Plant RBR proteins are phosphorylated in cell cycle-phase dependent manner and the B" regulatory subunit containing OsPP2A holoenzyme mediates the dephosphorylation of OsRBR1

Summary of the PhD thesis

Ping Yu

Supervisor: Dr. Horvath Gabor

Biological Research Centre, Hungarian Academy of Sciences

Institute of Plant Biology

University of Szeged

Szeged

2015

SUMMARY

Introduction

The ability of the pRB (retinoblastoma protein) to inhibit cellular proliferation is counterbalanced by the action of CDKs. In quiescent and early G1 cells, the RB protein in non-phosphorylated and efficiently E2F binding state. The pRB-E2F complex down-regulate the activity of E2F and preventing E2F-dependent transcription. pRB became phosphorylated as cell progress towards to the S phase. Phosphorylation of pRB inactivates the pRB-E2F complex and releases E2F, allowing the expression of E2F-regulated genes required for cell cycle progression and S phase entry. The initial phosphorylation of pRB is most likely catalyzed by Cyclin D-CDK4 or Cyclin D-CDK6 complexes. Subsequently, Cyclin E-CDK2 complexes phosphorylate the pRB protein further, increasing the phosphorylation level. During mitosis, pRB is rapidly dephosphorylated by a specific mitotic phosphatase that removes the phosphate group from the pRB protein, thus restoring the dephosphorylated or hypophosphorylated form of the pRB protein at the start of the G1 phase.

Phosphorylation of plants RBR (RB-related protein) also occurs in a cell-cycle-dependent manner. The highest phosphorylation state occurs during the G1-S transition and is maintains till mid/late S and G2 phases. Considering the phylogenetic conservation of several cell cycle regulation elements (CDKs; cyclins) between animals and plants, this phosphorylation also results in an inactivation of RBR and the subsequent release of E2F-DP transcription factors in their active form.

Protein phosphatases types 1 and 2A (PP1and PP2A) have been implicated in the control of cell cycle events in yeast and mammalian cells and have been characterized as RB or RB-like protein phosphatase. Yan et al. (1999) proposed that PP1 is involved in the direct dephosphorylation of pRB while protein phosphatase 2A (PP2A) is involved in the dephosporylation components of pathways that play a role in the regulation of G1 cyclin-dependent kinase activity. PP2A may also have an important cell division regulatory role, since its activity contributes to the control of mitotic kinases and microtubule organization in alfalfa (Ayaydin et al., 2000).

PP2A consists of three distinct functional components: a structural core subunit (PP2Aa/PR65), a catalytic subunit (PP2Ac) and a B regulatory subunit. A and C subunits usually bind each other to form

the core enzyme $PP2A_D$. The binding of different B subunits to the AC core enzyme generates a broad variety of holoenzymes thereby regulating phosphatase activity and substrate specificity (Janssens and Goris, 2001). The ability of the PP2A holoenzyme to dephosphorylate specific intracellular proteins depends on its activity and appropriate intracellular localization, which in turn is largely determined by the nature of its regulatory subunit.

In a previous yeast two-hybrid screen in our lab, a gene product from *Medicago truncatula* was identified as interactor of the full-length MsRBR11 protein. This protein shares 39% identity and 57% homology with the murine PR59 PP2A regulatory subunit, and strongly associates with OsRBR1 but not with OsRBR2 (Lendvai et al., 2007). It is interesting to note that a similar selectivity occurred among Murine RBs. Only the p107 protein strongly interacts the PR59 PP2A B'' regulatory subunit while pRB did not show association (Voorhoeve et al., 1999b). This analogous behavior also supports the assumption that plant RBRs belonging to different subfamilies (see 1.2.1) have different roles in the regulation of plant cell division and differentiation (Dudits et al., 2011).

In our work, the rice homologue of the *MsPP2A B''* gene, *OsPP2A B''*, has been isolated. The pair wise interaction studies confirmed that the OsPP2A B'' regulatory subunit interacts specifically with the OsRBR1 protein.

Aims of study

Initially we were looking for the specific antibodies for non-phosphorylated and phosphorylated forms of RBR proteins of *Medicago* and rice, which will help to monitor the protein during the cell cycle of cultured cells and detect the presence of the proteins in all other experiments. For a deeper understanding of the molecular basis of these divergent functions of plant RBR proteins it is essential to know the cell cycle phase-dependent changes in levels and post-translational modifications of these proteins.

The results of previous work indicated the specific interaction between OsPP2A B" and OsRBR1. We were wondering which domain within the OsRBR1 essential for the formation of OsPP2A B"-OsRBR1complex and if the EF-hand of OsPP2A B" important for the binding. In addition, for the OsPP2A B" regulatory subunit, we were interested in its post-transcriptional modification.

To sum up, we focused on the following points:

1) Measure the cell cycle phase-dependent variation in the amounts of the phospho-MsRBR11 and the phospho-OsRBR1 proteins, which helps to understand the differences in the phosphorylation of RBR proteins in monocots and dicots.
2) To analyse the interaction between OsRBR1 and the OsPP2A B" regulatory proteins *in vivo* and *in vitro*.
3) To understand which domains in both OsRBR1 and the OsPP2A B'' regulatory proteins are important for their interaction.
4) Based on the results of the computer-assisted phosphorylation site analysis, to study the effect of phosphorylation on the function of the OsPP2A B'' regulatory protein:
    - identify the phosphorylated amino acid residues;
    - study the effect of phosphorylation on OsRBR1 binding and phosphatase
    - activity using non-phosphorylatable and phosphorylation-mimicking mutants.

Material and Methods

- Construction of *Oryza sativa* var. japonica cv. UNGGI-9 cDNA libraries for yeast two hybrid screening
- Yeast two-hybrid screens, pairwise interaction analysis and the interaction quantitation (β-galactosidase activity).
- Plant suspension culture synchronization (alfalfa and rice) and flow cytometry.
- GST- and His-tagged proteins purification
- Protein phosphorylation (deletion mutants and site-directed mutants of B" regulatory subunit)
- Immuoprecipitation
- Protein phosphatase activity (OsPP2A heterotrimeric holoenzyme contains different B" regulatory subunit: wild type, non-phosphorylatable and phosphorylation-mimicking mutants) under different conditions

Results and short discussion

Our work started with the testing of the specific antibodies for MsRBR1/OsRBR1and phospho-MsRBR1/OsRBR1 detection. The anti-AtRBR1 antibody produced against the recombinant C-terminal fragment of the Arabidopsis AtRBR1 protein consisting of 236 amino acids (Horváth et al., 2006). The antigen competition experiment supports the use of this antibody in the alfalfa experimental system. Since the NVYV**S**PLR**S** motif in the C-terminus of the alfalfa MsRBR1 protein may correspond to the NIYI**S**PLK**S** motif of the human pRB protein which has a conserved 807/811serine (S) phosphorylation site. It has the possibility that the level of phospho-MsRBR1 can be monitored using an antibody produced against the phosphorylated pRB peptide containing Ser807/811. Experiments carried out to phosphorylate the recombinant His_6-tagged C-terminal fragment of the MsRBR1 protein, and then monitored the phosphorylation using the antibody and the incorporation of ^{32}P in parallel. Two parameters agree each other quite well; this means the antibody can be used to detect the phosphorylated MsRBR1 protein. Similar experiments carried out on rice found that the monoclonal antibody produced against the recombinant N-terminal fragment of the OsRBR1 and the human anti-phospho-pRB antibody can be used in rice experimental system as well.

Probed by those antibodies, we checked the variation of RBR and phospho-RBR proteins in alfalfa and rice cultured cells with cell-cycle dependent manner. Total amount of MsRBR1/OsRBR1 barely changed throughout the cell cycle, while phosphorylated forms of MsRBR1/OsRBR1 proteins showed clearly cell cycle dependent changes.

Since OsRBR1 associates with the OsPP2A B" regulatory subunit both *in vivo* and *in vitro*, we were wondering about the segment of the binding domain in OsRBR1 necessary for the formation of the complex. Yeast two-hybrid experiments between a set of deletion mutants of OsRBR1 and the OsPP2A B" regulatory subunit demonstrated that an intact B pocket domain is essential and sufficient for the interaction. The C terminus of OsRBR1 seems to be unnecessary for interaction.

The sequence analysis of the identified OsPP2A B" (*Os10g476600*) revealed that it contained two EF-hand domains which may potentially regulate its function by Ca^{2+}-binding. To determine if the EF-hand domain is necessary for the formation of the OsRBR1/OsPP2A B" complex, yeast two-hybrid experiments were carried out with the wild type OsRBR1 and a series of delete mutants of OsPP2A B"

(just mimus C-terminal, minus C-termianl and EF2, mimus C-termianl and two EF-hand, fragmeng only with C-termianl). It was clearly shown that none of the truncated mutants can bind to OsRBR1. This result confirms the importance of the C-terminal region of OsPP2A B" but also verified that the C-terminal itself does not sufficient for combination. The result showed that the full length OsPP2A B" is strongly self-activated, which may be related to the acidity character of the C-terminal region.

On the basis of all the yeast two-hybrid data we can safely conclude that the interaction between the OsRBR1 and OsPP2A B" proteins requires an intact B pocket domain of the RBR protein and the integrity of B" regulatory subunit. Such a finding supports the hypothesis that the dephosphorylation of plant RBR proteins is influenced by the increase in intracellular Ca^{2+}; responding in this way to extracellular stimuli, like e.g. environmental stress factors. Impairment of the interaction between the B" regulatory subunit and OsRBR1 by low concentration of Ca^{2+} concentrations provides another piece of evidence in support of this hypothesis.

Co-immunoprecipitation and recombinant protein phosphorylation experiments demonstrated that the OsPP2A B" regulatory subunit could be phosphorylated by PSTAIRE motif containing CDKs. Computer-assisted analysis revealed the presence of three putative CDK phosphorylation sites in the N-terminal region of the protein. A proteomic method proved that Ser95 and Ser119 can be phosphorylated but could not prove the Ser102 because of technique problem; phosphorylation studies on a series of deletion mutants confirmed the Ser95 and Ser119, and verified Ser102 at the same time.

Elimination of protein phosphorylation by serine to alanine exchange is a quite popular method used to verify putative phosphorylation sites. A series of site-directed mutants of the OsPP2A B" regulatory subunit was created. The results of mutants' phosphorylation showed that single mutants, double mutants, and triple mutant phosphorylated in apparently different levels. The experiment confirmed three predicted phsophorylation sites, and also revealed that there are no more potential phosphorylation sites within the OsPP2A B" regulatory subunit.

The triple alanine mutant (TMutA) and phosphorylation mimicking mutant (TMutE) were used to check the PP2A holoenzyme complex assembly and the interaction with OsRBR1. The results agreed with those of Davis (2005): mutations of the phosphorylation sites, by either ablating the phosphrylation site or producing a phosphorylation mimic, had no apparent affect on binding of PR70 to the PP2A core dimer (A and C subunits). Data from pull down assays and yeast two-hybrid

interaction assays between pBD-Gal4-OsPP2A B" mutants and pAD-Gal4-OsRBR1 also supported the observation that these site-directed mutations did not affect the association between B" and the catalytic subunit, but non-phosphorylation of B" does impact on the interaction to OsRBR1. It seems that phosphorylation of B" regulatory subunit does not play a role in the combination of PP2A holoenzyme but influence the affinity of OsRBR1.

Under standard condition and low concentration of free Ca^{2+} condition, TMutA contained PP2A holoenzyme showed lower phosphatase activity to $PP2A_{Twt}$ while $PP2A_{TtmE}$ showed higher phosphatase activity than the $PP2A_{Twt}$, which means that the phosphorylation of B" indeed elevates the activity of the PP2A heterotrimeric holoenzyme. This experiment also indicated the effect of Ca^{2+} on the activity of PP2A, e.g. proved the importance of post-transcriptional regulation of the B regulatory subunit.

Publications

Ábrahám E*, **Yu P*,** Farkas I, Darula Z, Varga E, Lukács N, Ayaydin F, Medzihradszky KF, Dombrádi V, Dudits D, and Horváth GV. (2015) The B" regulatory subunit of protein phosphatase 2A mediates the dephosphorylation of rice retinoblastoma-related protein-1. Plant Molecular Biology 87: 125-141.

*: Equal contribution

IF: 4,257

Ábrahám E, Miskolczi P, Ayaydin F, **Yu P**, Kotogány E, Bakó L, Ötvös K, Horváth GV, and Dudits D. (2011) Immunodetection of retinoblastoma-related protein and its phosphorylated form in interphase and mitotic alfalfa cells. Journal of Experimental Botany 62: 2155-2168.

IF: 5,364

Yu P, Ábrahám E, Darula Z, Dudits D, Horváth VG (2011)
The Role of Phosphorylation in the Function of RBR-interacting Phosphatase Subunit. ACTA BIOLOGICA SZEGEDIENSIS 55: 189-193.

Conference paper/lecture/poster

Ábrahám E, **Yu P,** Farkas I, Darula Zs, Varga E, Lukács N, Ayaydin F, Medzihradszky KF, Dombrádi V, Dudits D, and Horváth GV.(2014) The role of the B" subunit of PP2A phosphatase complex in the dephosphorylation of *Oryza sativa* retinoblastoma-related protein-1. Biokémia 38: pp. 63-63.

Farkas I., Ábrahám E., **P Yu**, Dombrádi V., Dudits D., Horváth VG.(2010) A protein foszfatáz 2A B" regulátor alegységével kölcsönható fehérjék vizsgálata rizsben. Biokémia 34: pp. 33-33.

Ábrahám E, **Yu P,** Ayaydin F, Darula Zs, Farkas I, Lukács N, Dudits D and Horváth GV.(2010) Plant phosphatases, cyclin-dependent kinases and retinoblastoma-related proteins: let's dance together Lecture on Straub-Days, BRC HAS

Farkas I., Ayaydin F., Kókai E., Ábrahám E., **Yu P**., Dombrádi V., Dudits D., Horváth VG.(2009) Rizsből származó protein foszfatáz 2A B" regulátor alegység szerepének vizsgálata rizsben és dohányban, Biokémia 33, pp. 25-25.