Theses of the Ph.D. dissertation

Features of the plant NRP proteins: from phosphatase inhibition to heat stress tolerance

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Szeged
2013

INTRODUCTION

Even small environmental changes can induce gene expression or repression of hundreds of genes in plants, contributing to their endless developing. Regulation of such a synchronized event has to employ chromatin remodelling – a process that involves posttranslational modifications of histones.

One of the putative proteins involved in the regulation of histone modification patterns is NRP1 ("Nucleosome assembly protein 1-related protein") in Arabidopsis thaliana (At NRP1). This protein belongs to the NAP1 family of potential histone chaperones, which possess a central conserved NAP domain, and a highly acidic C-terminal domain. Histone chaperones play a crucial role in nucleosome assembly and are thought to be necessary for prevention of nonproductive aggregation between highly positive charged histones and highly negative charged DNA.

NAP1 represents the primary chaperone of H2A and H2B and is highly conserved from yeast to human. Mammalian NAP1 proteins have multiple roles, including nucleosome assembly, histone trafficking and the regulation of cell cycle and transcription.

In plants, the first cDNA encoding NAP1 was reported in soybean. Later studies revealed that tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*) NAP1 proteins also belong to multigene families. The different members of the tobacco and rice NAP1 group proteins have distinct subcellular localizations and appear to have specific functions. Loss-of-function allelic triple

mutants of the three ubiquitously expressed Arabidopsis NAP1 genes, *nap1;1*, *nap1;2* and *nap1;3* show a normal growth phenotype, and only exhibit detectable defects in DNA repair under stress conditions.

In addition, plants contain genes encoding more distantly NAP1-related proteins (NRPs), which form a distinct phylogenic group more closely related to the animal SET/I_2^{PP2A} proteins. SET was identified first as the product of a translocated gene in acute undifferentiated leukemia. In biochemical assays, SET/I_2^{PP2A} proteins stimulate replication of the adenovirus genome and inhibit protein phosphatase 2A (PP2A). The SET/I_2^{PP2A} protein was also identified in protein complexes with histone acetylation and methylation enzymes, with B-type cyclins, with a granzyme A–activated DNase, and with transcription factors.

Although its human counterpart is a well characterized protein, we have little information on the plant NRP proteins. Arabidopsis thaliana SET-orthologs, named as NAP1-related protein

NAP1-related protein 1 and 2 (NRP1 and NRP2) have important roles in cell cycle regulation and root meristem formation, in addition to their potential histone chaperone function. The Arabidopsis *nrp1-1 nrp2-1* double mutants exhibit extreme sensitivity against genotoxic stress and impaired somatic homologous recombination. Furthermore, the expression of the *Medicago sativa* NRP gene has been found to be upregulated by the auxin analogue 2,4-dichlorophenoxyacetic acid during somatic embryogenesis.

METHODS

• Plant and cell culture maintenance, stress induction

• Arabidopsis protoplast isolation and transformation

• Phosphatase activity measurement

•Co-immunoprecipitation and chromatin immunoprecipitation (ChIP)

• Genetic engineering methods, GATEWAY technology

• RNA isolation, cDNA synthesis

• Real-time polymerase chain reaction

• Bacterial protein expression and purification, antibody production

• Plant protein purification, Western blot analysis,

• Immunolocalization

• Fluorescence loss in photobleaching (FLIP)

• Confocal laser scanning microsopy

RESEARCH OBJECTIVES

The animal homologues of the NRP1 protein act *in vitro* and *in vivo* as protein phosphatase 2A (PP2A) inhibitors. Analysis of deletion mutants revealed that the region between amino acids 25-119 of the human SET/I2PP2A protein is indispensable for inhibition. As this region exhibits high similarity between the plant and the human proteins, our first aim was to investigate the phosphatase inhibitor capacity of plant NRPs. The Drosophila SET/I_2^{PP2A} protein has been

shown to be accumulated at active loci marked by histone H3 Ser10 phosphorylation during heat shock. Therefore we tested the effect of plant NRP proteins on histone H3 dephosphorylation and on gene expression during heat response. Our aim was to investigate At NRP1 overexpressing and *nrp1-1 nrp2-1* double mutant plants, particularly considering their heat stress tolerance.

RESULTS

1. The purified Arabidopsis NRP1 protein and the purified *M. sativa* NRP protein inhibited the phosphatase activity of rabbit skeletal muscle PP1 and PP2A catalytic subunit ($PP1_C$ and $PP2A_C$) preparations.
2. Immunoprecipitated Arabidopsis PP2A dephosphorylated the phospho-Ser10 residue of histone H3, and this reaction was inhibited by the purified At NRP1 in a dose dependent manner.
3. NRP proteins interact with $PP2A_C$ and with histone H3 phosphorylated at the 10th serine residue (histone $H3_{(pSer10)}$) *in vivo*, as verified by co-immunoprecipitation.
4. The absence of NRP proteins in *nrp1-1 nrp2-1* Arabidopsis mutants has no effect on the overall level of histone $H3_{(pSer10)}$.
5. By the use of chromatin immunoprecipitation, we showed that heat-induced gene expression is associated with histone $H3_{(pSer10)}$-bound chromatin in *Arabidopsis thaliana*.

6. NRPs, however, do not directly mediate heat-induced gene expression, since there was no difference in the expression levels of heat shock protein (HSP) genes between the double mutant and the wild type plants.
7. Based on immunolocalization and western blot fractionation experiments, we can say that
    a. At NRP1 is localized in the nucleus regardless of heat treatment
    b. At ambient temperature (25°C), it is in the soluble fraction of the nucleus. Since it is a very small protein, it may diffuse out of nuclei during their isolation
    c. At 45°C At NRP1 binds to other proteins (chromatin) strongly, therefore stays in the nucleus during subcellular fractionation.
    d. Other types of stresses including heat stress at 37 °C, salt stress or heavy metal stress did not cause the nuclear immobilization of At NRP1.
8. Overproduction of At NRP1 leads to a slightly elevated tolerance against severe heat stress.
9. At NRP1 overexpressing plants do not exhibit altered overall phenotype or altered histone H3($_{pSer10}$) level as compared with the null segregant plants.

DISCUSSION

Arabidopsis and Medicago NRPs were found to inhibit animal PP2A activity *in vitro*. The phosphatase inhibitory activity of human SET/I_2^{PP2A} was reported for a 20 kDa truncated form, while in the present study full length plant proteins were assayed. Phosphatase activities were determined with either phosphorylase a or phosphohistone substrates. The recombinant Arabidopsis thaliana NRP1 (At NRP1) protein was able to inhibit the activity of both PP1 (protein phosphatase 1) and PP2A (protein phosphatase 2A) enzymes (purified from rabbit skeletal muscle) on both substrates in a dose dependent manner. However, there was a considerable difference in their efficiency: the At NRP1-mediated inhibition was more efficient on the catalytic subunit of PP2A (PP2Ac) than on PP1. The results also indicated that the phosphatase inhibition power of NRP1 is substrate specific, the protein acts more effectively on phosphohistone substrates *in vitro*, as compared with phosphorylase a. Similar results were obtained with a purified *Medicago sativa* NRP protein indicating that this inhibitory activity of plant NRP proteins is likely a general feature.

We tested the effect of At NRP1 on immunoprecipitated *Arabidopsis thaliana* PP2A as well, using histone $H3_{(pSer10)}$ substrate (histone H3 phosphorylated at the tenth serine residue). When increasing concentrations (0.5-2 μM) of purified At NRP1 were added to the phosphatase reaction, the dephosphorylation reaction was inhibited in a dose dependent manner *in vitro*. An unrelated His tagged protein

(His-Ms ROP6) had no effect on the phosphatase, proving that the inhibition was not caused by the basic tag of the fusion proteins.

In order to support the assumption that NRPs can serve as PP2A inhibitors *in vivo*, pull down assays were carried out. PP2A catalytic subunit antibody co-immunoprecipitated NRP proteins from Arabidopsis as well as from Medicago cell extracts. The presence of the Arabidopsis NRP protein in histone $H3_{(pSer10)}$ antibody-precipitated chromatin was also demonstrated. These assays clearly indicate the potential interaction of NRPs with PP2A and histone $H3_{(pSer10)}$ *in planta*.

NRP proteins have been reported to possess histone chaperone-like properties, to influence cell division, gene silencing, and sensitivity against DNA damaging agents. Most of these functions require nuclear localization of the NRP proteins and indeed, we detected fluorescent protein-NRP fusions in the nuclei. Our results indicate, however, that under normal conditions NRPs are not chromatin-bound but soluble: they are readily released from the nuclei during their isolation and are highly mobile within the nuclei as determined by a FLIP assay (fluorescence loss in photobleaching). Interestingly however, these proteins get strongly bound to the chromatin following short heat-treatment of cells/seedlings. Only strong heat-treatment ($>42^0$C) of Arabidopsis cells resulted in the nuclear retention of NRPs, in contrast to the untreated, or mild-heat (37^0C) treated cells. Immobilization of these proteins seemed to be persistent: nuclei of heat-treated cells still keep NRPs at 42 hours after the treatment. Other stress treatments, including salt, heavy

metal, genotoxic and UV stress, did not resulted in the nuclear retention of NRPs.

We tested the effect of deletion and overexression of At NRP1. Neither the mutant, nor the overexpressing plant lines showed differential levels of histone $H3_{(pSer10)}$, as compared with the wild type or null segregant control plants. The At NRP1 overexpressing transgenic plants did not show any apparent phenotype, however, they possess slightly elevated heat tolerance capacity.

Taken together, our data show that in plants NRPs are among the mobile, soluble nuclear proteins that can form stable complex with the chromatin in response to heat. They inhibit the serine/threonine phosphatase activity of PP1 and PP2A enzymes in a substrate-specific manner. Nevertheless, Arabidopsis NRP proteins interact with PP2A and histone $H3_{(pSer10)}$ based on co-immunoprecipitation experiments, suggesting a role in the regulation of histone dephosphorylation.

PUBLICATIONS

[1]**Bíró, J**., Farkas, I., Domoki, M., Otvös, K., Bottka, S., Dombrádi, V., & Fehér, A. (2012). The histone phosphatase inhibitory property of plant nucleosome assembly protein-related proteins (NRPs). *Plant physiology and biochemistry: PPB / Société française de physiologie végétale*, *52*, 162–8.

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[1] This publication served as the basis of the dissertation