Evolutionarily conserved 14-3-3 proteins have important functions as dimers in numerous cellular signaling processes, including regulation of transcription. Yeast 14-3-3 proteins, known as Bmh, inhibit a post-DNA binding step in transcription activation by Adr1, a glucose-regulated transcription factor, by binding to its regulatory domain, residues 226 to 240. The domain was originally defined by regulatory mutations, ADR1<sup>c</sup> alleles that alter activator-dependent gene expression. Here, we report that ADR1<sup>c</sup> alleles and other mutations in the regulatory domain impair Bmh binding and abolish Bmh-dependent regulation both directly and indirectly. The indirect effect is caused by mutations that inhibit phosphorylation of Ser230 and thus inhibit Bmh binding, which requires phosphorylated Ser230. However, several mutations inhibit Bmh binding without inhibiting phosphorylation and thus define residues that provide important interaction sites between Adr1 and Bmh. Our proposed model of the Adr1 regulatory domain bound to Bmh suggests that residues Ser238 and Tyr239 could provide cross-dimer contacts to stabilize the complex and that this might explain the failure of a dimerization-deficient Bmh mutant to bind Adr1 and to inhibit its activity. A bioinformatics analysis of Bmh-interacting proteins suggests that residues outside the canonical 14-3-3 motif might be a general property of Bmh target proteins and might help explain the ability of 14-3-3 to distinguish target and nontarget proteins. Bmh binding to the Adr1 regulatory domain, and its failure to bind when mutations are present, explains at a molecular level the transcriptional phenotype of ADR1<sup>c</sup> mutants.
cose-repressed genes in multiple ways, including inhibiting a repressor’s function (32–37); inducing the expression, promoting the activation, and facilitating the promoter binding of a transcription activator (38, 39); and activating transcription initiation and stabilizing the nascent transcript (40). In response to low glucose, Snf1 activates two transcription factors, Cat8 (Zn cluster DNA binding) and Adr1 (Zn finger DNA binding) (41), that function both independently and in combination to activate the expression of glucose-repressed genes (42). Snf1 regulates the activity of Cat8 at the transcriptional and posttranscriptional levels, whereas it regulates Adr1 uniquely at the posttranscriptional level (43).

A role for Bmh in glucose repression was discovered after it was found to be associated with Reg1 (27), a subunit of the PP1-type protein phosphatase Glc7, which dephosphorylates and inactivates Snf1. Snf1 is apparently partially active in the presence of glucose when Bmh is absent, leading to constitutive activation of glucose-repressed genes (43). Although some details of the mechanism of regulation of Adr1 activity by Bmh are known, how the two interact has not been determined. Surprisingly, there is no such study available for yeast 14-3-3 and its client. Therefore, the residual and motif preferences of yeast 14-3-3 are unknown.

In the present study, we mapped the minimal Bmh binding region on Adr1 and found that it colocalizes with ADR1’ alleles, residues 226 to 239. Extensive mutational analysis distinguishes two regions that are important for Bmh binding: a core region consisting of residues immediately flanking Ser230, the site of phosphorylation, and a distal region that participates in Bmh binding without affecting Ser230 phosphorylation. This result suggests the direct involvement of residues outside the 14-3-3 motif in interacting with Bmh. The inability of monomeric Bmh to bind and regulate Adr1 activity suggests that the distally located residues, including Tyr239, might be providing a contact with the second subunit of the Bmh dimer to accomplish stable and functional interaction between Adr1 and Bmh.

### Materials and Methods

#### Yeast strains and growth of cultures.

All the strains used in this study are listed in Table 1. Epitope tags were introduced according to previously published methods (46, 47). Yeast cultures were grown in either yeast extract-peptone medium or synthetic medium lacking the appropriate amino acid or uracil for plasmid selection. The repressed cultures contained 5% glucose, and the derepressed cultures contained 0.05% glucose. Cultures of yeast were grown at 30°C. The bmh1-170 allele is temperature sensitive for growth at 37°C, but not at 30°C. However, it displays the same defect in Adr1-dependent gene expression at both temperatures (44), so 30°C was employed to avoid introducing temperature stress.

#### Plasmid constructs.

All pOBD2-based vectors were made by cloning the corresponding coding sequence into the TRP1-CEN4 vector, pOBD2 (48), using gap repair methods (49, 50), where proteins were expressed as N-terminal Gal4 DNA binding domain (Gal4DBD) fusions, as described by Parua et al. (44). PCR fragments were generated using forward and reverse primers that contained homology to the vector sequences flanking the polylinker region of pOBD2, as well as homology to ADR1. The Ncol and PvuII-digested pOBD2 plasmid (51) and a PCR fragment were used to transform Pf69-4a to Trp” prototrophy. Plasmid DNA from Trp” transformants was rescued and sequenced to confirm that recombination had produced the correct in-frame gene fusion using primers OBDSF and OBDSR. Western analysis with an anti-Gal4DBD monoclonal antibody (R55C1; Santa Cruz Biotechnology) was used to confirm the synthesis of a fusion protein of the correct size.

#### Preparation of protein extracts from yeast cells.

Protein extracts from yeast cells were prepared following the procedure described by Parua et al. (44). In brief, 50 to 100 ml of yeast cell culture grown to an OD600 of ~1 was used for protein extraction. Cells were collected by centrifugation at 1,600 x g for 5 min at 4°C in a Sorvall RC3B plus centrifuge, washed once with 15% glycerol containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and resuspended in an equal volume of chromatin immunoprecipitation (ChiP) lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) containing protease and phosphatase inhibitors (Sigma). The cells were broken with glass beads in a Savant FP120 FastPrep machine with two disruption cycles of 45 s at a speed setting of 4.0. The unbroken cells and debris were pelleted by centrifugation in a microcentrifuge at 13,600 x g for 10 min. The clarified extract was collected in a fresh microcentrifuge tube containing 1 mM PMSF and 1X phosphatase inhibitor and used in subsequent experiments.

#### GST pulldown assays.

Glutathione S-transferase (GST) pulldown assays were done following the protocol described by Parua et al. (44). GST-Bmh1 wild-type (Bmh1-wt) and mutant fusion proteins were expressed from pGEX-3X-BMH1 and pKP92, respectively, in Escherichia coli BL21(DE3). The fusion proteins were immobilized on glutathione-Sepharose 4B beads as described by the manufacturer (GE Healthcare Life Sciences). Pulldown assays were performed using 30 to 40 µg of glutathione-Sepharose 4B-coupled GST fusion proteins and yeast extract containing ~2 µg of total proteins in 1X PBST (phosphate-buffered saline containing 0.1% Tween 40), 1X protease inhibitor, and 1X phosphatase inhibitor (Sigma). The suspension was incubated at 4°C for 1 h with continuous mutation. The beads were then pelleted by centrifugation at

### Table 1 Strains used in the study

| Name    | Genotype                        | Reference |
|---------|---------------------------------|-----------|
| Pf69-4a | MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADEl2 met2::GAL7-IanZ | 78        |
| YLL908  | W303 MATα bmh2Δ::kanmx          | 79        |
| YLL1087 | W303 MATα bmh2Δ::kanmx          | 79        |
| PPV7    | YLL1087 REG1-13MYC::NatMx       | This study|
Targeting gene expression at 30°C as at 37°C (method described by Collart and Oliviero (37°C). Cells were harvested by centrifugation at 5,000 g for 1 min, washed three times with 1× PBS, and resuspended in 60 μl of 2× LDS-NuPAGE sample buffer, followed by heating at 95°C for 5 min. Fractions collected at different steps of the pulldown assays were analyzed by SDS-PAGE and Western blotting.

**mRNA isolation and qRT-PCR.** Total RNA was extracted from strains grown in either repressing or derepressing medium using the acid phenol method described by Collart and Oliviero (52). Residual DNA in the RNA preparation was removed by treatment with DNase I (Ambion) following the manufacturer’s recommendations. cDNA synthesis was performed with SuperScript III (Invitrogen) following the manufacturer's protocol. cDNA preparation was removed by treatment with DNase I (Ambion) following the manufacturer's recommendations. cDNA synthesis was performed with SuperScript III (Invitrogen) following the manufacturer’s protocol. Quantitative real-time reverse transcription (RT)-PCR (qRT-PCR) for measuring mRNA levels was performed using a 1:300 dilution of the cDNA. A standard curve was generated from triplicates and analyzed in duplicate.

**β-Galactosidase assays.** β-Galactosidase assays were performed as described by Guarente (53). Cells were grown at 30°C in selective medium to an A600 of 1.0. The reported values (in Miller units) are the averages of the results for three to five transformants.

**Protein expression and purification from E. coli.** Bmh1 wild type and mutants were expressed in E. coli as an N-terminal GST tag from the vectors pGEX-3X-BMH1 and pKP92, respectively. Protein purification was done following the protocol described by Parua et al. (54). Briefly, 2 μg of E. coli-expressed and purified wild-type and mutant Bmh1 was incubated with 0.1% glutaraldehyde (Santa Cruz Biotechnology) in a reaction buffer containing 20 mM Tris-acid, with Ser in place of Pro at position 2 (FIG 1A). Residues 227-232 (RRASFS) match motif I (RRX[pS/T]XP, where X is any amino acid), with Ser in place of Pro at position +2 (FIG. 1A). Residues 254 to 260 (RVKFSTP) perfectly match consensus motif II (RXX X[pS/T]XP) (2) (FIG. 1A). To determine whether they are important for Bmh binding, we performed GST pulldown assays using

![FIG 1](https://example.com/fig1.png)

**RESULTS**

Bmh binding region and Adr1' mutations colocalize to amino acids 226 to 240. Sequence analysis suggests that the Bmh binding region of Adr1, residues 215 to 260, consists of two blocks of conserved amino acids, 226 to 240 and 254 to 260, each of which contains 14-3-3 motif-like sequences (44). Residues 227 to 232 (RRASFS) match motif I (RRX[pS/T]XP, where X is any amino acid), with Ser in place of Pro at position +2 (FIG. 1A). Residues 254 to 260 (RVKFSTP) perfectly match consensus motif II (RXX X[pS/T]XP) (2) (FIG. 1A). To determine whether they are important for Bmh binding, we performed GST pulldown assays using...
E. coli-expressed GST-Bmh1 and yeast extracts prepared from cells expressing various Gal4DBD-Adr1 variants encompassing the amino acid 215 to 260 region of Adr1 (Fig. 1A). As shown in Fig. 1B, the shortest protein fragment retained with GST-Bmh1 contained amino acids 226 to 240, whereas the fragment (residues 240 to 280) containing consensus motif II (RVKFSTP at 254 to 260) was not pulled down with GST-Bmh1. An independent experiment using Gal4DBD-Adr1 fusion variants with or without the 254-to-260 region encompassing residues 215 to 310 of Adr1 suggests that the amino acid 254 to 260 is not required for binding (see Fig. S1 in the supplemental material). These results suggest that amino acids 226 to 240 of Adr1 are necessary and sufficient for Bmh binding. Surprisingly, all 21 Adr1 mutations found genetically were localized within this region (55–57), suggesting that the region has a unique function.

Next, we asked whether this minimal Bmh binding region (amino acids 226 to 240) is sufficient to cause Bmh-mediated inhibition of transcription factor activity. We generated a Gal4DBD-fused Adr1 variant encompassing Adr1 amino acids 226 to 240, the Bmh binding region, and the cAD (amino acids 260 to 360), and its activity was evaluated in both BMH1 wild-type and bmh1-ts (bmh1-170) mutant strains expressing a Gal4DBD-fused Adr1 variant with the indicated mutations. (GS) represents a six-amino-acid linker consisting of alternating Gly-Ser residues. Cultures of yeast were grown at 30°C. The bmh1-170 allele displays the same defect in Adr1-dependent gene expression at 30°C as at 37°C (44). The expressed fusion cassette is diagramed at the top. β-Galactosidase activity is expressed as Miller units with standard deviations generated from three separate experiments using three different biological replicates.

| ADR1c Mutant | BMH1 | bmh1-ts |
|--------------|------|---------|
| WT           | 1.4  | 25.2    |
| R229K        | 11.9 | 35.4    |
| A229P        | 12.0 | 27.0    |
| S230A        | 14.7 | 21.9    |
| S230D        | 19.0 | 32.3    |
| F231S        | 11.4 | 26.5    |
| S232R        | 12.5 | 26.8    |
| A233G        | 8.9  | 20.3    |
| A233T        | 11.8 | 21.1    |
| Y239C        | 16.0 | 29.0    |
| Y239N        | 11.2 | 24.7    |
| Vector       | 0.3  | 0.3     |

FIG 2 Effect of Adr1 mutation(s) on Bmh binding and Bmh-mediated regulation of transcriptional activity. (A) Adr1 (amino acids 226 to 240) wild-type sequence and amino acid substitutions. The underlined residues are genetically identified ADR1c alleles. (B) (Left) GST pulldown results after immunoblotting (Western blotting [WB]) with anti-Gal4DBD antibody. GST pulldown was done using E. coli-expressed GST-Bmh1 and a yeast extract (CE)-containing Gal4DBD-Adr1 fusion variant with the indicated mutations. FT, flowthrough; P, pellet. (Right) Ser230 phosphorylation status. The results were obtained after Western blotting of the CE with anti-pSer230 antibody. ND, not determined; Wt, wild type. (C) lacZ reporter assays from plasmid pHIZ18 (GAL1op-CYC1-lacZ) (53) in BMH1 wild-type and bmh1-ts (bmh1-170) mutant strains expressing a Gal4DBD-fused Adr1 variant with the indicated mutations. (GS) represents a six-amino-acid linker consisting of alternating Gly-Ser residues. The expressed fusion cassette is diagramed at the top. β-Galactosidase activity is expressed as Miller units with standard deviations generated from three separate experiments using three different biological replicates.

E. coli-expressed GST-Bmh1 and yeast extracts prepared from cells expressing various Gal4DBD-Adr1 variants encompassing the amino acid 215 to 260 region of Adr1 (Fig. 1A). As shown in Fig. 1B, the shortest protein fragment retained with GST-Bmh1 contained amino acids 226 to 240, whereas the fragment (residues 240 to 280) containing consensus motif II (RVKFSTP at 254 to 260) was not pulled down with GST-Bmh1. An independent experiment using Gal4DBD-Adr1 fusion variants with or without the 254-to-260 region encompassing residues 215 to 310 of Adr1 suggests that the amino acid 254 to 260 is not required for binding (see Fig. S1 in the supplemental material). These results suggest that amino acids 226 to 240 of Adr1 are necessary and sufficient for Bmh binding. Surprisingly, all 21 Adr1 mutations found genetically were localized within this region (55–57), suggesting that the region has a unique function.

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all of the mutant proteins showed constitutive reporter activity in the region of Adr1. As we observed that all of the mutations disrupted phosphorylation of Ser230 and indicated that most of the ADR1c mutations disrupted phosphorylation of Ser230 and thereby prevented Bmh binding. The results for the Y239N mutants were quite surprising and interesting. Despite a level of Ser230 phosphorylation comparable to that of the wild-type, the substitution completely abolished binding, indicating that Y239 plays an important role in Bmh binding, either by taking part in interaction directly or by influencing the peptide orientation in the target binding cleft(s) of Bmh. Therefore, it was important to study how distally located Y239 influences Bmh binding without affecting Ser230 phosphorylation. Furthermore, this impairment of binding of Y239 mutants was not due to the influence of its very C-terminal location in the context of the amino acid 226 to 240 peptide, as we obtained consistent results using a construct encompassing amino acids 226 to 250 of Adr1 and Ser230 phosphorylation were examined by Western blot analysis of the CE using anti-pSer230 antibody. Protein expression was monitored by Western blotting with anti-Gal4DBD antibody. Adr1 (amino acids 226 to 250) variants were expressed as an N-terminal Gal4DBD fusion protein.

Fig. 2B (right), A229P and A233G were very weakly phosphorylated and Y239F was significantly phosphorylated at Ser230. Despite a level of Ser230 phosphorylation comparable to that of the wild type, the substitution completely abolished binding, indicating that Y239 plays an important role in Bmh binding, either by taking part in interaction directly or by influencing the peptide orientation in the target binding cleft(s) of Bmh. Therefore, it was important to study how distally located Y239 influences Bmh binding without affecting Ser230 phosphorylation. Furthermore, this impairment of binding of Y239 mutants was not due to the influence of its very C-terminal location in the context of the amino acid 226 to 240 peptide, as we obtained consistent results using a construct encompassing amino acids 226 to 250 of Adr1 and Ser230 phosphorylation were examined by Western blot analysis of the CE using anti-pSer230 antibody. Protein expression was monitored by Western blotting with anti-Gal4DBD antibody. Adr1 (amino acids 226 to 250) variants were expressed as an N-terminal Gal4DBD fusion protein.

(A) GST-pulldown profile of Gal4DBD-Adr1 fusion variants in the context of the amino acid 226 to 250 region of Adr1. Pulldown was done using E. coli-expressed GST-Bmh1 and yeast cell extract (CE) expressing a Gal4DBD-Adr1 fusion peptide with the indicated amino acid substitutions. After washing, beads were boiled and used as the pellet (P) fraction. (B) The effects of various mutations on Ser230 phosphorylation were examined by Western blot analysis of the CE using anti-pSer230 antibody. Protein expression was monitored by Western blotting with anti-Gal4DBD antibody. Adr1 (amino acids 226 to 250) variants were expressed as an N-terminal Gal4DBD fusion protein.

(C) β-Galactosidase activity was measured from the reporter plasmid pHZ18 in Bmh1 wild-type and bmh1-ts (BMH1-170) mutant strains expressing Gal4DBD-Adr1 fusion protein with the indicated mutations. Cultures of yeast were grown at 30°C. The bmh1-170 allele displays the same defect in Adr1-dependent gene expression at 30°C as at 37°C (44). The error bars represent the standard deviations in three separate experiments using three different biological replicates.

Fig. 3 Effects of Adr1 mutations on Bmh binding and Bmh-mediated regulation of the activity of Adr1. (A) GST-pulldown profile of Gal4DBD-Adr1 fusion variants in the context of the amino acid 226 to 250 region of Adr1. Pulldown was done using E. coli-expressed GST-Bmh1 and yeast cell extract (CE) expressing a Gal4DBD-Adr1 fusion peptide with the indicated amino acid substitutions. After washing, beads were boiled and used as the pellet (P) fraction. (B) The effects of various mutations on Ser230 phosphorylation were examined by Western blot analysis of the CE using anti-pSer230 antibody. Protein expression was monitored by Western blotting with anti-Gal4DBD antibody. Adr1 (amino acids 226 to 250) variants were expressed as an N-terminal Gal4DBD fusion protein. (C) β-Galactosidase activity was measured from the reporter plasmid pHZ18 in Bmh1 wild-type and bmh1-ts (BMH1-170) mutant strains expressing Gal4DBD-Adr1 fusion protein with the indicated mutations. Cultures of yeast were grown at 30°C. The bmh1-170 allele displays the same defect in Adr1-dependent gene expression at 30°C as at 37°C (44). The error bars represent the standard deviations in three separate experiments using three different biological replicates.

Insights into Bmh-Adr1 Interactions

FIG 3

Insights into Bmh-Adr1 Interactions

Role of residues other than Adr1c in Bmh binding. No mutation was found by genetic screening in the region between Ala233 and Tyr239. To reveal their role, if any, in Bmh binding and inhibition of Adr1, we generated Gal4DBD fusion proteins with amino acids 226 to 250 of Adr1 that had substitutions for each residue from Q234 to S238, as indicated in Fig. 2A. GST pulldown assays were performed using glutathione-Sepharose 4B-immobilized GST-Bmh1 and yeast extract expressing the above-mentioned fusion peptides. As shown in Fig. 3A, S235A and S238A substitutions abolished Bmh binding, whereas three other substitutions (Q234A, A236G, and S237A) retained Bmh binding ability. Interestingly, three additional Y239 substitutions (including two aromatic ones), Y239A, Y239W, and Y239F, disrupted Bmh binding (Fig. 3A), suggesting that the hydroxyl group

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None of the non-ADR1c mutations had an effect on Ser230 phosphorylation (Fig. 3B), suggesting the direct involvement of Tyr239, Ser235, and Ser238 in Bmh binding. The activities of the mutants were consistent with their Bmh binding profiles (Fig. 3C).

A dimerization-deficient Bmh is unable to bind Adr1. We asked whether dimerization-deficient Bmh could bind and regulate Adr1 activity. Several approaches have been used to generate dimerization-deficient 14-3-3 proteins (1, 16–20, 22, 25, 63–67). One such dimer-incapable 14-3-3 protein, introduced by Tzivion et al. (17), contained 7 nonconservative amino acid replacements in the N-terminal region (E5L, I12AE, Y82REKIE to K5Q, Q12QR, Q82RENIQ; mutated residues are underlined). To examine the interaction between dimerization-deficient Bmh and Adr1, we introduced seven mutations (D7L, I14AE, Y87RSKIETE to R7, Q14NK, N87RSNIETQ) in the dimeric interface of Bmh1 to disrupt the dimer. To examine whether the mutations had disrupted dimer formation, glutaraldehyde cross-linking was performed using E. coli-expressed and purified wild-type and mutant Bmh1 in Bmh1 wild-type and bmh1-ts (bmh1-170) mutant strains. ADH2 mRNA was normalized to ACT1 mRNA. Cultures of yeast were grown at 30°C, and Ser238 in Bmh binding. The activities of the mutants were consistent with their Bmh binding profiles (Fig. 3C).

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Probing of the Bmh binding region in Adr1 to understand the yeast 14-3-3 sequence motif(s). The novel mode of interaction between Bmh and Adr1 suggests that other yeast proteins might employ a similar strategy. We asked whether other yeast 14-3-3 targets also contain a Bmh binding motif containing a long stretch of amino acids between the phosphorylated Ser/Thr and a distal Tyr. A computational approach followed by a binding assay allowed us to identify other proteins with a similar motif that were bound by Bmh. First, we searched in the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/) for yeast proteins sharing the sequence pattern RX2-3[pS/T]X4-10Y, where X is any amino acid and the subscript number denotes the number of residues allowed. We found 6,252 sequence hits representing 3,308 unique sequence entries after searching 5,886 proteins in SGD. Then, we asked how many of the 270 Bmh targets found by Kakiuchi et al. (68) were present in the SGD-extracted protein list. There were 204 Bmh targets with the sequence RX2-3[pS/T]X4-10Y.
TABLE 2 Adr1-like sequence pattern hits (SGD and PhosphoGRID)*

| Protein | Pattern | RX_1[pS/T]X_10Y | RX_2[pS/T]X_4-10Y | Position (pS/T) |
|---------|---------|-----------------|-------------------|-----------------|
| Accl    | RAVVSDSL5Y | 1157            |                   |                 |
| Cki1    | RPGVRSYESGYV | 9               |                   |                 |
| Nup60   | RRATVPSAPY | 10              |                   |                 |
| Pbe2    | RTSTSSHY | 38              |                   |                 |
| Nnk1    | RANSXDITY | 65              |                   |                 |
| Reg1    | RTRSMGGLDEY | 75              |                   |                 |
| Nap1    | RLGSLQVGDSGY | 76             |                   |                 |
| Nth1-b  | RRGSBEDDTY | 83              |                   |                 |
| Boi2    | RAKSTKRIY | 118             |                   |                 |
| Cdc23-a | RKHSHPMKRY | 135             |                   |                 |
| Cdc23-b | RRSLSNLNSAY | 151             |                   |                 |
| Cdh1-a  | RPPSSVGSASLTY | 193            |                   |                 |
| Sfl1-a  | RRKNSSNNQNY | 220            |                   |                 |
| Mhp1    | RSKTESEY | 222             |                   |                 |
| Gip2    | RSKSVHDHQAPVKY | 221         |                   |                 |
| Frt1    | RRRSAGSFIDY | 228            |                   |                 |
| Adr1    | RRASAFAAQQSY | 230           |                   |                 |
| Jsn1    | RSQNNASSDY | 275             |                   |                 |
| Npr1    | RJSGHFAHSPGTQSY | 317       |                   |                 |
| Bni5    | RRSSLNKY | 340             |                   |                 |
| Atg13   | RANSFPEOQWSKVVKY | 355        |                   |                 |
| Mgp1    | RSTGGTNMILGY | 381           |                   |                 |
| Cyk3    | RARTLTSDKHPRY | 391           |                   |                 |
| Haa1-a  | RSRSFHHPPANEY | 439          |                   |                 |
| Prp3    | RSHSQPHETQFY | 492           |                   |                 |
| Kin2-a  | RTNSFVTGEYY | 501           |                   |                 |
| Haai-b  | RRSVDNHYR | 506             |                   |                 |
| Msb1    | RRDSSAPDNNQGYY | 538          |                   |                 |
| Ydr18c  | RSTSSNHFNTVNY | 573           |                   |                 |
| Msd3    | RLSSGSGILNDY | 565           |                   |                 |
| Kap1-a  | RGGTTTVQHPGQAY | 624          |                   |                 |
| Mpt5    | RHFSFLPANAY | 662           |                   |                 |
| Fp1k    | RTNSFVTGEYY | 676           |                   |                 |
| Kin1-a  | RAVDFVPFGAKPSY | 677          |                   |                 |
| Pmd1    | RSSVPSPPV | 785             |                   |                 |
| Kin1-b  | RAKSVGHHARLESKY | 791         |                   |                 |
| Kap1-b  | RRLSMEQFKGNYV | 827          |                   |                 |
| Akl1    | RGKSKRNNY | 884             |                   |                 |
| Kin1-c  | RKTSTETY | 986             |                   |                 |
| Sec1-c  | RTNSAISQSPVNY | 701          |                   |                 |
| Kap2-a  | RSDINNNSRNKNDTY | 88          |                   |                 |
| Tco89-a | RVLTHDGLTDNDY | 52           |                   |                 |
| Syt1-a  | RRRTRTVDFDY | 275           |                   |                 |
| Sec1-b  | RGHSTSISY | 849             |                   |                 |
| Sec1-c  | RSRRTNASSQSPVNY | 699         |                   |                 |
| Ms4     | RRKKSITTDIPDNNY | 558         |                   |                 |
| Kap1-c  | RDFTFTPVSQHRY | 526          |                   |                 |
| Msn2    | RQRASLPLIBDLSY | 451          |                   |                 |
| Syt1-b  | RRRKTVDFDY | 277             |                   |                 |
| Sfl1-b  | RKNSQNYNCQ | 221             |                   |                 |
| Igd1    | RKRSSFKEKFDIDY | 175          |                   |                 |
| Kap2-b  | RRSIDINNNSRNKNDTY | 88         |                   |                 |
| Fin1    | RRRMSPECGLK | 74            |                   |                 |
| Tco89-b | RRVLTVDGTLNDY | 52           |                   |                 |
| Cdh1-b  | RRPSYTVGDYR | 50             |                   |                 |
| Ask10   | RKSSSTTY | 627             |                   |                 |

*The table lists peptides sharing an Adr1-like sequence pattern (RX_1[pS/T]X_10Y) from a search in SGD with filtering by PhosphoGRID. Phosphorylated residues (Ser or Thr) are in boldface, and their positions are also indicated. Sequences that have +2 Pro are shaded.

a, b, and c indicate various peptides from the same protein.

How significant is that enrichment of high-throughput Bmh target sequences with a consensus sequence pattern? To examine this, we used Fisher’s exact test (see Table S3 in the supplemental material) and calculated a P value of 1.667e-11. By searching the database in PhosphoGRID (http://www.phosphogrid.org/) to find Bmh targets containing either phosphorylated Ser or Thr in the search motif, we found 56 matches from 43 different proteins that contain an Adr1-like consensus sequence (Table 2). These matches were grouped into two classes based on mode I and mode II motif characteristics, i.e., whether two or three residues were present between the N-terminal Arg and the phosphorylated residue. For yeast 14-3-3 targets other than Adr1, Bmh binding sites have been determined experimentally only in neutral trehalase, Nth1, and the protein kinase Yak1 (69, 70). In Nth1, Ser60 and Ser83 play equally important roles in Bmh binding and activity modulation (69). Interestingly, both sites lack +2 Pro, as in the Bmh binding site in Adr1. One of the two above-mentioned Bmh binding sites of Nth1 containing Ser83 was in our final list. In Yak1, autophosphorylated Ser335 plays an important role in Bmh binding, together with an unidentified site in the N terminus (70). Ser335 in Yak1 has a Pro at the +2 position and closely matches a mode II 14-3-3 motif. Interestingly, our list was enriched with several known 14-3-3 targets, although the Bmh recognition sequence is unknown. Surprisingly, only six of them share +2 Pro.

To examine whether the matched sequences are Bmh targets, we performed GST pulldown assays using yeast-expressed Gal4DBD fusion proteins containing a matched sequence. To determine which sequences to examine in more detail, all of them were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and their phylogeny was determined using EBI Web Service (http://www.ebi.ac.uk/Tools/webservices/services/phylogeny/clustalw2 PHYLOGENY), aligning them to a family on the basis of sequence homology and the number of residues present between the phosphorylated residue and Tyr (see Fig. S3A and B in the supplemental material). GST-pulldown assays were performed on one known Bmh target representing each family in this phylogeny. We chose seven sequences, including Adr1, and the corresponding coding sequence (CDS) was cloned into a plasmid and expressed in yeast as a Gal4DBD fusion protein. Alignment of all seven is presented in Fig. 5A. As shown in Fig. 5B, six out of the seven sequences we tested showed Bmh binding. We conclude that many yeast 14-3-3 targets share a sequence feature that has a Tyr residue outside the phosphomotif.

**DISCUSSION**

14-3-3 proteins regulate numerous signaling processes by interacting with their phosphorylated targets (1–3). An unbiased phosphopeptide library search revealed two consensus 14-3-3 motifs, motif I (RXX-pS/T-Xp and motif II (RXX-pS/T-Xp) (7). The third motif (carboxy terminal region) has been observed in few 14-3-3 targets, e.g., plant H2-ATPase (8, 9). However, the Pro located at position +2 of the phosphorylation site occurs in only about one-half of known 14-3-3 binding motifs (71). The Bmh binding motif in Adr1 also lacks (+2) Pro. Ser is present at the +2 position and, surprisingly, replacement of that Ser with Pro reduced the binding. It is unknown whether yeast 14-3-3 or Bmh prefers Pro at the two Bmh binding sites (69, 72). Interestingly, both sites lack +2 Pro, suggesting that Bmh Ser83 positions +2 Pro outside the Bmh binding site. This is the first report documenting the involvement of residues outside the yeast 14-3-3 motif. However, the involvement of residues located distally from the phosphorylation site has been documented structurally and...
biochemically for a few targets in higher eukaryotes (73–75). Lys943 in the H+/H-ATPase is located 10 residues upstream of the 14-3-3 binding motif, and it has been shown to be involved in direct binding with the N-terminal region of the second 14-3-3 monomer. This interaction explains why dimerization of both 14-3-3 and the H+/H-ATPase target is indispensable for binding and activity regulation (75). Cdc25b may represent another example of a site with distally located interactions, but there is no structural study to corroborate that explanation. Although the evidence demonstrates that distally located Ser238 and Tyr239 play important roles in Bmh binding and regulation of the activity of Adr1 without affecting Ser230 phosphorylation, we do not yet know how these two residues influence Bmh binding. One possibility is that Adr1’s interaction with Bmh resembles the interaction of H+/H-ATPase with 14-3-3. If that is the case, the distal portion of Adr1’s Bmh binding region would emerge from the target binding cleft of one monomer, and S238 and Y239 would interact with the nearest surface of the second 14-3-3 monomer, as shown in the proposed model (Fig. 6). This model assumes that a Bmh dimer would be important for efficient interaction with Adr1, and indeed, a dimerization-deficient Bmh was unable to bind Adr1. Structural studies need to be done to gain insight into the role of the distal region in binding and inhibition of Adr1 activity. An important recognition region located distal to the canonical 14-3-3 motif might help explain how these proteins distinguish target from nontarget proteins.

An intriguing but unexplained observation is the presence of a second predicted 14-3-3 binding motif between residues 254 and 260 (44). We did not detect Bmh binding to this site. Because it is highly conserved in Adr1 orthologs in other yeast species (76), we speculate that phosphorylation of S258 may occur under some physiological conditions and promote Bmh binding and an additional avenue of regulation.

In conclusion, these studies provide a molecular understanding of the altered regulatory properties of ADR1c alleles first isolated by Ciriacy (58) and later expanded by Denis et al. (55). These alleles were the earliest demonstration, together with mutations in the GAL system, that eukaryotic gene regulation involved trans-acting regulatory factors. They are also the only genetically isolated and characterized mutations we are aware of that define a binding site for 14-3-3 proteins. The unusual ability to isolate such mutants was due to unique properties of the motif in providing stringent inhibition of gene expression when Bmh is bound to the site. Inhibition apparently occurs at a step after Adr1 has bound its cognate promoters (45), but the mechanism is unknown. Inhibition of a post-Adr1 binding step is also suggested by the observation that Adr1c activators show a reduced requirement for and enhanced recruitment of some transcriptional coactivators (77).

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