Ssk1p Response Regulator Binding Surface on Histidine-Containing Phosphotransfer Protein Ypd1p

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Ypd1p, a histidine-containing phosphotransfer protein, plays an important role in a branched His-Asp phosphorelay signal transduction pathway that regulates cellular responses to hyperosmotic stress in Saccharomyces cerevisiae. Ypd1p is required for phosphoryl group transfer from the membrane-bound Sln1p sensor histidine kinase to two downstream response regulator proteins, Ssk1p and Skn7p. To investigate the molecular basis for interaction of Ypd1p with these response regulator domains, we used an approach that coupled alanine-scanning mutagenesis of surface-exposed residues in Ypd1p with a yeast two-hybrid interaction screen. Mutated residues that adversely affected the interaction of Ypd1p with the C-terminal response regulator domain of Ssk1p were identified and found to cluster on or near the αA helix in Ypd1p. Our results, supported by analysis of a modeled complex, identify a binding site on Ypd1p for response regulators that is composed of a cluster of conserved hydrophobic residues surrounded by less conserved polar residues. We propose that molecular interactions involving Ypd1p are mediated primarily through hydrophobic contacts, whereas binding specificity and strength of interaction may be influenced by select polar side chain interactions.

In Saccharomyces cerevisiae, a multistep His-Asp phosphorelay system is involved in cellular responses to osmotic stress. The Sln1p, Ypd1p, and Ssk1p proteins constitute an expanded “two-component” regulatory system and serve to control a downstream Hog1p-dependent mitogen-activated protein (MAP) kinase cascade (25, 32, 36, 38, 42). The omsoregulatory effect of this signaling pathway is to increase intracellular glycerol levels in order to counteract hyperosmotic shock (3, 4, 6, 26).

The plasma membrane-bound osmosensor Sln1p is related to the large family of bacterial two-component histidine kinases but is considered a hybrid protein because it has an additional response regulator domain at the C terminus. Under normal osmotic conditions, Sln1p is believed to be in a kinase-active state and catalyzes ATP-dependent autophosphorylation of a conserved histidine residue within its cytoplasmic histidine kinase domain (25, 42). The phosphoryl group is subsequently transferred to a specific aspartic acid residue within the C-terminal response regulator domain (Sln1p-R1). Ypd1p, also known as a histidine-containing phosphotransfer (HPt) protein, is then required for phosphoryl group transfer from Sln1p-R1 to the C-terminal response regulator domain of Ssk1p (Ssk1p-R2) (16, 22, 36). Phosphorylation of Ssk1p prevents its interaction with Ssk2p/Ssk22p (MAP kinase kinase kinases), and the MAP kinase cascade remains inactive. However, when cells are under hyperosmotic stress, Ssk1p is dephosphorylated through a mechanism that is currently not well understood, which allows it to interact with and activate the Hog1p MAP kinase cascade (35).

Ypd1p can also shuttle phosphoryl groups to the C-terminal response regulator domain of Skn7p (Skn7p-R3) (14, 22).

Skn7p has been implicated in a number of cellular processes, including transcriptional regulation of cell cycle genes (5, 30), cell wall synthesis (2, 7, 8), calcineurin signaling via the Crz1p transcription factor (48), and osmotic and oxidative stress responses (20–22, 29, 37). However, phosphorylation of Skn7p from Sln1p-Ypd1p apparently is not required for oxidative stress responses or for calcineurin signaling (21–23, 29, 48).

Previous studies have shown that a Ypd1p/Ssk1p-R2 complex can be physically detected both in vivo (36) and in vitro (14). The phosphorylated lifetime of Ssk1p-R2 is dramatically stabilized by this interaction, an effect that appears to be specific for Ssk1p because it was not observed with the Sln1p-R1 or Skn7p-R3 response regulator domains (14). Indeed, binding specificity has been shown to reside with the HPt domain in other multistep His-Asp phosphorelay pathways (34), as well as the analogous H-containing dimerization/phosphotransfer domains of sensor histidine kinases (27, 49).

The crystal structure of Ypd1p revealed an elongated single-domain protein composed of a four-helix bundle core elaborated upon with two additional α-helices and a short 3_10-helix (39, 50). We previously postulated that the region of the molecule composed of the αA, aC, and αD helices would constitute a docking surface for response regulator proteins (14, 50). In the present study, we address this hypothesis by identifying critical residues on the surface of Ypd1p that are important for Ypd1p/Ssk1p-R2 interactions.

MATERIALS AND METHODS

Materials. Custom-synthesized oligonucleotide primers and DNA modification enzymes were obtained from Invitrogen. Pfu and Pfu Turbo polymerases were purchased from Stratagene. Antibodies against the Ssk1p-R2 domain and Ypd1p were raised in rabbits (Cocalico Biologicals, Inc.). Materials for the yeast two-hybrid analysis (ProQuest kit) were obtained from Gibco-BRL/Invitrogen. Chlorophenol red β-n-galactopyranoside (CPRG) was obtained from Boehringer Mannheim. Media used for bacterial and yeast cultures were from Difco. All chemicals used were of ultrapure grade.

Construction of vectors. For detection of protein-protein interactions between Ypd1p and the response regulator domain of Ssk1p, two plasmids were con-
structed for use in the ProQuest yeast two-hybrid system. Two synthetic oligonucleotides were used in the amplification of the YPD1 gene fragment, a 5' primer to which a SalI restriction site was added and a 3' primer to which a NotI restriction site was added. The PCR mix (100 μl) contained both primers (50 μM each) and YPD1 cDNA template (100 ng), deoxynucleoside triphosphates (200 μM each), and Pfu DNA polymerase (2.5 U). The gene fragment for full-length Ypd1p (167 amino acids) was amplified by PCR, digested with SalI and NotI restriction enzymes, and cloned into the pDBLeu vector. The resulting vector, pDBLeu-YPD1, expresses a Gal4p DNA binding domain (DBD)-Ypd1p fusion protein in S. cerevisiae. The gene fragment encoding the response regulator domain of Ssk1p (amino acids 495 to 712) was prepared similarly but was cloned into pCr16. The resultant vector, pCr6-Ssk1p, expresses a Gal4 activation domain (AD)-Ssk1p-R2 fusion protein in S. cerevisiae.

Alanine-scanning mutagenesis. Selected amino acid residues on the surface of Ypd1p were mutated with the QuikChange method (Stratagene) (33). Two oligonucleotide primers carrying the selected mutation were included with the pDBLeu-YPD1 plasmid template in a PCR. After 16 rounds of amplification with Pfu Turbo, the reaction mixture was digested with DpnI to destroy the wild-type template. The reaction mixture was subsequently transformed into Escherichia coli DH5α competent cells. Plasmid DNA was isolated from selected transformants, and mutations were confirmed by DNA sequencing.

Yeast two-hybrid assay. Plasmids encoding the AD-Ssk1p-R2 and DBD-Ypd1p fusion proteins were cotransformed into the host strain, MaV203, with the polyethylene glycol-lithium acetate method (1). Transformation mixtures were plated onto synthetic complete (SC) medium lacking leucine and tryptophan and grown at 30°C for 48 h. Three individual colonies of each mutation were selected and assayed for β-galactosidase activity. Each colony was used to inoculate 2.5 ml of SC medium lacking leucine and tryptophan and grown at 30°C overnight. One milliliter of each culture was then used to inoculate 5 ml of YPAD medium (1% yeast extract, 2% peptone, 0.01% adenine sulfate, 2% dextrose, pH 6.0) and then grown at 30°C until an optical density at 600 nm (OD600) of 1.0 was reached.

Cells were harvested from 1.5-ml aliquots and washed twice with 1.0 ml of assay buffer (100 mM HEPES, 150 mM NaCl, 5 mM l-aspartate, 1% bovine serum albumin, 0.05% Tween 20, pH 7.3). Cell pellets were resuspended in 100 μl of assay buffer and lysed by repeated rounds of immersion in liquid nitrogen and incubation in a 37°C water bath. Assay buffer (900 μl) containing 2 mM CMPS was added to the cell lysate. The reaction mixture was incubated at room temperature overnight. The reaction was then quenched by addition of 250 μl of 6 mM ZnCl2, and the total reaction time was recorded. Cellular debris was removed by centrifugation at 13,400 × g. The OD574 of the supernatant was determined. The OD574 of the supernatant was recorded. β-Galactosidase units were calculated with the equation β-galactosidase units = 1,000 × OD574 × V × OD600, where V is the time (in minutes) and V is the volume and optical density (at 600 nm) of the initial aliquot. β-Galactosidase activity for each of the mutants was compared to that of wild-type Ypd1p. For each mutant, three independent cultures were assayed in triplicate, and the standard deviation from the mean was determined.

Immunoblot analysis. Yeast cell concentrations were normalized to the optical density at 600 nm of the culture, and appropriate aliquots were taken. Cells were lysed as described above, and 4× sample buffer containing 0.25 M Tris (pH 6.8), 40% glycerol, and 8% sodium dodecyl sulfate (SDS) was added. The samples were loaded onto an SDS–10% polyacrylamide gel and electroblotted to 100 V for 30 min. Following electrophoresis, the samples were electroblotted onto polyvinylidene difluoride membranes (Millipore) in transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol, and 0.1% SDS. Membranes were probed with a Ypd1p-specific antibody followed by an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin secondary antibody and then visualized with the enhanced chemiluminescence detection system from Amer sham Biosciences.

Modeling of Ypd1p–response regulator interactions. Based on shape and electrostatic complementarity and the incorporation of available biochemical data, Ypd1p (PDB code 1QSP) and CheY (PDB code 2CHE) were docked together with the program FTDock (18). The final model of the complex was identified from multiple predictions (10,000) generated by FTDock. All predictions which resulted in a distance between the H64 of Ypd1p and D57 of CheY of greater than 6 Å were excluded, because the proximity of these two active-site residues is a prerequisite for phosphotransfer between Ypd1p and CheY.

Since hydrophobic residues were identified in the present study as being involved in the association of Ypd1p with the Ssk1p-R2 response regulator domain, models of the Ypd1p/CheY complex that allowed I13, I17, M20, F27, L31, and E58 of Ypd1p to be within a distance of 6 Å from (any residue of) CheY were selected. Only two models of the complex were left after this step. One model was eliminated because it positioned hydrophilic residues close to the hydrophilic patch on the surface of Ypd1p outlined by the above residues. The single model that remained (FTDock prediction solution no. 5424) was used for the following discussion.

RESULTS

Mutations that affect Ypd1p/Ssk1p-R2 interactions. Based on the available crystal structure of Ypd1p (39, 50), specific residues on the surface of Ypd1p in the vicinity of the phosphorylatable histidine residue (H64) were changed to alanine to create a library of Ypd1p mutants. Surface-exposed alanine or glycine residues were not subjected to mutagenesis. The yeast two-hybrid system was originally devised to identify proteins that physically interact with each other in vivo (9, 11, 12). In this study, a yeast two-hybrid assay was employed to investigate mutations that disrupt protein–protein interactions (44, 45, 47). Selected residues were mutated to alanine for several reasons. First, in the absence of structural information, alanine-scanning mutagenesis has proven to be an effective approach for identifying protein–protein interaction sites (46). Second, Ypd1p is an all-helix protein, and alanine has a propensity for forming α-helices. Third, mutation of only surface-exposed residues would be the least likely to have an adverse effect on the structural integrity of Ypd1p. Finally, we reasoned that by mutating residues to a side chain with lesser volume (i.e., alanine), we could reduce the possibilities of obtaining “false-positives” (due to steric hindrance) in our interaction screen. The Ypd1p alanine substitution mutants were screened in vivo with a yeast two-hybrid assay. In addition, several previously characterized Ypd1p mutants (G68Q, Q66A, and Q66L) that showed impaired phosphotransfer activity (16) were also included in the interaction screen.

To test the viability of the assay, the interaction between wild-type Ypd1p and Ssk1p-R2 was compared to that of various controls. Our results indicate that Ypd1p and Ssk1p-R2 interact relatively weakly with one another, roughly twofold greater than the weakest interacting control pair provided with the ProQuest system, the human E2F1 and retinoblastoma (Rb) proteins (44). The strength of the interaction between each of the Ypd1p alanine mutants and Ssk1p-R2 was determined in the same manner. For each of the alanine mutants, the level of β-galactosidase activity was determined with a highly sensitive CPRG assay (10) and is reported relative to that of the wild-type Ypd1p control (Fig. 1).

The level of protein expression for each of the alanine substitution mutants was checked against that of endogenous Ypd1p to ensure that differences in β-galactosidase activity were not due to differential expression or protein stability of the mutants. Immunoblot analysis showed equivalent steady-state levels of all of the Ypd1p fusion proteins (Fig. 2).

A total of 37 Ypd1p mutants were screened with the yeast two-hybrid assay and assigned to one of four classifications: little or no change in interaction, moderately disrupting, severely disrupting, and increased interaction (Fig. 1). Those showing 75 to 100% of wild-type interaction (T41, T42, R48, E53, N55, and T57) had little or no effect on the interaction of Ypd1p with Ssk1p-R2. A substantial number of mutations (T12, S19, D23, Q45, E58, N61, H64, K67, S70, W80, E83, Q86A, Q86L, and R90) exhibited 25 to 74% of the wild-type interaction and were classified as moderately disrupting.
teen residues (I13, E16, M20, D24, F27, L31, Q38, D60, F65, G68Q, S69, L73, and Q76) had a profound effect in terms of disrupting the interaction between Ypd1p and Ssk1p-R2. These residues appear to be crucial for protein-protein interactions and were categorized as severely disrupting (less than 25% of the wild-type interaction). Intriguingly, three Ypd1p mutants (I17, Q34, and N87) displayed a twofold enhancement in binding to Ssk1p-R2.

Molecular surface analysis. To identify the putative response regulator binding site on Ypd1p, the residues tested in the yeast two-hybrid assay were mapped onto the molecular surface of Ypd1p with the graphics program GRASP (31) (Fig. 3). From the ribbon representation of the tertiary structure (Fig. 3A) and the view of the molecular surface (Fig. 3B) of Ypd1p, it was apparent that the severely disrupting mutations clustered together and covered portions of the αA, αB, and αC helices. Of the 14 mutations that had this effect, all but 2 fell in the vicinity of αA. These two exceptions, D60A and G68Q, were located on opposite sides of the site of phosphorylation (H64). In the view presented in Fig. 3, glycine 68 is located one helical turn above H64 on αC of Ypd1p and is thought to facilitate phosphoryl transfer by contributing to the solvent accessibility of H64 (16). The other mutation, D60A, is located one helical turn below H64.

Results from the in vivo interaction assay and analysis of the
molecular surface of Ypd1p indicate that the region of Ypd1p used for binding Ssk1p-R2 potentially covers about 750 Å². The size of this recognition site was estimated by considering only residues that, when mutated, resulted in less than 25% of the wild-type interaction. The Ypd1p/Ssk1p-R2 interaction identified here is relatively small in comparison to other protein-protein interfaces that have been characterized structurally (17, 24), but is consistent with proteins that interact with each other in a reversible manner. Interestingly, mutations that severely disrupted the interaction with Ssk1p-R2 were clustered between H64 and the αA helix of Ypd1p and were not centered on the site of phosphorylation. Mutations that resulted in a moderate disruption of the interaction between Ypd1p and Ssk1p-R2 primarily surrounded the area marked by the severely disrupting mutations.

**DISCUSSION**

**Response regulator binding site on Ypd1p.** Close examination of the response regulator binding surface on Ypd1p revealed a large hydrophobic patch (approximately 690 Å²), which was surrounded by polar and charged residues (Fig. 4B). The location of the hydrophobic patch closely overlapped the location of the severely disrupting mutations identified in our in vivo interaction assay.

The sequence alignment of Ypd1p with other HPt homologs (Schizosaccharomyces pombe Mpr1p, Candida albicans Ypd1p, Arabidopsis thaliana AHP2, Ditycystosium discoideum RdeA, and Escherichia coli ArcB) revealed eight residues that were conserved in HPt domains and contributed to the hydrophobic patch (Fig. 4A). These residues mainly reside on αA (I13, I17, and M20), the N-terminal portion of αB (F27 and L31), and the C-terminal end of αC (G68, L73, and G74). In addition, we postulate that A71 and A72 (which were not subjected to mutagenesis) are also part of the hydrophobic patch involved in general binding of HPt domains to response regulators. Mutational studies performed on the C-terminal HPt domain of ArcB (ArcB') from E. coli also revealed that several conserved residues in a similarly located hydrophobic patch exhibited decreased phosphotransfer activity, which the authors suggested may be attributed to a loss of interaction between ArcB' and its cognate response regulator ArcA (19, 28).

The results from the yeast two-hybrid assay support the
importance of the hydrophobic patch. Of the eight conserved hydrophobic residues identified by the sequence alignment, six resulted in a severe loss of interaction (I13, M20, F27, L31, G68, and L73) and one (I17) resulted in enhanced binding with Ssk1p-R2. If the hydrophobic patch is indeed needed for general recognition of response regulator domains, what allows Ssk1p-R2. If the hydrophobic patch is indeed needed for gen-

Unexpectedly, three mutations (I17A, Q34A, and N87A) resulted in a twofold enhancement in binding between Ypd1p and Ssk1p-R2. At this point, we can only speculate about the reasons for these observations. For example, mutation of residues Q34 and N87 to a more hydrophobic alanine residue might have resulted in a larger hydrophobic binding surface for the response regulator. Alternatively, mutation of I17, Q34, and N87 to alanine, a residue with a smaller side chain volume, could possibly reduce steric clashes at the protein-protein interface, thus allowing the two proteins to form a tighter complex. It is also possible that the corresponding interacting residues on the surface of Ssk1p-R2 could have chemical properties that allow better interaction with alanine at these three positions on Ypd1p. Further studies which should help to distinguish among these or other possibilities are under way.

Theoretical docking study sheds further light on Ypd1p-response regulator interactions. The bacterial chemotaxis protein CheY is a well-characterized single-domain response regulator with a doubly-wound $\alpha_1\beta_3$ tertiary fold (40, 41), typical of all other response regulator domain structures that have been determined to date. We had demonstrated previously that Ypd1p can interact with CheY in vitro phosphoryl transfer experiments (15). Thus, in order to further investigate the possible mode of interaction between Ypd1p and response regulators, a theoretical complex between Ypd1p and CheY (40) was constructed based on shape complementarity, electrostatic considerations, and the results obtained from the yeast two-hybrid interaction assay. A theoretical model of the complex was created using the FTDOCK program, which evaluates the surface shape and electrostatic complementarity of two monomers and docks them together as rigid bodies (13).

In the modeled Ypd1p-CheY complex, the contacts that are observed involve the $\alpha_A$, $\alpha_B$, and $\alpha_C$ helices of Ypd1p and the first $\alpha_1$ plus two loop regions ($\beta_1-\alpha_1$ and $\beta_5-\alpha_5$) of CheY (Fig. 5). The majority of the interactions seen are hydrophobic in nature and include three residues from $\alpha_A$ in Ypd1p (I113, I17, and M20) and two residues near the N terminus of $\alpha_B$ (F27 and L31) that were implicated in both the yeast two-hybrid screen and the HPt domain sequence alignment as being important for Ssk1p-R2 interactions. Residues from the $\alpha_C$ helix in Ypd1p make specific contacts to residues in CheY located in the $\beta_1-\alpha_1$ and $\beta_5-\alpha_5$ turn regions. Completely consistent with the results from our yeast two-hybrid interaction screen, residues located between H64 and helix A in Ypd1p form a patch that define an interaction surface for CheY in the modeled complex. However, two Ypd1p residues

FIG. 5. Theoretical complex between Ypd1p and CheY. A theoretical model of a docked complex between Ypd1p and the bacterial response regulator CheY was created with the program FTDOCK (18). (A) The complex shows that the $\alpha_1$ helix of CheY (red) aligns in a parallel fashion with the $\alpha_C$ helix of Ypd1p (with the H64 side chain protruding out) and makes additional contacts to Ypd1p via loop regions $\beta_1-\alpha_1$ and $\beta_5-\alpha_5$ (shaded red). The areas of Ypd1p involved in making contacts to CheY involve $\alpha_A$, the N-terminal portion of $\alpha_B$, and the C-terminal portion of $\alpha_C$ (shaded yellow), an interaction surface consistent with experimental results from our analysis of Ypd1p/Ssk1p-R2 interactions. (B) A 90° rotation of the modeled complex further reveals the areas of Ypd1p involved in binding the response regulator CheY.

(D24 and D60) important for Ssk1p-R2 interaction fall just outside the area of interaction in the model with CheY. It is possible that these residues are involved in specific interactions with Ssk1p-R2.

Comparisons to Spo0B-Spo0F complex. The crystal structure of the complex between Spo0B and Spo0F from Bacillus subtilis was the first complex to reveal details regarding interactions between a histidine-containing phosphotransfer protein and a response regulator (51). Although the structures of response regulator domains deposited in the Protein Data Bank (http://www.rcsb.org) show little differences in global three-dimensional structure, there are significant differences between the structures of the HPt proteins Spo0B and Ypd1p. Each Spo0B monomer consists of two domains, an N-terminal $\alpha$-helical hairpin domain and a C-terminal $\alpha/\beta$ domain containing a $\beta$-sheet and two $\alpha$-helices (43). Unlike other HPt proteins, Spo0B forms a stable dimer via its N-terminal do-
main. This results in the formation of a central four-helix bundle, which is flanked on either side by the C-terminal α/β domains. The site of phosphorylation (H30) within each monomer is located in the middle of the α helix.

Results from our yeast interaction screen indicate that the αA helix of Ypd1p is critical for complex formation. However, Spo0B does not have a helix that corresponds to αA of Ypd1p. Instead, the α1 and α2 helices of Spo0B form a more extended antiparallel helical hairpin compared to Ypd1p. The additional surface area created by this extension is mostly hydrophobic. This allows Ypd1p and Spo0B to use similar surface characteristics to bind response regulators. Moreover, the α helix of the Spo0F response regulator was nearly parallel to the four-helix bundle of Spo0B in the complex (51) and is located between the helices corresponding to αB and αC of Ypd1p. The same interaction is observed in our theoretical complex between Ypd1p and the bacterial response regulator CheY. The same interaction is seen in the Spo0B-Spo0F complex. Although the structures of the HPt proteins are very different, the interactions with response regulators may show the same general characteristics.

Conclusions. Data from the in vivo interaction assay and the theoretical complex between Ypd1p and CheY suggest that a hydrophobic patch located around the αA helix of Ypd1p is needed for proper recognition and binding of response regulators. The majority of the mutations that resulted in a severe disruption of interaction between Ypd1p and Ssk1p-R2 involved αA and portions of the αB and αC helices nearby. Other residues located outside the hydrophobic patch presumably impart specificity for one response regulator over another. Our interpretation of a modeled complex between Ypd1p and CheY suggests that the N-terminal α helix of response regulators is involved in making the most contact with the HPt protein.

Overall, our experimental results coupled with insight gained from the study of a modeled Ypd1p-CheY complex support the notion that complexes between HPt domains and multiple response regulators will have common areas of interaction. However, additional studies are necessary to identify specific binding determinants on Ypd1p for recognition of Snlp1-R1 and Skn7p-R3 and to determine the effect that protein phosphorylation has on binding specificity and strength of interaction.

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