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AtMSI4 and RbAp48 WD-40 repeat proteins bind metal ions

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Abstract The mammalian RbAp48 protein is the most extensively studied member of the conserved family of Msi1-like WD-40 repeat proteins, which are components of complexes involved in the assembly and modification of chromatin. We have isolated a plant homolog of RbAp48, AtMSI4. By metal affinity chromatography, zinc blotting and atomic absorption analysis, we demonstrate that purified recombinant RbAp48 and AtMSI4 proteins bind 3–4 metal ions per molecule of protein. Metal competition assays indicate a preference for zinc. Both N- and C-terminal halves of RbAp48 and AtMSI4 display zinc binding activity, suggesting it is an intrinsic property of the propeller structures likely to be formed by these proteins. Metal binding might mediate and/or regulate protein-protein interactions which are functionally important in chromatin metabolism.

Key words: Arabidopsis thaliana; Metal binding; Chromatin assembly factor; WD-40 repeat

1. Introduction

Proteins containing WD-40 motifs occur in multicomponent complexes involved in signal transduction, cell cycle control, cell fate determination, transcription, cytoskeletal organization, pre-mRNA splicing and vesicular trafficking [1], and are implicated in protein-protein interactions [2]. The Msi1-like family of WD-40 repeat proteins has been the focus of numerous recent studies. MSI1 is a negative regulator in the *Saccharomyces cerevisiae* RAS-cAMP signaling pathway; its overexpression suppresses heat shock sensitivity, reduces cAMP levels of RASVal19 and ira1 mutants and suppresses the snf4 mutant [3,4]. Disruption of MSI1 leads to increased ultraviolet radiation sensitivity and reduced telomeric silencing [5].

Many Msi1-like proteins occur in complexes promoting the assembly and modification of chromatin: for example, Msi1, RbAp48 and p55 are components of chromatin assembly complexes of yeast, humans and *Drosophila* respectively [5–7]. The *Drosophila* p55 protein also is an integral subunit of a multiprotein ATP-dependent nucleosome remodeling factor (NURF) [8] and an Msi1 homologue from yeast, Hat2p, is a regulatory subunit of the major cytoplasmic histone acetyltransferase [9]. RbAp48 is a subunit of human histone deacetylase HD1 [10]. RbAp48 and its homolog, RbAp46, have been identified as components of sequence specific Mad-Max-mSin3A transcriptional repressor complexes [11], and both proteins associate with the retinoblastoma (Rb) protein in vitro and in vivo [12,13]. Although Msi1-like proteins have been strongly implicated as histone chaperones for histone acetylases, histone deacetylases and CAF proteins, their specific role in Rb binding and suppression of the Ras pathway and snf4 mutations remains unclear.

The crystal structure of a WD-40 repeat protein – the Gβ-subunit of adenyl cyclase – reveals seven highly conserved WD-40 repeats corresponding to seven propeller blades [14,15]. Each propeller blade consists of a small four-stranded twisted β sheet. Given the conservation of amino acid sequence among WD-40 repeat proteins, it is likely that members of the Msi-like family also form similar propeller structures.

Protein structural domains are frequently stabilized by metal ions, particularly zinc [16], and this occurs in a wide variety of proteins with regulatory functions, particularly DNA binding proteins. Zinc binding allows polypeptide chains to fold into structures well suited for participating in macromolecular interactions. Some of these domains (like RING and LIM) are involved in mediating protein-protein interactions [16,17].

We and others have isolated the genes for plant homologs of Msi1-like WD-40 repeat proteins ([18], Kenzior and Folk, unpublished). Comparative analysis of these proteins has revealed a great degree of functional and structural similarity. Serendipitously, we have observed that both AtMSI4 and RbAp48 possess metal binding properties and are capable of coordinating at least three atoms of zinc per molecule of protein. We suggest this property may be involved in the capacity of these proteins to associate with other proteins.

2. Materials and methods

2.1. Plasmids

Expression plasmid pET-48NB, containing the full length RbAp48 protein, was generously provided by E.-H. Lee. pETAtMSI4 contains the entire coding sequence for AtMSI4 between the *Nde*I and *Bdl*II sites of pETh3a (Kenzior and Folk, unpublished).

2.2. Purification of RbAp48 and AtMSI4 proteins

The RbAp48 and AtMSI4 recombinant proteins were expressed in *Escherichia coli* BL21(DE3)pLYS by standard methods [19]. Four hours after induction cells were chilled on ice, harvested and washed once with TEB buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol 0.1% NP-40) and sonicated in TEB containing 1 mM phenylmethylsulfonyl fluoride. Cell debris and inclusion bodies were pelleted by centrifugation and washed three times in TEB buffer. The resulting pellet was dissolved in 6 M guanidinium-HCl in TEB and then refolded by stepwise dialysis against 3, 1.5, 0.75, 0.4, 0.2 M guanidinium-HCl in TEB. The residual guanidinium-HCl was removed by extensive dialysis against three changes of TEB. The RbAp48 and AtMSI4 proteins were >98% pure as judged by SDS-PAGE and Coomassie staining (Fig. 1A). Protein concentration was determined using the molar extinction coefficient calculated from the amino acid sequence (ExPASy molecular biology WWW server of the Swiss Institute of Bioinformatics) and Bradford dye assay [20] using bovine serum albumin as a standard. To minimize zinc contamination only the highest purity reagents were used; all solutions were prepared using high grade water, and glassware used for metal analysis was

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washed with 30% nitric acid and thoroughly washed with deionized water.

2.3. Thrombin digestion

Preparations of RbAp48 and AtMSI4 were digested with thrombin (Sigma) at a 50:1 ratio for 6 h in TEB buffer supplemented with 2 mM $CaCl_2$.

2.4. Metal analysis

Preparations of RbAp48 and AtMSI4 were analyzed for zinc content by atomic absorption spectroscopy using a 'Spectr AA 30' flame atomic absorption spectrometer (Varian, Australia), as described by Falchuk et al. [21]. Dilutions from a zinc standard (1000 ppm, Fisher) were used for spectrometer calibration. Prior to analysis, proteins (~200 μg) were dialyzed against 2000 volumes of 10 mM Tris pH 7.5, 50 mM NaCl, 2 mM β-mercaptoethanol, 0.1 mM $ZnCl_2$ for 24 h, then against four changes (24 h each) of the same buffer without $ZnCl_2$. Zinc levels in the final three changes of dialysis buffer were below the limit of detection (0.05 ppm).

2.5. Polyacrylamide gel electrophoresis and zinc blotting

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [22]. Proteins were electroblotted onto polyvinylidene (PVDF) membrane (Immobilon-P, Millipore) or nitrocellulose membrane using a semidry electroblotting system (Bio-Rad). For metal competition assays, 3 μg of each protein in metal binding buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 5 mM β-mercaptoethanol) was blotted onto nitrocellulose membranes using a slot blot manifold. After protein transfer, filters were incubated in metal binding buffer for 1 h and probed for 30 min with 10 μCi/ml $^{65}ZnCl_2$ in metal binding buffer. The filters were then washed with metal binding buffer for 30–40 min, with four changes of buffer. In competition experiments, competing metal ions were included in the metal binding buffer during all steps. After washing, the filters were exposed to film using an intensifying screen. Immobilized proteins were subsequently detected by staining the membrane with 0.1% amido black in 10% acetic acid, 40% methanol.

2.6. In vitro transcription-translation

mRNA was transcribed from linearized plasmids using a T7 RNA polymerase transcription system (Promega) and translation was carried out using a rabbit reticulocyte lysate (Promega) with [^{35}S]-methionine (NEN).

2.7. Immobilized metal chelate affinity chromatography (IMAC)

Iminodiacetic acid-agarose charged with Ni(II)SO_4 was used for protein binding assays. The resin was equilibrated with IMAC-5 (20 mM Tris, pH 7.9, 0.5 M NaCl, 10% glycerol, 5 mM imidazole) for native protein binding and Urea-5 (20 mM Tris, pH 7.9, 0.5 M NaCl, 10% glycerol, 5 mM imidazole, 6 M urea) for denatured protein binding. Bound proteins were eluted with Urea-100 buffer (20 mM Tris, pH 7.9, 0.5 M NaCl, 10% glycerol, 100 mM imidazole, 6 M urea). Where indicated the resin was additionally washed with Urea-20 buffer (20 mM Tris, pH 7.9, 0.5 M NaCl, 10% glycerol, 20 mM imidazole, 6 M urea).

3. Results

3.1. Metal affinity chromatography of RbAp48 and AtMSI4

While studying interactions between purified RbAp48 and AtMSI4 proteins and target proteins immobilized on Ni(II) charged IDA agarose, we observed that both proteins bind Ni-IDA resin tenaciously, even in buffers containing 5 mM imidazole (IMAC-5) with 6 M urea. Significant quantities of these proteins bound to a Ni column in 6 M urea, 20 mM imidazole, but are eluted with 100 mM imidazole or 50 mM EDTA. Neither RbAp48 nor AtMSI4 was retained by un-

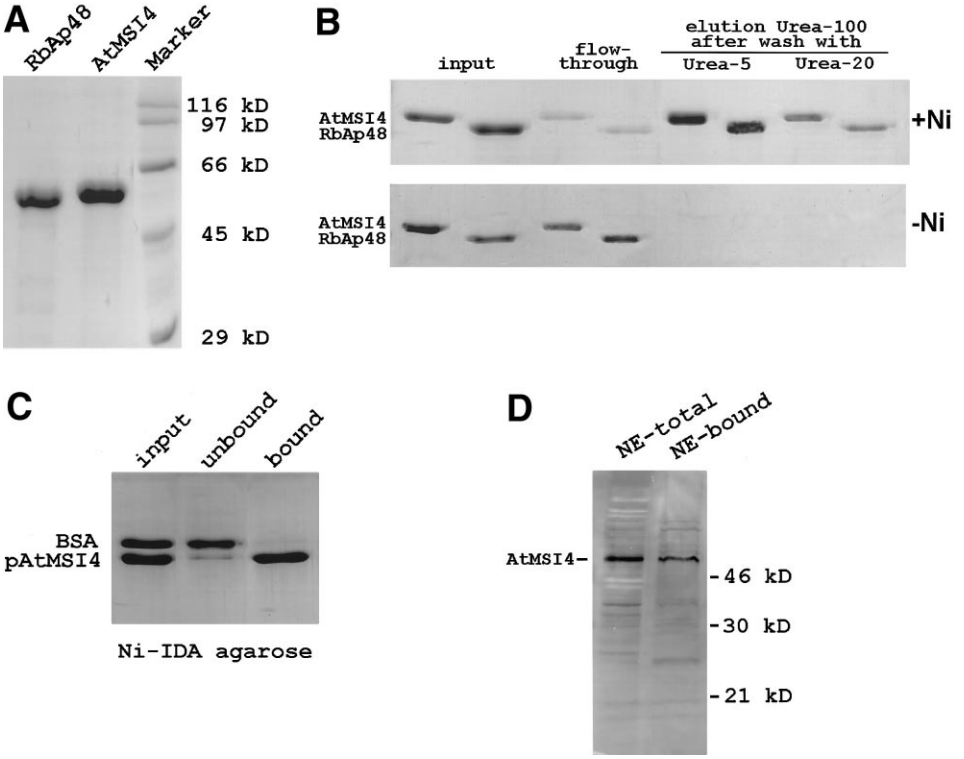

Fig. 1. AtMSI4 and RbAp48 proteins specifically bind to the Ni-IDA agarose column. A: Purified recombinant AtMSI4 and RbAp48 proteins. B: Metal affinity chromatography of recombinant AtMSI4 and RbAp48 proteins. Purified proteins were loaded on a Ni-IDA column washed with Urea-5/Urea-20 buffer and eluted with Urea-100 buffer. Eluted proteins resolved on a 10% SDS gel and stained with Coomassie blue. C: AtMSI4 specifically binds to the Ni-IDA resin in the presence of BSA. D: AtMSI4 from *Arabidopsis thaliana* nuclear extract binds to Ni-IDA column. Nuclear extract from *Arabidopsis* cell suspension culture was loaded on a Ni-IDA column washed with Urea-5 buffer and eluted with Urea-100 buffer. Total nuclear extract and eluted proteins were separated on a 10% SDS-PAGE gel, transferred to PVDF membrane and probed with affinity purified AtMSI4 specific antibody.

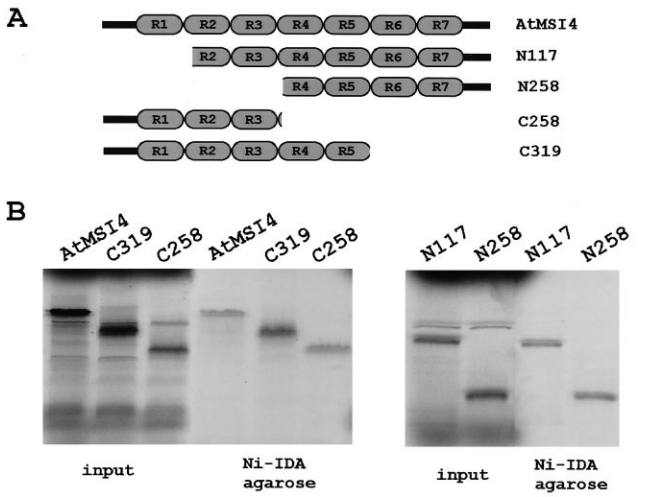

Fig. 2. Analysis of the distribution of the metal binding sites in AtMSI4 protein. A: Structure of the AtMSI4 deletion mutants analyzed in this study. B: Ni-IDA-agarose binding of in vitro translated N- and C-terminal deletion mutants of AtMSI4. In vitro translated proteins were loaded on a Ni-IDA column washed with Urea-5 buffer and eluted with Urea-100 buffer. Eluted proteins were separated on 10% SDS gel and detected by autoradiography.

charged IDA resin (Fig. 1B). To confirm the binding specificity, AtMSI4 was mixed with bovine serum albumin (BSA) (a weak zinc binding protein), and the binding assay was repeated. AtMSI4 was retained by the Ni-IDA agarose under conditions in which BSA was not (Fig. 1C). We also found that unpurified AtMSI4 protein could be absorbed from crude nuclear extracts by Ni-IDA agarose (Fig. 1D).

Although these observations indicate that RbAp48 and AtMSI4 proteins possess metal binding properties, their amino acid sequences do not suggest known metal binding motifs. To localize the metal binding sites of AtMSI4, a set of N-terminal and C-terminal deletions was constructed (Fig. 2). The mutant proteins were produced by in vitro translation. Ni-IDA agarose binding assay of the translated proteins under denaturing conditions (Urea-5 buffer) demonstrated that both N-terminal and C-terminal halves of AtMSI4 retained the capacity to bind to the resin in Urea-5 buffer, suggesting that AtMSI4 possesses multiple sites capable of metal binding.

3.2. Atomic absorption analysis of RbAp48 and AtMSI4

Since RbAp48 and AtMSI4 are nuclear proteins, zinc is a likely candidate for the metal they are capable of binding. Atomic absorption analysis was used to confirm the metal binding properties and to determine the metal ion binding capacity of the recombinant proteins purified to homogeneity. Residual metal ions were stripped from the proteins during the guanidinium-HCl denaturation step used for their purification, as the zinc content of purified proteins was below the limit of detection (data not shown). The resulting protein preparations were dialyzed against buffer containing 0.1 mM $ZnCl_2$ for 24 h, then subjected to extensive dialysis against four changes of the same buffer without zinc during 96 h. Aliquots of the proteins were subjected to atomic absorption analysis after each 24 h period. As shown in Fig. 3, both proteins bound metal, even after 4 days of dialysis, while samples of dialysis buffer after each dialysis change showed

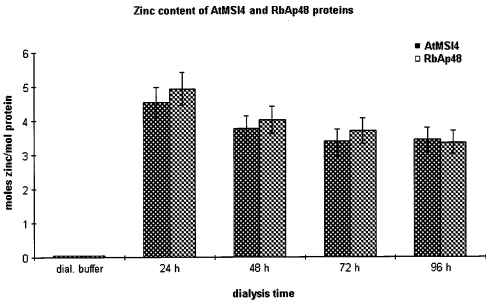

Fig. 3. Atomic absorption analysis of the zinc content of AtMSI4 and RbAp48 proteins.

no detectable zinc after the first day of dialysis. The molar ratio of bound zinc was calculated using a mass of RbAp48 of 46 704 Da and of AtMSI4 of 50 611 Da. These data indicate that RbAp48 binds 3–3.5 mol of zinc and AtMSI4 binds 3–3.2 mol of zinc (average of three repeats).

3.3. $^{65}Zn(II)$ blotting assay

The zinc binding activity of RbAp48 and AtMSI4 was further investigated using a $^{65}Zn(II)$ blotting assay [23], a frequently used procedure for the analysis of metal binding properties of proteins [24–27]. The RbAp48 and AtMSI4 proteins before or after digestion with thrombin were resolved by SDS electrophoresis and transferred to PVDF membrane, denatured in 6 M guanidinium-HCl and renatured in metal binding buffer, and then probed with ^{65}Zn. Under these conditions (Fig. 4) autoradiography indicated ^{65}Zn binding to the full-length RbAp48 and AtMSI4 proteins, as well as to several thrombin digestion products. Strong binding also was observed to several of the marker proteins, e.g. carbonic anhydrase and insulin, which are known for their metal binding capacity. However, no binding was detected for lysozyme and ovalbumin, which are not metalloproteins. The relative binding efficiency of different polypeptides could not be quantitatively interpreted, as it may reflect differences in efficiency of transfer, renaturation or binding conditions.

The specificity of the zinc binding activity of RbAp48 and AtMSI4 was addressed by testing the ability of different di-

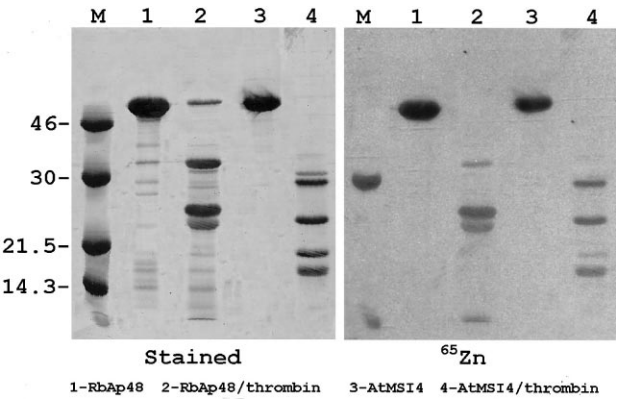

Fig. 4. AtMSI4 and RbAp48 proteins and fragments after thrombin digestion bind ^{65}Zn. Purified AtMSI4 and RbAp48 were subjected to thrombin digestion. Intact proteins and products after digestion were loaded on 15% acrylamide SDS gel and then transferred to a PVDF membrane and probed with ^{65}Zn as described in Section 2. Zinc binding was detected by autoradiography and amido black staining.

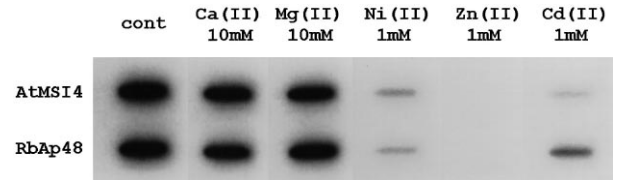

Fig. 5. Competition for binding by divalent metal ions. Samples containing 3 μg of AtMSI4 and RbAp48 proteins were transferred to a PVDF membrane preincubated in metal binding buffer and probed with ^{65}Zn for 1 h either in the presence of competing ions (as indicated) or in their absence (control). Filters were washed in three changes of metal binding buffer with the corresponding ion and autoradiographed.

valent cations to compete for ^{65}Zn binding. We utilized a slot blot modification of the binding assay. The RbAp48 and AtMSI4 proteins were blotted directly on PVDF membrane preincubated in metal binding buffer and then incubated with ^{65}Zn in metal binding buffer in the presence of different competitor ions (Fig. 5). As anticipated, ^{65}Zn binding was totally abolished by 1 mM $ZnCl_2$, while addition of 10 mM Ca(II) and Mg(II) had no effect on ^{65}Zn binding. Zn binding was selectively competed by 1 mM Ni(II) and Cd(II), although to a lesser extent. These results confirm that only ions with a coordination chemistry similar to zinc are efficient competitors of ^{65}Zn binding to RbAp48 and AtMSI4.

4. Discussion

Metals in proteins serve catalytic, structural and regulatory purposes [28]. Residues that directly bind Cu, Fe, Mn and Zn ions include imidazole nitrogens (H), carboxylates (D/E), sulfur (C/M), hydroxyl (S/T/Y) and carbonyl oxygens. Zinc typically coordinates with four ligands in proteins [29] and zinc binding sites in metalloproteins generally can be divided into two categories: conformational binding sites, which include residues widely separated in the polypeptide chain (which are brought together by tertiary structure), and structural binding elements formed by residues proximal to each other in the polypeptide chain.

The results of metal chelate chromatography, atomic absorption analysis and ^{65}Zn blotting experiments demonstrate that human RbAp48 and *Arabidopsis* AtMSI4 are metalloproteins which can bind at least three atoms of zinc or other metals with similar coordination chemistry. RbAp48 and AtMSI4 contain seven WD-40 repeats with short N- and C-terminal extensions and exhibit 31% amino acid identity and 50% similarity (Fig. 6). Consequently, we expect that these proteins might use conserved amino acid residues for metal coordination. Amino acid sequence analysis does not reveal obvious zinc binding motifs in these proteins, but this is not surprising, as many metal binding sites do not conform to consensus motifs [30–32]. Among the conserved residues are eight His (at least one in each WD-40 repeat), numerous acidic (Glu and Asp) and one Cys. Both the N- and C-terminal halves of RbAp48 and AtMSI4 possess metal binding properties, and their binding to a metal affinity column under denaturing conditions suggests that one or more metal binding sites are resistant to denaturation and therefore might be structural. Metal binding sites might localize completely within a WD-40 repeat or, alternatively, several WD-40 repeats may contribute to metal coordination.

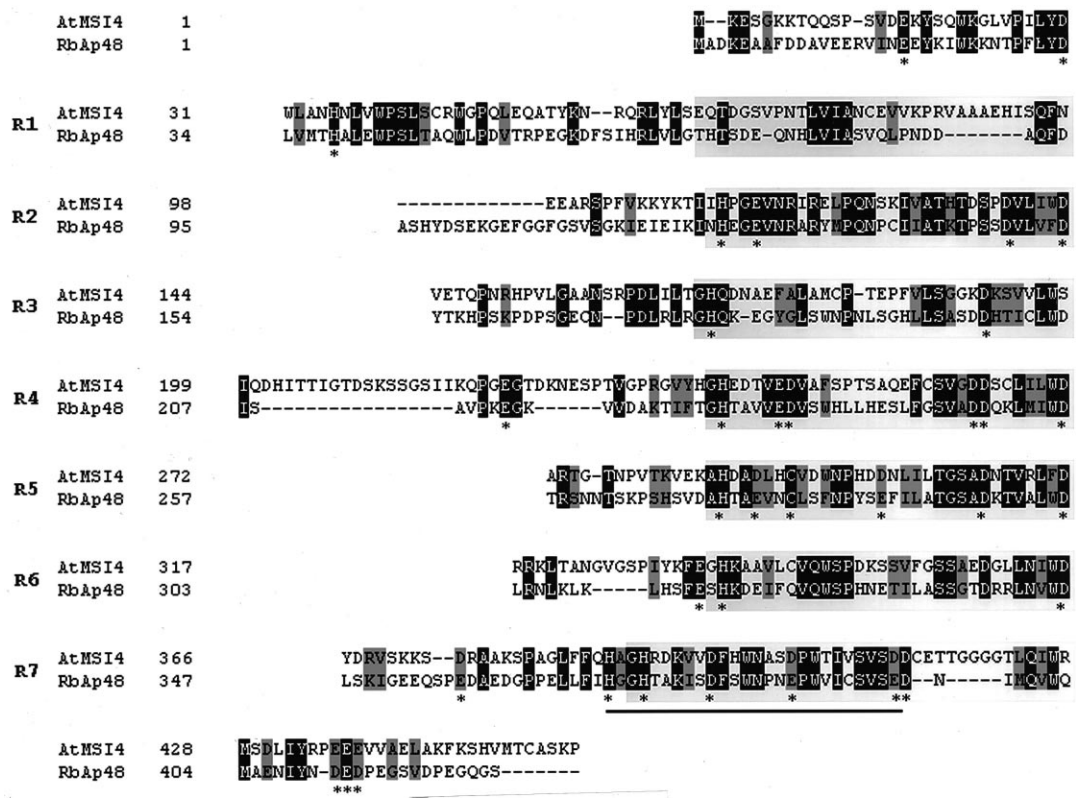

Fig. 6. Comparison of the amino acid sequences of *Arabidopsis* AtMSI4 with human RbAp48. Dashes indicate gaps in homology, asterisks conserved amino acids capable of zinc coordination. Potential zinc binding site is underlined.

In metal affinity chromatography, histidine functions as the predominant ligand [33]. The affinity of RbAp48 and AtMSI4 for Ni-IDA agarose suggest that each protein uses one or more His residues for metal coordination. Possibly, WD-40 repeat R7 may serve as a potential site for metal binding. Its sequence **HA/GGHX$_5$DX$_{16}$D/E** is conserved not only between RbAp48 and AtMSI4 but among all known Msi-like proteins (MSI1, RbAp46, AtMSI1–3, L.eMSI1, yeast Hat 2p, *Drosophila* p55). The absence of a sulfur coordinating ligand in one or several of the zinc binding sites is suggested by the metal competition data, as cadmium was a weaker competitor than zinc in binding assays (Fig. 5). Cadmium ions have a greater affinity for sulfur coordinating atoms than zinc [30].

Structural zinc binding protein domains typically mediate nucleic acid binding and protein-protein interactions. RbAp48 and AtMSI4 do not bind to DNA-cellulose, even under very mild conditions (Kenzior nd Fold, unpublished). In contrast, Msi-like proteins have been proposed to function as structural platforms facilitating interactions between proteins involved in chromatin metabolism. We suggest that coordination of metals may stabilize or modulate the tertiary structure of certain WD-40 domains so as to generate binding surfaces for protein-protein interaction, or may even mediate protein-protein interaction with target proteins such as histones, histone acetylases, histone deacetylases, chromatin assembly factors and Rb. A recent study of the histone deacetylase HDAC1 indicates that it is a metalloenzyme. Critical histidine and aspartic acid residues required for enzymatic and structural integrity of HDAC1 have been identified, and alteration of a number of these residues causes decreased interaction with RbAp48 [34].

To our knowledge, RbAp48 and AtMSI4 are the first WD-40 domain proteins to be shown to possess metal binding capacity; however, other proteins which adopt propeller-like structures can bind metals. Galactose oxidase possesses a unique mononuclear copper binding site essential for catalysis: the cupric ion resides on the solvent accessible surface of the β propeller domain and is coordinated by two histidines, two tyrosines and acetate ion [35]. The N-terminal portion of an integrin α subunit is composed of seven repeats of about 60 amino acids each, arranged in a structure that is predicted to fold into a β propeller domain. A putative Ca^{2+} binding motif is present in repeat 4 in some integrins and in repeats 5–7 in all integrins [36]. Integrin ligands and a putative Mg^{2+} ion are predicted to bind to the upper surface of the β propeller. The divalent cations Mg^{2+} or Ca^{2+} are required for ligand binding, either through bridging interactions between integrin and ligand [37] or through induction of conformational changes in the integrin that unmasks sites involved in ligand recognition [38].

Whether metal binding capacity is a unique feature of the Msi family of WD-40 domain proteins is yet to be explored. Conservation of structural features and biophysical characteristics of all WD-40 repeat proteins suggest that other WD-40 proteins should possess this capability.

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