Thesis of Ph.D. dissertation

Identification of interacting partners of the PP4 phosphatase and investigation of its substrate-recognition mechanism

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Introduction

Reversible protein phosphorylation is one of the most common post-translational modifications in eukaryotes and is essential in the regulation of various physiological processes. The phosphorylation state of the proteins is determined by the precisely coordinated action of protein kinases and protein phosphatases. While protein kinases have long been one of the best-known families of enzymes, the knowledge available about protein phosphatases is limited.

PP4 phosphatase, a member of the PPP family of Ser/Thr phosphatases, most commonly functions as a three-subunit holoenzyme. The holoenzyme consists of a catalytic (PP4c), a structural (R2), and a regulatory (R3, Falafel in *Drosophila*) subunit, the latter being responsible for substrate recognition and binding. PP4 is an essential phosphatase that plays a central role in the regulation of the cell cycle, cell division, differentiation, apoptosis, DNA repair, multiple signalling pathways, the immune system and metabolism. Both PP4 deficiency and excessive activity can cause severe cell division disorders, which can lead to the development of various cancers.

Despite their role in cell homeostasis, in the case of most PPPs we still do not know how they select their substrates. For intensively researched phosphatases such as PP1, PP2A, and PP2B, conserved binding motifs that determine phosphatase specificity (directly or indirectly) are already known. These binding motifs belong to the so-called short linear motifs (SLiM). SLiMs are located in essentially disordered parts of proteins, usually a short section of about 10 amino acids in length, within which are a few evolutionarily highly conserved amino acids.

SLiMs are not always cis-acting elements, therefore their presence is not necessarily sufficient to dock phosphatases. It is not yet fully understood, but presumably the environment and protein structure of SLiMs may also influence this property. Knowledge of these motifs provides an opportunity to precisely manipulate the interaction between PPPs and their substrates, which helps us to better understand their function in different processes.

Aims of this study

PP4 is an essential regulator of cellular function. It interacts with a variety of proteins through its different regulatory subunits, but at the beginning of our work, the mechanism of its substrate recognition was not yet known. To investigate the substrate recognition of PP4 with the use of *Drosophila* as model organism, we set the following objectives:

1.) identify new interactional partners of PP4 with the use of the conserved domains of the Falafel subunit

2.) determine the interactional surface between the domains of Falafel and their novel binding partners

3.) determine the conserved substrate recognition motif

By examining the objectives listed above, not only we can better understand the role of PP4 in the regulation of cellular homeostasis and possible ways of its substrate recognition, but the identification of a

conserved substrate recognition motif would also greatly facilitate the identification of potential PP4 substrates.

Materials and methods

DNA constructs and cloning

The cDNAs of the proteins used in the experiments were obtained from the *Drosophila* Gene Collection (Berkeley *Drosophila* Genome Project, *Drosophila* Gold Collection). Classical (restriction) and Gateway techniques were used to generate DNA constructs used in *in vivo* and *in vitro* experiments.

Maintenance of *Drosophila* strains and collection of embryos

The *white*^{1118} *Drosophila* strain was maintained under normal conditions and early syncytial embryos were collected for mass spectrometry experiments.

Generation of stably transfected D. Mel-2 cell lines

Drosophila cell lines producing various transgenic proteins were generated by the use of Cellfectin II reagent according to the manufacturer's protocol (Gibco).

Protein production and purification

Probe proteins used in *in vitro* experiments were produced in *E. coli* and purified to homogeneity using Glutathione Sepharose 4B beads.

Affinity-purification followed by mass-spectrometry

To identify the interacting partners of Falafel, embryonic lysates and purified probe proteins were incubated together, non-specifically binding proteins were removed by several washing steps. After digestion with trypsin, samples were analysed by mass spectrometry.

In vitro binding experiment and autoradiography

To identify the real physical interacting partners, probe proteins were incubated with ^{35}S-methionine-labeled proteins produced by an *in vitro* transcription/translation (IVTT) system. After denaturing gel electrophoresis and gel drying the presence or absence of interactions were detected by autoradiography. To determine the interactional surfaces, smaller overlapping sections were generated from each protein and then examined as described above.

Site directed mutagenesis

Double amino acid substitution mutants (AxxA and AxPA) of the FxxP and MxPP motifs and L70A and L69A mutants of the Drosophila and human EVH1 domains were generated using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies).

Co-immunoprecipitation from D. Mel-2 cells

For *in vivo* interactional experiments, transiently transfected D. Mel-2 cells were collected. Proteins were isolated from the cell lysates using GFP-trap beads (Chromotek) and then the samples were analysed by Western-blot.

Western-blot

Samples were separated on denaturing gels and blotted onto PVDF membrane. After blocking, membranes were incubated with the appropriate primary and secondary antibodies. Light from the cleavage of the HRP substrate was detected using a gel documentation system.

Summary of the results

To identify the novel interactional partners of the PP4 phosphatase, we purified N-terminally GST-fused EVH1 and Smk-1 form *E. coli* cells and incubated it with lysates of early syncytial *Drosophila* embryos. Affinity purification followed by mass spectrometric analysis identified 40 proteins as potential new interaction partners of PP4.

We performed *in vitro* binding experiments to identify the proteins which are real physical interaction partners of the EVH1 and Smk-1 domains. GST-EVH1 and GST-Smk-1 purified from bacterial cells to homogeneity and immobilized on affinity beads were used as bait proteins. Our prey proteins were produced by a coupled *in vitro* transcription/translation system and labelled with ^{35}S-methionine for autoradiographic detection. Using this method, we were able to identify eight proteins (Prp16, Psc, Incenp, Sowah, Stwl, Centrobin, Miranda and CG8478) as EVH1 interacting partners and eight proteins (Licorne, Nipsnap, RfC4, Zwilch, ZW10, spindle A, γTub23C, and Grip75), which specifically interact with the Smk-1 domain of Falafel.

One of the main objectives of our research was to investigate the modes of the substrate recognition of PP4 phosphatase and to identify a

possible conserved substrate recognition motif. As a first step, we determined the shortest possible interaction surface between each domain and the newly defined interaction proteins. Shorter, overlapping sections of the proteins were generated, and the fragment of each protein required for binding to the EVH1 or Smk-1 domain was identified by *in vitro* binding experiments.

Two experimentally validated *Drosophila* proteins (CENP-C and Miranda) bound by the EVH1 domain of Falafel are known from the literature. Both proteins contain a short motif (FxxP), which is also present at multiple sites in the interaction proteins we identified (except CG8478), suggesting this motif mediates recognition by PP4. A paper was published in late 2019 in which Ueki et al. identified the substrate recognition motif of the human PP4 phosphatase. Their observations are consistent with our findings in *Drosophila*. In addition to the FxxP motif, an MxPP motif was also described by them, so we extended our further studies to this motif as well. For most of our proteins, both motifs occurred at multiple sites in the amino acid sequence, so we replaced the F and P or M and P amino acids at the first and fourth positions of the motifs with alanines by site-directed mutagenesis. Based on our results, each protein has a prominent FxxP or MxPP motif, the mutation of which leads to a large attenuation or complete disappearance of the EVH1 interaction.

We demonstrated that leucine at 70th position of the EVH1 domain of Falafel has a prominent role in the binding of interacting proteins. Substitution of this amino acid to alanine, the EVH1 domain completely lost its binding capacity to the tested *Drosophila* proteins. We have also shown that not only substrate binding through EVH1, but also the essential role of

this leucine is highly conserved among species. While the EVH1 domain of human SMEK1 was able to bind the tested *Drosophila* proteins, replacement of conserved leucine with alanine again led to complete loss of binding.

Based on our results, the recognition and binding of interaction partners through the Smk-1 domain is independent of the EVH1 domain, and none of the proteins interacting with Smk-1 bind to the EVH1 domain. Of the Smk-1 interaction proteins, only ZW10 carries two FxxP motifs, however, their mutation has no effect on the interaction based on our *in vivo* co-immunoprecipitation experiments. Determining how the binding of interaction proteins happens through Smk-1 requires further investigation.

In the framework of an international cooperation, we have shown that PP4 plays an important role in the phosphoregulation of BAF during cell division. PP4 together with CENP-C and BAF form an interdependent, positive feedback network at the centromere. Knockout of any component directly or indirectly affects the function and localization of the other two proteins. Based on our binding experiments, we know that BAF does not bind to either domains of Falafel, however, the PP4/Flfl complex has been shown to dephosphorylate BAF. Consequently, the recognition of the PP4 substrates might be achieved not only directly, through its conserved domains, but also, in a third, indirect way, through a HUB protein, which in the case of BAF is CENP-C.

List of publications **MTMT identifier: 10060623**

Publications associated with the Ph.D. dissertation:

Karman, Z.; Rethi-Nagy, Zs.; Abraham, E.; Fabri-Ordogh, L.; Csonka, A.; Vilmos, P.; Debski, J.; Dadlez, M.; Glover, D.M.; Lipinszki, Z.: *Novel perspectives of target-binding by the evolutionarily conserved PP4 phosphatase.* OPEN BIOLOGY 10: 200343. (2020) (IF: 4,931)

Torras-Llort, M.; Medina-Giró, S.; Escudero-Ferruz, P.; Lipinszki, Z.; Moreno-Moreno, O.; **Karman, Z**.; Przewloka, M.R.; Azorín, F.: *A fraction of barrier-to-autointegration factor (BAF) associates with centromeres and controls mitosis progression.* COMMUNICATIONS BIOLOGY 3:1 Paper: 454 (2020) (IF: 4,165)

Other scientific publications:

Olah, Z.; Bush, A.I.; Aleksza, D.; Galik, B.; Ivitz, E.; Macsai, L.; Janka, Z.; **Karman, Z**.; Kalman, J.; Datki, Z.: *Novel in vivo experimental viability assays with high sensitivity and throughput capacity using a bdelloid rotifer.* ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY 144 pp. 115-122., 8 p. (2017) (IF: 3,974)