iron inhibition of Php4 function. This observation was supported by two experimental results. First, grx4Δ cells in which either a full-length grx4+ allele or the GRX domain coding sequence was reintegrated regained the capacity to upregulate isa1+ gene expression in response to iron (Fig. 6). In contrast, the deletion of the grx4+ gene resulted in constitutively active Php4, thereby maintaining the target gene isa1+ in a constant state of repression (Fig. 6) (27). Second, in cells harboring a php4Δ grx4Δ double deletion, coexpression of VN-Php4 and VC-GRX domain produced BiFC signals in the cytoplasm of iron-replete cells, indicating that the GRX domain can associate with Php4 and that it contributes to its inactivation via its relocalization from the nucleus to the cytoplasm. Therefore, based on results reported here, it would appear that S. pombe Grx4 functions as an iron sensor for Php4. Furthermore, the presence of the GRX domain is sufficient for iron inhibition of Php4 function. Interestingly, the results also showed that the GRX domain was sufficient to allow recognition of the Php4-Grx4 complex by the exportin Crm1 (27) since the Php4-GRX domain was exported in response to iron.

Yeast two-hybrid analysis showed that the TRX domain of Grx4 binds Php4 in a constitutive manner. Two Php4 regions were involved in the Php4-TRX domain association. The first region was relatively short and encompassed the last 40 C-terminal residues of Php4 (residues 253 to 295), while the second region was larger and involved amino acid residues 55 to 218 of Php4. These data strongly suggest that the TRX domain functions as a docking domain for the association between Grx4 and Php4. Although the precise amino acid residues responsible for the interaction between the TRX domain and Php4 must await finer mapping analysis, the Cys59 residue located within the TRX domain was found to be required for this interaction.

Previously it was shown that Grx4 is required for inhibition of Php4 function in response to iron. The question thus arises concerning the mechanism of this inhibition. As opposed to the TRX domain, the GRX domain of Grx4 interacted in an iron-dependent manner with Php4. Under high-iron conditions, the GRX domain associated with Php4 through a minimal domain encompassing amino acid residues 188 to 254 of Php4. This minimal C-terminal region of Php4 contains two Cys residues located at positions 221 and 227. Interestingly, these two Cys residues are highly conserved in other Php4-like proteins, including HapX from Aspergillus nidulans, Hap3 from Candida albicans, HapX from Aspergillus fumigatus, and HapX from Cryptococcus neofo-
mains (13, 14, 19, 44). Substitution of these two Cys residues (Cys221 and Cys227) by alanine residues abolished the association between the LexA10Grx4244 and VP16153Php4254 fusion proteins (Fig. 4). Similarly, substitution of Cys172 to alanine in the GRX domain of Grx4 disrupted the interaction between this domain and Php4. One model that could explain these observations would be that the GRX domain forms a [2Fe-2S] cluster with Php4. This putative [2Fe-2S] cluster is located in the CGFS motif of Grx4, Cys221 and Cys227 of Php4, and the cysteine residue of one molecule of glutathione (GSH). At this time, however, the model remains speculative, and the mechanism by which Grx4 communicates the presence of iron to Php4 needs further investigation. Experiments are under way to investigate this mechanism. It is important to mention that, in support of the model, a number of studies have revealed that CGFS-type monothiol glutaredoxins can act as scaffolds for the delivery of [2Fe-2S] clusters to acceptor proteins (1, 16, 38). Under conditions of excess iron, Grx4 would acquire an [2Fe-2S] cluster that would in turn trigger the interaction between the GRX domain and the C-terminal region (residues 188 to 254) of Php4. This interaction between the GRX domain and Php4 would induce an inhibitory conformational change that would disrupt the Php4/Php2/Php3/Php5 heteromeric complex, leading to Php4 release and inactivation (Fig. 8). The Grx4-mediated inactivation of Php4 would lead to its recruitment by the exportin Crm1 and then to its subsequent export from the nucleus to the cytoplasm (27). Conversely, under conditions of iron starvation, the GRX domain would dissociate from the C-terminal portion (residues 188 to 254) of Php4, resulting in the ability of Php4 to bind the Php2/Php3/Php5 heterotrimeric complex and thereby repressing the transcription of its target genes (Fig. 8). Because the TRX domain of Grx4 is constitutively associated with Php4, other cellular components may be required to communicate the cellular iron levels to the GRX domain of Grx4. These components may participate in signaling and/or delivery of a putative [2Fe-2S] cluster that would be inserted into the GRX domain of Grx4 to inactivate Php4. Further studies are needed to decipher the detailed mechanism by which Php4 is inactivated by the monothiol glutaredoxin Grx4.

ACKNOWLEDGMENTS

We are grateful to Gilles Dupuis and William Home for critically reading the manuscript and for their valuable comments. We are indebted to Won-Ki Huh (Institute of Microbiology, Seoul National University) for the gift of pFAsa-VN173-KANMX6 and pFAsa-VC155-KANMX6 plasmids. P.V. and M.J. were recipients of studentships from the Faculté de Médecine et des Sciences de la Santé of the Université de Sherbrooke and the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT), respectively. This study was supported by Natural Sciences and Engineering Research Council of Canada grants MOP-238238-2010-15 and MOP-396029-2010-DAS to S.L.

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