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PPEF/PP7 protein Ser/Thr phosphatases

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Abstract PPEF/PP7 represents one of the five subfamilies of the PPP protein Ser/Thr phosphatases. Studies published in recent years point to a role of plant PP7 at a crossroad of different pathways of light and stress signalling. In animals, PPEFs are highly expressed in sensory neurons, and *Drosophila* PPEF phosphatase, rdgC, is essential for dephosphorylation of rhodopsin. Expression profiling suggests that mammalian PPEF may play a role in stress-protective responses, cell survival, growth, proliferation, and oncogenesis. Despite structural similarities of the catalytic domains and the fact that some of these phosphatases are involved in light perception both in animals and in plants, the plant and non-plant representatives of this group have distinct domain architecture and appear not to be orthologues.

Keywords Signal transduction · Protein phosphorylation · Enzyme evolution · Calcium · Calmodulin · Domain structure · Nuclear localisation

Introduction

Protein Ser/Thr phosphatases of the PPP family are found universally in eukaryotes [1–4], and are also present in archaea and eubacteria [5–7]. On the basis of the extent of structural similarity of their catalytic domains, eukaryotic PPP phosphatases are subdivided into five major clades: (1) PP1 [8]; (2) PP2A (including closely related PP4 and PP6) [9–13]; (3) PP2B (calcineurin, sometimes designated as PP3) [14–16]; (4) PP5 [17, 18]; and (5) PPEF/PP7 [19]. The first four PPP subfamilies have been extensively investigated and are either already widely employed [82, 83] or considered as promising drug targets, both in humans [79] and for development of antiparasitic drugs [4, 80, 81]. In a stark contrast, the PPEF/PP7 subfamily remains largely unexplored.

The term PPEF (protein phosphatases with EF-hand domains) was introduced by two groups who cloned these phosphatases from mammals [20, 21]. The term PP7 was used by another group who cloned one of them independently [22], since the last human PPP phosphatase identified by that time was PP6. At the same time, we used the term PP7 to designate novel phosphatases identified in plants [23]. Although designation of the animal and plant phosphatases as PP7 resulted from a fortuitous coincidence, phylogenetic analysis of their catalytic domains suggested that they indeed together form a separate clade [19, 24, 25]. PPEF/PP7, together with PP5, probably diverged from PP1, PP2A and PP2B early in eukaryotic evolution [1]. Phosphatases related to PPEF/PP7 have subsequently been identified in a number of other eukaryotic lineages (reviewed in [4]). Except in plants, they are characterised by the presence of EF-hand domains and will be referred to here as PPEF. Plant representatives of the subfamily have distinct domain architecture, different regulatory regions and will be referred to as PP7.

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PPEF phosphatases

Domain architecture

Catalytic domains of PPEFs are flanked by extended N- and C-terminal domains, which are thought to have

regulatory roles. In addition, inserts of various lengths are present in the middle of their catalytic domains (Fig. 1). Animal PPEFs contain up to five motifs with sequence similarity to EF-hand consensus, yet only two or three of them are likely to be functional [20–22, 26]. In PPEFs from unicellular organisms, the number of functional EF-hands is generally higher and amounts to six EF hands in *Phytophthora*. PPEFs from kinetoplastids lack any functional EF-hand motifs, although their C-terminal domains preserve overall sequence similarity with other PPEF phosphatases [27]. Thus, a high variability in the number and arrangement of EF-hand motifs and the presence of degenerate EF-hands appear to be a typical feature of PPEF phosphatases. This may indicate variations in the PPEF regulation by Ca^{2+} mediated by their C-terminal domains.

A calmodulin-binding site of the IQ type [28] conserved in PPEFs was predicted [19] and confirmed experimentally for *Drosophila* and human PPEFs [29, 30]. IQ motifs are conserved in PPEFs from all eukaryotes, with two exceptions: (1) in kinetoplastids, it is replaced by a domain containing Zn finger motifs [4], and (2) in a dinoflagellate *Karenia brevis*, the N-terminal domain shows no significant similarity to any other proteins. PPEFs are N-terminally acylated, which may play a role in their targeting to membranes [27, 31].

PPEF regulation

The presence of calmodulin-binding site and EF-hand domains suggests that PPEFs are under dual control by Ca^{2+}. This would be reminiscent of another PPP subfamily, calcineurins, which are regulated by calmodulin, and by another EF-hand protein, calcineurin B [15]. Calmodulin binding to rdgC increases its phosphatase activity and is essential for rhodopsin dephosphorylation in vivo [29]. Although the ability of the IQ motifs of human PPEF1 and PPEF2 to bind calmodulin in vitro has been demonstrated [30], calmodulin reportedly has no effect on phosphatase activity of recombinant human PPEF1 [22]. PPEF1 activity requires Mg^{2+} and is stimulated by unphysiologically high Ca^{2+} concentrations [22], which agrees with our direct measurements of Ca^{2+} binding to the EF-hand domain of human PPEF1 (M. Kutuzov, N. Bennett, unpublished work). Thus, the

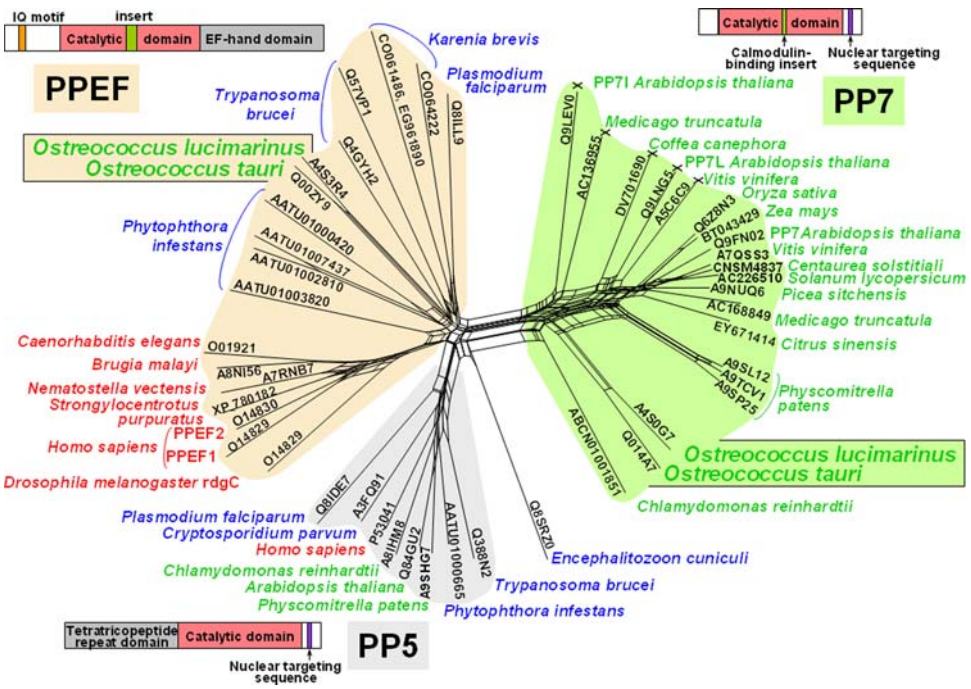

Fig. 1 Structural relationships between the catalytic domains of PPEF, PP7 and PP5 phosphatases from various eukaryotic lineages. Sequences start 5 AA residues before the conserved GDXHGQ motif and end 4 AA after the conserved SAPNY motif. Analysis was performed using Neighbor-Net [78]. Gap sites were excluded. The PPEF, plant PP7 and PP5 subfamilies are shaded in different colours, and their representative domain architectures are shown. Species are colour-coded as follows: *red* animals; *green* plants; *blue* various unicellular eukaryotes. The following groups are represented: animals (nematodes: *Brugia malayi*, *Caenorhabditis elegans*; arthropods: *Drosophila melanogaster*; chordates: *Homo sapiens*; cnidarians: *Nematostella vectensis*; echinoderms: *Strongylocentrotus purpuratus*), apicomplexans (*Cryptosporidium parvum, Plasmodium falciparum*); dinoflagellates (*Karenia brevis*); kinetoplastids (*Trypanosoma brucei*); microsporidians (*Encephalitozoon cuniculi*); oomycetes (*Phytophthora infestans*). *Ostreococcus* species, which have both PPEF and plant-type PP7 phosphatases, are shown in *larger font* and are *colour shaded*. *X* Isoforms predicted to be inactive or to have reduced activity. Accession numbers for each sequence are indicated

role of Ca^{2+} in regulation of human PPEFs remains unclear. The affinity of the EF-hand domain of *C. elegans* PPEF for Ca^{2+} is higher, but still largely beyond the physiological range [31].

A preliminary report suggests that PPEF2 is phosphorylated in vivo at a Ser residue in a long insert in the middle of its catalytic domain (see Fig. 1), and that this site binds 14-3-3ζ protein in a phosphorylation-dependent manner [32]. Functional role of PPEF2-14-3-3ζ interaction is not known.

PPEF functions

RdgC from *Drosophila* was the first discovered PPEF phosphatase [26]. RdgC is rhodopsin phosphatase, and its absence results in a light-dependent apoptosis of photoreceptor cells and in retinal degeneration, triggered by accumulation of hyperphosphorylated rhodopsin [26, 33–35]. Observation of a Ca^{2+}-activated rhodopsin phosphatase activity in mammalian photoreceptor cells in vitro prompted a hypothesis that a mammalian homologue of rdgC may exist and play a similar role in dephosphorylation of rhodopsin, or may even play a more general role in regulating G protein-coupled receptors (GPCRs) [36]. Subsequent identification of mammalian rdgC homologues, PPEF1 and PPEF2, and the findings that they are specifically expressed in sensory neurons, in particular in photoreceptor cells (PPEF2) [20–22, 31], seemed to lend credence to their possible role as GPCR phosphatases. However, mice lacking both PPEF1 and PPEF2 showed no signs of photoreceptor apoptosis or retinal degeneration [37, 38]. CaBP4, an EF-hand protein that regulates Ca^{2+} channels of photoreceptor synapses, was suggested as a possible PPEF substrate [39], but this has not been rigorously tested.

Antisense oligonucleotide-mediated knockdown of PPEF in sea urchin was found to result in endoderm and oral ectoderm defects during larval development [40]. Since *Drosophila* and mice lacking PPEF phosphatases do not show obvious morphological defects, a similar role of PPEFs in development appears not to be conserved at least in arthropods and vertebrates. A pro-survival role of mammalian PPEF2 has been suggested by a siRNA-based screening [41].

The presence of PPEF phosphatases in unicellular eukaryotes [24, 27, 42] indicates an ancient origin of these enzymes, predating the appearance of developed GPCR signalling as found in metazoans. Such ancient origin and considerable conservation in evolution [4] would imply the involvement of PPEFs in some basic functions of the eukaryotic cell. In line with this notion, *Trypanosoma* PPEF may be positively involved in cell growth [27].

Insights into possible PPEF functions from expression profiling

In *Caenorhabditis*, *Drosophila* and mammals, PPEF expression was mainly detected in various sensory neurons [20–22, 26, 31], suggesting a specific role in sensory signalling. However, more recent large-scale expression profiling experiments [43, 44] show that PPEF phosphatases are expressed wider than previously anticipated. Thus, PPEF1 expression is several-fold higher in the testis than in the regions originally reported. Above average expression is also observed: for PPEF1, in the uterus, spinal cord, appendix, bone marrow and B lymphoblasts; for PPEF2, in the testis, oocytes, skeletal and at least some smooth muscles, cardiomyocytes, veins, umbilical cord, appendix, spleen, thymus, tonsil, pharynx mucosa, bone marrow (especially erythroblasts), B-lymphocytes and peripheral blood monocytes. In order to gain insight into possible functions of human PPEF phosphatases, we compiled the publicly available data from microarray experiments (Table 1). These data show that *PPEF* expression is modulated by the conditions that affect the following cellular functions: (1) Expression of PPEF phosphatases is responsive to apoptosis-inducing or apoptosis-inhibiting treatments, and is elevated in the cells resistant to apoptosis (Table 1, Section 1), which is compatible with a role in cell survival; (2) a positive correlation exists between PPEF expression and cell growth, proliferation or oncogenic transformation (Table 1, Section 2); and (3) *PPEF* expression is also affected by expression of proteins implicated in differentiation (Table 1, Section 3).

Thus, some of PPEF functions may be related to apoptotic and oxidative stress responses (in agreement with the data of MacKeigan et al. [41]), cell proliferation, oncogenesis and differentiation. *PPEF* expression is also affected by several peptidases (Table 1): presenilin-related peptidase hIMP1, involved in differentiation; Tmprss6, thought to participate in iron sensing and a dipeptidyl-peptidase DPP3, which induces antioxidant response. This suggests that cleavage of an upstream component may be involved in regulation of *PPEF* expression. An intriguing observation is that *PPEF1* expression is affected by PTEN depletion in three different cell lines, which strongly suggests that its expression is regulated downstream of PTEN.

Plant PP7 phosphatases

Domain architecture

Unlike PPEFs, PP7 (except pseudophosphatase isoforms, see below) do not have extended flanking domains. Two regulatory regions have been identified in PP7: a positively

Table 1 Expression profiling of human PPEF phosphatases

Cell line	Description	Conditions	PPEF1	PPEF2	Reference
1. Apoptotic and oxidative stress responses					
$CD4^+$ T-cells		SDF-1[a]	+	++	[62]
HCT116	Colon cancer	miR-34a[b]	−	(+)	[63]
SW480	Colon cancer	miR-34a[b]	(−)	− −	[64]
SKOV3	Ovarian adenocarcinoma	Vincristin resistance	++	±	[65]
HT29	Colon cancer	Methotrexate resistance	++	±	[66]
IMR-32	Neuroblastoma	ARE[c] activation	±	++	[67]
2. Cell proliferation and oncogenesis					
NK-YS	Lymphoma/leukaemia	Epstein–Barr virus (EBV) infection	ND	+	[68]
B-lympho-blastoid cells		EBNA-2[d]	++	±	[69]
Primary human mammary epithelial cells		RhoA over-expression[e]	+	+	[70]
DAOY	Medulloblastoma	Bmi1/Mel18 depletion[f]	±	−	[71]
3. Differentiation					
Embryonic stem cells		SOX17[g]	−	+	[72]
MCF10A	Mammary epithelial cells	YAP[h]	(−)	+	[73]
HEK 293	Embryonic kidney fibroblasts	Inactive mutant of hIMP1[i]	+	−	[74]
Endometrial stromal cells		PKA activation[j]	ND	+	[75]
4. Other links					
HepG2	Liver carcinoma	Tmprss6[k]	+	±	[76]
A431	Epithelial carcinoma	PTEN depletion	− −	±	[77]
SKBR3	Breast cancer	PTEN depletion	(−)	(+)	
HCC827	Lung cancer	PTEN depletion	+	±	

Designations of PPEF1 and PPEF2: ± = below 1.5-fold; (−) (+) = 1.5- to 2-fold; − + = 2- to 4-fold; − − ++ = >4-fold; *ND* no data

[a] Ligand for a chemokine receptor CXCR4; promotes $CD4^+$ T cell survival via phosphatidylinositol 3-kinase and MAPK pathways

[b] MicroRNA that is directly transactivated by p53 and promotes apoptosis

[c] Antioxidant response element (ARE) induces transcripts of proteins that have antioxidant and neuroprotective roles

[d] Transcription factor essential for EBV-mediated growth transformation

[e] Expression of RhoA results in immortalization and preneoplastic transformation; relative to control with Rho T37A mutant, which is unable to bind effectors

[f] Depletion of these transcriptional regulators results in inhibition of proliferation and tumour suppression

[g] Transcription factor implicated in endoderm determination and maintenance

[h] Coactivator of several transcription factors that promote epithelial-to-mesenchymal transition

[i] Peptidase of the presenilin family, implicated in cell differentiation and development

[j] Activation of the protein kinase A pathway is required for decidualisation of endometrial stromal cells, which is a prerequisite for implantation

[k] Transmembrane peptidase implicated in iron sensing; inactive peptidase mutant has no effect

charged insert in the catalytic domain and an atypical nuclear targeting sequence in the C-terminus (Fig. 1). Inserts in the catalytic domains have arisen in PP7 independently from PPEFs, since their exact positions differ. Comparison of the primary structures of the inserts between different plant species shows a gradual increase in the length of this region and accumulation of positively charged residues from unicellular algae to higher plants (our unpublished observation), which suggests evolution of a progressively more elaborate regulatory site. A pair corresponding to $K^{208}R^{209}$ in *Arabidopsis thaliana* PP7 is absolutely conserved between all catalytically active isoforms. The R209L mutation in *A. thaliana* results in a loss of function [45]. The ability of the R209L mutant to bind calmodulin has not yet been tested.

PP7 is a constitutively nuclear phosphatase [45–47]. Although canonical nuclear localisation signals (NLS) contain stretches of basic amino acids, the positively charged insert is dispensable for nuclear localisation of PP7 [46]. Instead, a conserved sequence in the C-terminus, which shows no similarity with canonical NLS, appears to be necessary for nuclear targeting of PP7 [46]. A homologous sequence is conserved in PP5 (which is also mostly nuclear in plasmodium [48], but can be both cytoplasmic and nuclear in animals) and is also required for its nuclear location [49]. Comparison of the nuclear

targeting sequences in PP5 and PP7 from a variety of species suggests a general consensus for both PP5 and PP7, characterised by the presence of several prolines and a FXAV motif ([49]; and our unpublished data). The presence of a common signal for nuclear targeting of phosphatases from organisms as diverse as metazoans, fungi, plants, apicomplexans and kinetoplastids raises an intriguing question about a putative receptor for this signal, which would be highly conserved throughout eukaryotes. Since nuclear translocation of PP5 can be regulated [50–53], while PP7 is constitutively nuclear [45–47], another interesting question is what are the structural features that determine this difference. In the conserved FXAV sequence, X = Glu or Asp in PP7, but is most often Ser or Thr in PP5 sequences (our unpublished observations). A negative charge might promote nuclear targeting, which in the case of PP5 might be introduced by Ser/Thr phosphorylation.

Two additional isoforms, designated as PP7I (inactive) and PP7L (long) [3], have additional N- and/or C-terminal domains. The N-terminal domain of PP7L shares sequence similarity with mutator-like transposases, while its C-terminal domain is enriched in acid residues and in Ser/Thr and does not show significant similarity to any other proteins (our unpublished data). The N-terminal domain of PP7I shares a weak similarity with transcription factors of the MADS family and some uncharacterised proteins from plants and other eukaryotes, while its C-terminal domain shows no similarity to any proteins (our unpublished data). We were only able to detect homologues of *Arabidopsis* PP7I and PP7L in some dicotyledonous species but not in other plant groups (see Fig. 1), which suggests their recent appearance in evolution. The basic inserts in PP7I and PP7L are not well conserved. Since the inserts may play an autoinhibitory role [54], and enzymatic activity of PP7I and PP7L is presumably impaired by substitutions in their catalytic centres, this may reflect the loss of the necessity to control enzymatic activity in these isoforms.

Regulation

In terms of regulation of PP7 phosphatase activity, the basic insert may play a dual role: it may act as an autoinhibitory region [54] and as a calmodulin-binding site [55, 56]. In contrast to calcineurins and PPEFs, where the calmodulin exerts positive effect on phosphatase activity, PP7 was found to be moderately inhibited by calmodulin, at least in an in vitro assay using recombinant proteins [55]. Whether the activity of endogenous PP7 is Ca^{2+}-dependent has yet to be verified.

P_i is a general competitive inhibitor of phosphatases. However, PP7 is inhibited by submillimolar P_i concentrations mainly non-competitively, which indicates allosteric regulation [57]. This unusual property suggests that it might play a role in sensing phosphate.

PP7 functions

Like PPEFs, *Arabidopsis thaliana* PP7 was suggested to function in sensory signalling [57–59], since it is highly expressed in a subset of stomata [58]. Indeed, in vivo studies using antisense RNA-mediated depletion, PP7 gene knockout and a spontaneous PP7 mutant revealed that this phosphatase is a positive regulator of signalling downstream of blue light receptors cryptochromes [47] and also controls amplification of phytochrome signalling [45]. PP7 was found to interact with nucleotidediphosphate kinase 2 (NDPK2), a positive regulator of phytochrome signalling [45]. Whether PP7 directly dephosphorylates any of the light receptors or NDPK2 remains an open question.

A separate study using PP7 knockout and overexpression identified its positive role in *A. thaliana* thermotolerance due to its interaction with heat shock transcription factor HSF and up-regulation of protective heat shock proteins [56]. A possible involvement of PP7 in salicylic acid-dependent defence signalling has also been suggested [45], which would be compatible with cell wall thickening upon stable overexpression of PP7-GFP in tobacco (our unpublished observations). Salicylic acid has been implicated not only in pathogen defence responses but also in thermotolerance [60]. Thus, available data point to a role of PP7 at a crossroad of different pathways of light and stress signalling.

Plant PP7 and PPEF are not orthologues

PPEF/PP7 are conserved in most eukaryotic phyla, with the notable absence in fungi [1, 4]. Previous phylogenetic analysis indicated that plant PP7 are most closely related to PPEFs [1, 24, 25], suggesting that the plant and non-plant enzymes are orthologous. This was also supported by (1) the absence of PPEFs in plants and the absence of plant-type PP7 in non-plant eukaryotes [1, 19]; (2) expression of these phosphatases in sensory cells both in animals [21, 22, 26, 31] and in plants [58]; and (3) a direct demonstration of the involvement of some of these enzymes in photoreception in animals [26, 33, 35] and in plants [45, 47].

However, as discussed above, the domain composition of the plant and non-plant enzymes is different, which would imply that these phosphatases have undergone a drastic rearrangement of their domain architecture in plants. To re-evaluate the assumption that PPEFs and plant PP7 are orthologues, we took advantage of much more representative sequence data now available and re-assessed the inventory of PP5, PPEF and PP7 phosphatases present in sequence databases. As expected, most retrieved

phosphatase sequences (except a phosphatase from a microsporidial parasite *Encephalitozoon cuniculi* [4]) clearly fall into the three clades corresponding to PP5, PPEF and plant PP7 (Fig. 1). Consistent with the previous observations, all plant-type PP7 are found within green plants, while PPEFs are present in various non-plant eukaryotes (Fig. 1). Nevertheless, we could detect co-existence of both bona fide PPEF and plant-type PP7 in two species of a unicellular green alga *Ostreococcus* (Fig. 1) and a related alga *Micromonas* sp. (data not shown). Although it cannot be ruled out that PPEFs may have been acquired by a particular group of green algae by horizontal gene transfer, this finding suggests that PPEFs and plant PP7 are likely not orthologous and co-existed in early *Viridiplantae*, followed by a loss of PPEFs in plants. Another line of evidence in favour of independent origin of PPEFs and plant PP7 is that the latter share with PP5 an atypical nuclear targeting signal [46, 49], which is absent in PPEFs.

Intriguingly, there seem to be certain parallels between PP7 and PP5 functions. Mammalian PP5 is also involved in cryptochrome signalling [61] and in heat shock factor regulation [50], and plant PP5 is involved, along with PP7, in phytochrome regulation [61]. This suggests that PP5 and PP7 may share similar functions, in line with their possibly common origin.

Conclusions

All known PPP phosphatases are multifunctional, and there is no reason to expect that this is not so for PPEF and PP7. Indeed, recent data indicate that plant PP7 play a role in such diverse processes as light perception, thermotolerance and possibly defence responses. While precise functions of PPEFs (except *Drosophila* rdgC) are not understood, their ancient origin suggests that, even if they do function as GPCR phosphatases in metazoans as it was hypothesised a decade ago, they are likely to have other more general roles in eukaryotic signalling, since well-developed GPCR signalling is only observed in metazoans. To this end, the expression profiling data suggest that mammalian PPEFs may participate in stress-protective responses and are likely to be positively involved in cell survival, growth, proliferation and oncogenesis. These considerations warrant the experimental assessment of the functions of this enigmatic group of protein phosphatases.

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