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Interaction with Cu^{2+} disrupts the RNA binding affinities of RNA recognition motif containing protein

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ABSTRACT

The glycine-rich proteins (GRP) containing RNA recognition motifs (RRM) are involved in the regulation of transcriptional and/or post-transcriptional events. Previous studies have established that GRP162 plays an important role in the restoration of fertility in Honglian cytoplasmic male sterile (HL-CMS) rice. In this study, the ion binding properties of rGRP162 were tested by isothermal titration calorimetry (ITC) and electrophoretic mobility shift assay (EMSA) was performed to test the interaction. Circular dichroism (CD) was carried out to detect the alteration of secondary structure in the presence and absence of Cu^{2+}. Furthermore, two RRM containing proteins, AtRBP45A and AtRBP47A, were expressed to validate the interaction. Results showed Cu^{2+} and Fe^{3+} bound GRP162, whereas Ca^{2+}, Mn^{2+}, Mg^{2+} and K^{+} did not. EMSA confirmed that interaction with Cu^{2+} interrupted the biological activity of GRP162 by disrupting the secondary structure of the protein based on the results of CD. Moreover, the RNA binding activities of rAtRBP45A and rAtRBP47A were also impaired in the presence of Cu^{2+}. Data suggest that Cu^{2+} in excess may disrupt RNA-binding proteins containing RRM that are essential for post-transcriptional regulation and may impair the development of plants or animals.

1. Introduction

Glycine-rich proteins (GRPs) belong to a large family of heterogenous proteins that are enriched in glycine residues [16]. In recent decades, glycine-rich RNA-binding proteins that contain one or more RNA recognition motifs (RRMs) at the N-terminus and a glycine-rich region at the C-terminus have been identified in a variety of organisms [4,12,25]. In mammals, the small protein rich in arginine and glycine (SRAG) was shown to be involved in the cell cycle [25]. In *Arabidopsis* and *Oryza sativa*, the diverse functions of GRPs were demonstrated in multiple pathways such as cell elongation, seed germination, seedling growth, flowering time transition, and stress tolerance under high salinity, drought, and cold stress conditions [10,11,14,20]. The functions of GRPs are complex and not yet completely elucidated [8]. The various sub-localizations of GRPs, nucleus and mitochondria, implied the complicated functions.

The glycine-rich regions have been proposed to be involved in protein–protein and protein–nucleic acid interactions [3]. Some evidence showed that the RRM have RNA-binding affinity for poly (U) [15,22]. Our previous study showed that GRP162 was involved in the restoration of fertility in Honglian cytoplasmic male sterile (HL-CMS) rice and interacted with protein and CMS RNA simultaneously [5,6]. The RNA recognition motif of GRP162, which is responsible for RNA binding affinity, was shown to be vital for restoration of fertility complex (RFC) during processing.

Metal ions involved in controlling multiple signaling pathways are essential for development in animals and plants. Iron (Fe^{3+}) is essential for plant growth, which is significantly impaired under Fe^{3+}-deficient conditions [7]. Copper (Cu^{2+}) is essential for plant growth but is toxic in excess [19]. Copper is widely distributed intracellular and actively transported into cells for physiological processes, including photosynthesis, mitochondrial respiration, and other functions [17]. Pathogens can overcome rice defense mechanisms by regulating host copper redistribution [24]. Magnesium (Mg^{2+}) is required for many enzymatic reactions, including those involving ATPases, kinases, the protein synthetic machinery, and polymerases [9]. Calcium (Ca^{2+}) is another key element for plant growth and development as a nutrient and second messenger [2,18].

In this study, isothermal titration calorimetry (ITC) was employed to study the ion-binding properties of rGRP162 and circular dichroism (CD) to study its secondary structure alterations. To validate the proposal that Cu^{2+} interrupted RNA binding activities of RRM containing proteins in general, *Arabidopsis* AtRBP45A and

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AtRBP47A were studied. These results suggest that the homeostasis of Cu^{2+} in the cell is important for the function of RRMs and post-transcriptional regulation.

2. Materials and methods

2.1. Expression and purification of rGRP162, rAtRBP45A and rAtRBP47A

The full-length cDNA of GRP162 was cloned into the pET32a, T7 expression vector and validated by sequencing (Novagen). AtRBP45A and AtRBP47A were cloned into pGEX-6p-1 and validated by sequencing, too (Novagen). The recombinant plasmids were introduced into BL21 (DE3) *Escherichia coli*, and the expression of the recombinant proteins was enhanced by induction with 1 mM IPTG when the absorbance at 600 nm was approximately 0.4. The cells were allowed to grow at 28 °C for an additional 5 h. The cells were collected by centrifugation, suspended in PBS buffer, lysed by sonication, and purified with the AKTA Prime plus protein purification system (GE Healthcare) as previously described [6]. The protein samples were separated by 10% SDS–PAGE and dialyzed twice against various buffers for further analysis at 4° for 2 days with gentle stirring. The dialyzed buffer should be treated by diethylpyrocarbonate (DEPC), when the recombinant proteins were further used for EMSA. The proteins were then quantified by the BCA method (Bio-Rad).

2.2. Electrophoretic mobility shift assay

The T7 promoter was engineered into the primer, and the DNA fragments were amplified using the primer pairs RNApF ggatcctaatacgactcactataggCTTGAATGATGCTATAAATCTCCATC and RNApR cccgtcgacATGGAAGACCGTTAGTCCCTCGGGTAGTG, with *atp6-orfH79* as a template. The RNAs were transcribed *in vitro* with the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific). The RNAs were gel-purified and labeled as previously described [6]. For the EMSA, dialyzed rGRP162, rAtRBP45A and rAtRBP47A were incubated with biotin labeled RNAs in 20 μL reaction mixtures that contained increasing concentrations of Cu^{2+}. Competitor RNAs and yeast tRNA were used as negative controls in this study. The samples were incubated at 37 °C for 30 min, separated by 5% native PAGE in 0.5× TBE buffer, and then transferred onto nylon membrane for hybridization and exposed to X-ray film (Fuji) for several minutes in a darkroom.

2.3. Isothermal titration calorimetry experiments

ITC experiments were performed at 298 K with a VP-ITC instrument from Microcal, Inc. The solutions were filtered and thoroughly degassed prior to the experiments to avoid air bubbles in the calorimeter. ITC measurements were performed by the titration of rGRP162 with various ions at 25° with stirring at 400 rpm. The buffers containing different ions were dissolved in 10 mM Tris buffer, 100 mM NaCl, pH 5.5, and the pH was readjusted with 0.1 M HCl. Subsequently, rGRP162 (50 μM rGRP162, 10 mM Tris buffer, 100 mM NaCl, pH 5.5) was titrated with various ions by 30 injections of 6 ml, and the heat evolved or absorbed data were collected. To correct the dilution and mixing effects, a series of control injections was performed, in which various ions were injected into the buffer alone. The heat signals of these controls were then subsequently subtracted from the raw data for each injection experiment. These data, corrected for the dilution, were further analyzed and fit to a curve using the MicroCal Origin Pro. 5.0 software.

2.4. Circular dichroism analysis

CD spectral data for rGRP162 were collected using a CD spectropolarimeter (Model J815, Jasco). 50 μM of rGRP162 in either PBS buffer or PBS buffer plus 6 mM Cu^{2+} was measured at 25°. The acquisition parameters were 0.5 nm resolution, 1.0 nm bandwidth, 0.5 s response, and 260–200 nm wavelength range at room temperature. The average spectra were collected from 200 nm to 260 nm three times. An estimate of the secondary structure was performed using the software jascow32 designed by the manufacturer (Jasco), which uses the Yang statistical algorithm.

3. Results

3.1. Protein expression and purification

The full-length cDNA of GRP162 was first isolated from the total cDNA of AK289192, Nipponbare (*O. sativa japonica*). Recombinant GRP162 was expressed in the T7 system and purified with a His-tag as described previously [6]. rGRP162 was detected by Western blot with an anti-His antibody, indicating that rGRP162 was highly pure and suitable for further studies (Fig. 1a and 1b). The results from SDS–PAGE suggested that the recombinant protein samples were approximately 98% purity. To remove imidazole and other contaminants such as ions and RNases, the recombinant rGRP162 was further dialyzed against various buffers and quantified using the bicinchoninic acid (BCA) method. DEPC-treated PBS buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 was used as the dialysis buffer for the electrophoretic mobility shift assay and circular dichroism measurements, and TS buffer containing 10 mM Tris, 100 mM NaCl at pH 5.5 was used as the dialysis buffer for ITC.

3.2. Ion binding properties of rGRP162

Isothermal titration calorimetry (ITC) was employed to screen many ions, including Fe^{3+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, and K^{+}. The results showed that Cu^{2+} and Fe^{3+} bound to rGRP162, whereas other ions did not (Fig. 2, Supplementary Figs. 1–4). The lack of interaction between rGRP162 and Ca^{2+}, Mn^{2+}, Mg^{2+}, and K^{+} suggested that these ions do not affect the function of GRP162 directly.

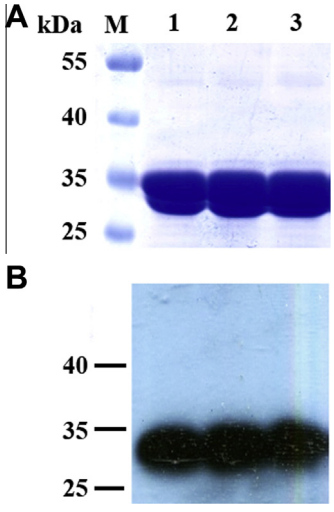

Fig. 1. Expression and purification rGRP162. (A) The recombinant proteins were loaded onto 10% SDS–PAGE gels and visualized by Coomassie blue staining. M, prestained marker SM0671 (Fermentas); 1–3 were purified rGRP162. (B) Western blotting with an anti-His tag antibody.

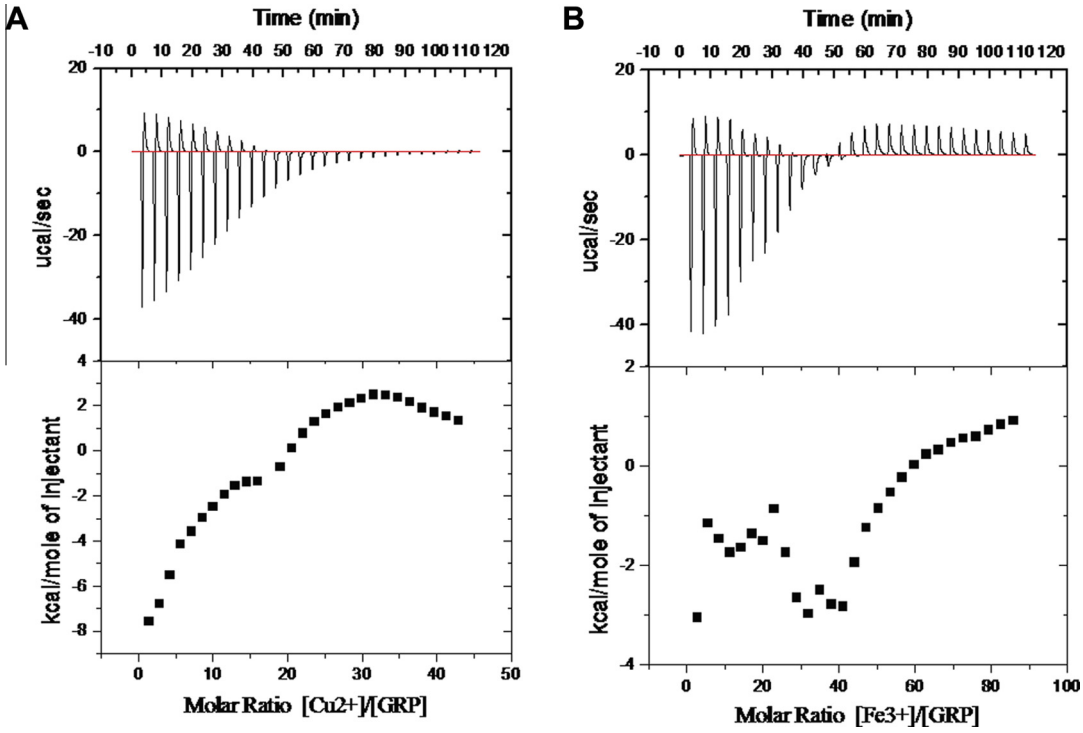

Fig. 2. ITC binding profile of rGRP162 with various ions. (A) The top panel shows the data obtained with automatic injections of Cu^{2+}. The bottom panel shows the integrated curve presented with the experimental points. (B) The top panel shows the data obtained with automatic injections of Fe^{3+}. The bottom panel shows the integrated curve presented with the experimental points.

True affinity data via heat measurement offers unique insight into biological processes. ITC can directly and unambiguously determine the number of binding sites (n) of a macromolecule. Thermodynamic parameters were calculated from the Gibbs free energy equation, and these data were analyzed and fit to a curve using the MicroCal Origin Pro. 5.0 software. An n value of three was optimal for the interaction between rGRP162 and Cu^{2+}, suggesting that rGRP162 possessed three potential binding sites for Cu^{2+} (Table 1) [23]. Therefore, quantitative analysis of the interaction confirmed that rGRP162 directly binds to Cu^{2+}.

3.3. The RNA binding affinity was disrupted by Cu^{2+}

The RRM of GRP162 plays an important role in the restoration of the fertility pathway and is responsible for the RNA binding of CMS transcripts in the HL-CMS line [6]. First, to test the hypothesis that Cu^{2+} or Fe^{3+} can enhance the RNA binding affinity of GRP162, an EMSA was performed. The data suggested that Cu^{2+} impaired the RNA binding affinity, whereas Fe^{3+} did not (data not shown). Consequently, EMSAs were performed with a fixed concentration of GRP162 and increasing concentrations of Cu^{2+}. Unexpectedly, rather than enhancing the RNA binding affinity, the presence of Cu^{2+} disrupted the RNA binding affinity of rGRP162 (Fig. 3). Even at the low concentration of 0.2 mM Cu^{2+}, the affinity was reduced. The results established that the homomultimer of rGRP162 was disrupted in the presence of Cu^{2+}. These results suggest that Cu^{2+} may be an inhibitor of the biological activity of rGRP162.

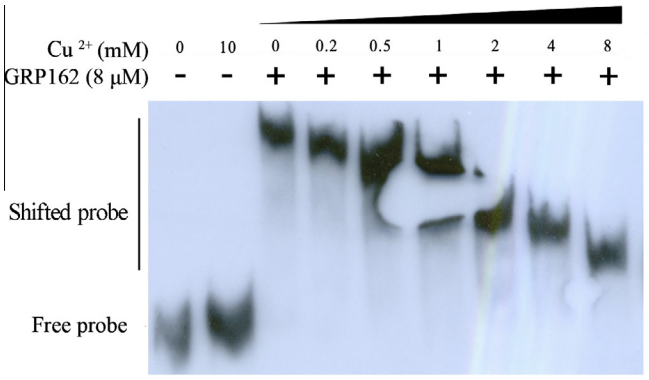

Fig. 3. RNA binding affinities analysis with EMSA. The EMSA using a fixed high concentration of GRP162 (8 μM) was performed with increasing concentrations of Cu^{2+} ranging from 0 to 10 mM. The negative control in lane 2 indicates that Cu^{2+} cannot degrade RNAs directly.

Table 1
Best-fit parameters of the ITC data to a sequential three-site binding curve for GRP162 based on the data in Fig. 2.

Sites (n)	Ka (M^{-1})	ΔH (kcal mol^{-1})
1	$(4.352 \pm 0.02) \times 10^4$	-21.34 ± 1.56
2	$(1.609 \pm 0.13) \times 10^3$	-141 ± 18.6
3	$(0.157 \pm 0.03) \times 10^3$	-2.56 ± 0.43

Ka is defined as the equilibrium binding association constant [23].
ΔH is defined as the molar enthalpy of each binding site [23].

3.4. Secondary structure was destroyed in the presence of Cu^{2+}

Since the secondary structure of RRMs is important for the RNA binding capability, we hypothesized that Cu^{2+} suppressed the RNA binding capability due to the destruction of the secondary structure of rGRP162. Circular dichroism was employed to investigate the secondary structure of rGRP162 in the presence and absence of Cu^{2+}. The structure of GRP162 was predicted using the software, SWISS MODEL (Automatic Modelling Mode) (http://swissmodel.expasy.org/) (Supplementary Fig. 5). Furthermore, the folding

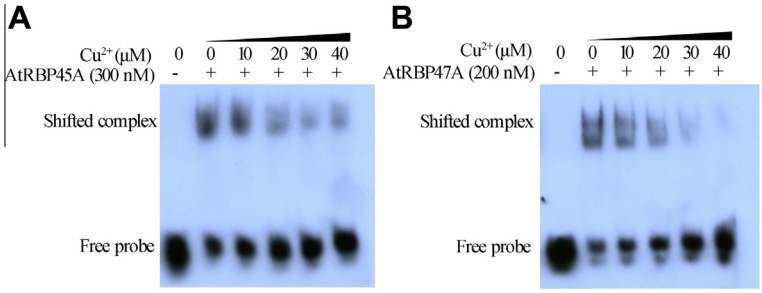

Fig. 4. RNA binding affinities analysis with EMSA. (A) The EMSA using a fixed high concentration of rAtRBP45A (300 nM) was performed with increasing concentrations of Cu^{2+} ranging from 0 to 40 μM. (B) The EMSA using a fixed high concentration of rAtRBP47A (200 nM) was performed with increasing concentrations of Cu^{2+} ranging from 0 to 40 μM. AU-rich RNA labeled with biotin at 5-end was used as a probe.

of GRP162 predicted by the SOPMA software (http://npsa-pbil.ibcp.fr) demonstrated 27.2% α-helix and 14.45% turn-sheet. The data from the circular dichroism spectra established that the recombinant protein was properly folded with approximately 30% α-helix, as visualized by the two typical negative α-helix peaks at 208 nm and 222 nm. Subsequently, the spectral constants of rGRP162 were detected in the presence of 6 mM Cu^{2+}. The results confirmed that the secondary structure of rGRP162 was greatly altered and that the typical negative peaks of the α-helix disappeared, suggesting that the nature secondary structure of rGRP162 was destroyed (Supplementary Fig. 6). Consequently, the RNA binding affinity was disrupted in the presence of Cu^{2+}. This result is also consistent with the results obtained in previous studies, which showed that the RRM is responsible for the RNA binding capability.

3.5. The RNA binding affinities of rAtRBP45A and rAtRBP47A were impaired

To make sure whether Cu^{2+} interrupted the RNA binding affinities of RRM containing proteins in general, AtRBP45A (NM_124872) and AtRBP47A (NM_103848) with RRM were studied. Previous report showed that AtRBP45A and AtRBP47A bound AU-rich RNA fragment [13]. Subsequently, AtRBP45A and AtRBP47A were cloned into pGEX-6p-1 for expression to validate the interaction, respectively. The recombinant proteins were purified with GST-tag described previously, and confirmed by Western blot. Results established that both of them were highly pure and suitable for further studies (Supplementary Figs. 7 and 8). Moreover, two recombinant proteins were dialyzed against DEPC-treated buffer for EMSA. EMSAs were performed with a fixed concentration of 300 nM rAtRBP45A and 200 nM rAtRBP47A and increasing concentrations of Cu^{2+}, respectively. Results showed the RNA binding affinities of these two RRM containing proteins were expectant reduced (Fig. 4a and b). No homomultimers of rAtRBP45A and rAtRBP47A were observed, and the RNA binding affinities was bona fide disrupted in the presence of Cu^{2+}.

4. Discussion

Copper is an important trace element in all organisms and is involved in several vital processes and Cu^{2+} is also important as the catalytic center of many enzymes and as a cofactor with redox capabilities [21,1]. It was proposed that GRPs play an important role in the regulation of transcriptional and post-transcriptional events [3].

In this study, we investigated the interaction between GRP162 and ions such as Fe^{3+} and Cu^{2+}. Despite the interaction between Fe^{3+} and GRP162 directly, Fe^{3+} did not enhance the RNA binding affinity of GRP162, and we cannot exclude the possibility that Fe^{3+} might be involved in other GRP162 functions, which will be elucidated in future. The interaction between Cu^{2+} and GRP proteins impaired the function of GRPs, which play an important role in the regulation of transcriptional and/or post-transcriptional events. Based on observations from CD spectra, our study determined that Cu^{2+} destroyed the secondary structure of GRP162 and disrupted its biological activity. Cu^{2+} may disrupt the RNA binding affinity by interacting with specific amino acids or by aggregation, and these possibilities should be investigated in further studies. Furthermore, two RRM containing proteins, AtRBP45A and AtRBP47A, were studied to validate the interaction between RRM and Cu^{2+}. Data confirmed the interaction greatly interrupted their RNA binding affinities, implied that Cu^{2+} impaired the biological function of RRM containing proteins in general. It can be proposed that Cu^{2+} in excess impairs plant or animal development by disrupting RNA binding proteins and possibly by other unknown pathways. These data suggest that the homeostasis of Cu^{2+} is important for either suppressing or activating the function of proteins in various biological processes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.006.

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