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ARTICLE

The role of phosphorylation in the function of RBR-interacting phosphatase subunit

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ABSTRACT Protein phosphatase 2A (PP2A) is a serine/threonine-specific phosphatase comprising a catalytic subunit(C), a scaffolding subunit (A), and a regulatory subunit (B). The B subunits are believed to be responsible for substrate specificity and localization of the PP2A complex. In plants, three families of B subunits exist, i.e. B (B55), B′, and B″. Here, we focused on one of the B″ regulatory subunit of rice. In yeast two hybrid analyses, the B″ regulatory subunit shows strong interaction with the rice retinoblastoma-related protein 1 (OsRBR1) but does not associate with OsRBR2. We created deletion mutants of OsRBR1 and the OsPP2A B″ regulatory subunit, the further pairwise assays revealed that the interaction between the OsRBR1 and OsPP2A B″ proteins needs an intact pocket domain of retinoblastoma-related protein and the presence of the EF-hands domains on the regulatory subunit. Computer-assisted analysis predicted the presence of three potential CDK phosphorylation sites in the amino-terminal region of the OsPP2A B″ regulatory subunit. The phosphorylation assay of the site-directed mutants, which created according to this prediction, has supported the potential phosphorylation. *In vivo* and *in vitro* assays indicated that the site-directed mutation of three phosphorylation sites can affect the phosphorylation of OsPP2A B″regulatory subunit and may affect the PP2A-linked phosphatase activity, but did not affect the binding to OsRBR1 and to the OsPP2A catalytic subunits.
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KEY WORDS

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The retinoblastoma tumor suppressor gene encodes a nuclear phosphoprotein (pRB) that regulates the G1 /S transition of the cell cycle. The active form of pRB binds and inactivates transcription factors, including members of the E2F family, whose target genes are necessary for S phase. The activity of pRB is regulated by phosphorylation/dephosphorylation cycles of serine and threonine residues. pRB is phosphorylated by members of the cyclin-dependent family of serine/threonine kinases whose active forms consist of a catalytic subunit (CDK) complexed with a cyclin partner (Weinberg RA 1995; Sherr, CJ 1996). The major kinases that phosphorylate pRB during G include cyclin E-CDK2, cyclin D-CDK4, and cyclin D-CDK6. The phosphorylation part of pRB is well documented, in contrast, little is known about the way in which phosphates are removed from pocket proteins during the cell cycle.

The retinoblastoma protein has been shown to interact directly with the protein phosphatase1 (PP1) catalytic subunit during the M phase of the cell cycle, when pRB is known to be dephosphorylated (Durfee et al. 1993; Nelson and Ludlow 1997). Besides PP1, Protein phosphatase 2A (PP2A) also plays a direct role in restricting the phosphorylation of pocket proteins through the cell cycle (Garriga et al. 2004).

PP2A is a serine/threonine-specific phosphatase comprising a catalytic subunit(C), a scaffolding subunit (A), and a regulatory subunit (B). The regulatory subunits determine the substrate specificity as well as the spatial and temporal functions of PP2A (YiGong 2009).

Two retinoblastoma-related (RBR) genes have been found in rice, OsRBR1 and OsRBR2. OsRBR2 is expressed mainly in differentiated cells, the function of the OsRBR1 gene may be related to cell division or cell cycle progression (Lendvai et al. 2007). The OsPP2A B" regulatory subunit, which was identified from our previous yeast two-hybrid screens, showed strong association with OsRBR1 but did not interact with OsRBR2. The PP2A protein phosphatase complex containing the identified B" regulatory subunit may have an important role in the dephosphorylation of rice RBR proteins during cell cycle progression and /or in response to extracellular stimuli. To verify this hypothesis, we designed several experiments to understand the role OsPP2A B" in detail. As a first approach we would have liked to analyze the role of CDK-mediated phosphorylation in the function of B" regulatory subunit.

Materials and Methods

Deletion mutagenesis of OsRBR1 and OsPP2A B" and site-directed mutagenesis of OsPP2A B" were conducted by PCR

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with corresponding primers, and PCR fragments were ligated into pET28a via convenient cleavage site. All the mutations were checked by sequencing.

For yeast two-hybrid experiments, the vector constructs pBD-GAL4 2.1/OsRBR1 and deleted versions were used as bait, and pGAD424/OsPP2A B" regulatory subunit and its mutants were used as prey. The cloning vectors pBD-GAL4 2.1, and pGAD424 were obtained from Clontech and Stratagene (La Jolla, CA, USA). The *Saccharomyces cerevisiae* yeast strain PJ69-4a (James et al. 1996) was transformed with the appropriate constructs, the transformants were grown on –Trp, –Leu and –Trp, –Leu, –His, –Ade medium, with monitoring of the activation of the HIS3 and ADE2 reporter genes. The protein association strength was quantified with the ONPG substrate of β-galactosidase, enzyme activity was determined according to Horváth et al. (1998).

Protein extracts from rice suspension cells were prepared according to Magyar et al. (1997). The purified recombinant His-tagged mutants of OsPP2A B" protein were phosporylated by the CDK complex bound on $p13^{SUC1}$ (Sigma). The reaction was monitored by detecting incorporated radioactive ^{32}P-labelled phosphate using Phosphor- Imager SI (Molecular Dynamics). Immunoprecipitations were done according to Ábrahám et al. (2011).

Results and Discussion

In order to identify which part of the OsRBR1 protein is required for the interaction with OsPP2A B" regulatory subunit, we performed deletion analysis. Like all the other pocket proteins, OsRBR1 can be divided into the N-terminal region, the pocket domain (include A, B domain and the spacer between A and B) and the C-terminal region. Deletion mutants containing the N-terminal region; N-terminal plus A domain; N-terminal, A domain plus spacer region; N-terminal plus whole pocket domain (C terminal deleted). The yeast two-hybrid experiment results showed that only the deletion mutant with intact pocket domain could interact with the OsPP2A B" regulatory subunit. A similar deletion experiment was done on OsPP2A B" regulatory subunit, and the OsPP2A B" mutant, which did not have the C-terminal EF-hand domain showed no association with OsRBR1 protein. Our experiments demonstrated that the interaction between the OsRBR1 and OsPP2A B" proteins needs an intact pocket domain of retinoblastoma-related protein and the presence of the EF-hands domains on the regulatory subunit.

Computer-assisted analysis of the OsPP2A B" amino acid sequence revealed the presence of three putative CDK phosphorylation sites in the N-terminal region of the protein. The following experiments showed that the CDK-cyclin complexes isolated from actively dividing rice suspension culture could phosphorylate both GST- and His-tagged OsPP2A B" protein.

Following this two sets of experiments were performed to verify phosphorylation sites. In the first one, four OsPP2A B" fragments containing different phosphorylation sites were created. The phosphorylation of those fragments supported also that the three potential CDK phosphorylation sites could be really phosphorylated. The second experimental set verified this result. A series of site-directed mutants of OsPP2A B" regulatory subunit were created, including three single, three double and one triple mutants. The double mutants showed lower phosphorylation levels than the wild-type and single mutants, and the triple mutant showed phosphorylation only at background level. The results of proteomic analysis also verified the phosphorylation of two out of three amino acid residues tested previously.

All the results of the yeast two-hybrid analyses, phosphorylation of site-directed mutants and immunoblotting demonstrated that the mutation of CDK phosphorylation sites of OsPP2A B" did not affect the interaction with OsRBR1 and OsPP2A catalytic subunits, however the OsPP2A holoenzyme containing OsPP2A B" triple mutant did not show any phosphatase activity. This suggests that the CDK-mediated phosphorylation has an activatory function on OsPP2A B" regulatory subunit containing PP2A phosphatase activity.

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