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MAP kinase phosphorylation of plant profilin

Apinun Limmongkon[a,1], Concetta Giuliani[a,1], Rudolf Valenta[b], Irene Mittermann[b], Erwin Heberle-Bors[a], Cathal Wilson[a,c,*]

[a] *Max F. Perutz Laboratories, University Departments at the Vienna Biocenter, Institute of Microbiology and Genetics, University of Vienna, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria*
[b] *Department of Pathophysiology, Vienna General Hospital, AKH, Medical University of Vienna, Vienna, Austria*
[c] *Unità di Nutrizione Sperimentale, Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione, Via Ardeatina 546, 00178 Rome, Italy*

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Abstract

Profilin is a small actin-binding protein and is expressed at high levels in mature pollen where it is thought to regulate actin filament dynamics upon pollen germination and tube growth. The majority of identified plant profilins contain a MAP kinase phosphorylation motif, P–X–T–P, and a MAP kinase interaction motif (KIM). In in vitro kinase assays, the tobacco MAP kinases $p45^{Ntf4}$ and SIPK, when activated by the tobacco MAP kinase kinase NtMEK2, can phosphorylate the tobacco profilin NtProf2. Mutagenesis of the threonine residue in this motif identified it as the site of MAP kinase phosphorylation. Fractionation of tobacco pollen extracts showed that $p45^{Ntf4}$ is found exclusively in the high-speed pellet fraction while SIPK and profilin are predominantly cytosolic. These data identify one of the first substrates to be directly phosphorylated by MAP kinases in plants.

Keywords: MAP kinase; MEK; Phosphorylation; Pollen; Profilin; Signaling

Mitogen-activated protein (MAP) kinase signaling pathways are major regulators of proliferation, differentiation, and stress effectors. The core of the MAP kinase signal transduction cascade is composed of a three-kinase module consisting of a MAP kinase kinase kinase, a MAP kinase kinase (or MEK), and a MAP kinase, that are sequentially activated after cellular stimulation [1]. Upon pathway stimulation, MAP kinases may translocate to the nucleus where they can control the activation of gene transcription through the phosphorylation of transcription factors [2], or they can target other protein kinases or membrane and cytosolic proteins [3]. Although this module appears to be conserved in all eukaryotes, novel combinations of molecules associated with the module may have arisen throughout evolution that govern processes that are particular to the lifestyle and morphological requirements of different organisms.

The MAP kinases $p45^{Ntf4}$ and SIPK are expressed in tobacco pollen grains and both are activated upon rehydration of the tobacco pollen [4,5]. In planta the mature pollen grain is dehydrated and is essentially in a quiescent state. When a pollen grain lands on a stigma it rapidly rehydrates, leading to cell swelling, reorganization of the cytoskeleton, and the emergence of a pollen tube that transports the sperm cells to the ovary [6]. The reorganization of the cytoskeleton involves the polymerization and re-orientation of actin filaments at the pore where the pollen tube eventually emerges, and the extension of actin cables along the growing pollen tube [7]. A number of actin-remodeling proteins are expressed in pollen, such as the actin-binding protein profilin [8,9], and actin-depolymerizing factors (ADF) and ADF-modulating proteins such as AIP1 [10]. The

* Corresponding author. Fax: +39 06 51494550.
E-mail address: wilson@inran.it (C. Wilson).
[1] These authors contributed equally to the work.

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pollen-specific expression of a subset of these gene families indicates the presence of a particular regulatory process in pollen that controls actin turnover.

Profilin is an actin monomer-binding protein that regulates the polymerization of actin filaments [11]. Apart from actin, profilin can also bind phosphoinositides and proline-rich proteins [12]. These features of profilin suggest that it may provide a link between signal transduction pathways and changes in cellular architecture [13], and profilin can be phosphorylated by protein kinase C [14]. A role for profilin in signaling in pollen has been proposed [15]. Actin polymerization is essential for pollen tube growth and profilin appears to be involved in this process [16].

Given that actin reorganization occurs upon pollen germination [7], that profilin is highly expressed in mature pollen grains [8,17,18], and that MAP kinases are rapidly activated after pollen hydration [4,5], we investigated if a link could exist between these elements by testing whether profilin might be a MAP kinase substrate.

Materials and methods

Plasmid constructs. The NtMEK2 and ntf4 constructs in pGEX expression vectors have been described previously [4,19]. RNA was isolated from tobacco flower buds using a Qiagen RNeasy Plant Mini Kit, and the SIPK cDNA was amplified by RT-PCR using the Enhanced Avian HT RT-PCR kit from Sigma, with the forward primer TTCCGGATCCAAAAATATGGATGGTTCTG (*Bam*HI site underlined) and the reverse primer CAATCTCGAGAGCAATTA TTCACATATG (*Xho*I site underlined). The cDNA was cloned *Bam*HI/*Xho*I into the expression vector pGEX-4T-1 (Amersham–Pharmacia Biotech).

In vitro mutagenesis. The profilin cDNA NtProf2 in the expression vector pET17b [20] was used for in vitro mutagenesis using MEGAPRIMER PCR [21] to change the threonine residue at position 114 (codon ACT) to alanine (codon GCT). A forward internal primer containing the desired mutation (underlined) ATGAGGAAC CAGTGGCTCCAGGACAGTGCAA and a reverse primer containing a *Kpn*I restriction site covering the 3′ end of the gene CGGGGTACCCTACTAATAGCCCTGGTCAACAAG (*Kpn*I site underlined) was used to amplify a fragment from the pET17b-NtProf2 clone [20]. The amplified fragment was isolated and used as a reverse primer with a forward primer containing an *Xba*I site CCACAACGGTTTCCCTCTAGAAATAATTTTG (*Xba*I underlined) that covered the *Xba*I site of the vector pET17b. The amplified product, using the NtProf2 cDNA as a template, was used to directly replace the NtProf2 cDNA in pET17b-NtProf2. All constructs were verified by sequencing.

Protein extraction and fractionation. Dry pollen or pollen cultured in germination medium for 5 min was ground in liquid nitrogen and then resuspended in extraction buffer as described [5]. The lysate was centrifuged for 10 min, 3500*g*, and an aliquot of the resulting supernatant was taken as the total extract. The rest of the supernatant was centrifuged at 100,000*g* for 1 h. The supernatant was taken as the cytosolic fraction. The pellet was washed in extraction buffer and then resuspended in the same extraction buffer. Nonidet-P40 was added to the resuspended pellet fraction to a concentration of 1%.

Recombinant protein isolation, immunoprecipitation, kinase assays. The expression and purification of GST fusion proteins [19] and of profilin from bacteria [20] were described previously. The anti-p45^{Ntf4} and anti-SIPK antibodies [4], anti-profilin antibody [22] for Western analysis and in vitro kinase assays [5] were as previously described, except for the use of 50 μg protein for Western analysis with the anti-p45^{Ntf4} and anti-SIPK antibodies and 25 μg with the anti-profilin antibody. Immunoprecipitation was also as described previously [5], except for a preclearing of the crude lysate before the high-speed centrifugation with 100 μl protein A–Sepharose for 30 min, and the use of 100 μg of protein for each immunoprecipitation reaction.

Results

Sequence features of plant profilins as MAP kinase substrates

The majority of plant profilin protein sequences show the presence of a P–X–T–P motif, which has been described as an optimal MAP kinase phosphorylation motif [3]. In a BLAST search of Swiss-Prot, over 50 plant profilins were found to contain this motif in the same position. This motif is shown for a number of profilin sequences from different plant species in Fig. 1A. In addition, the majority of plant profilins contain a motif (Fig. 1B) that matches a kinase interaction motif (KIM domain) that has been described for a large number of MAP kinase interacting proteins [23]. Due to their role as allergens, most known profilin sequences have been derived from pollen. *Arabidopsis thaliana* contains five profilin encoding genes, which show either constitutive expression or late pollen developmental-specific expression [17]. Interestingly, two of the constitutively expressed profilins contain a P–X–T–G motif (Fig. 1A). However, the presence of glycine at the P + 1 site can also serve as an acceptor site for MAP kinase phosphorylation [3].

Tobacco profilin2 is phosphorylated on threonine 114 by MAP kinases

We have shown previously that the MAP kinases p45^{Ntf4} and SIPK are activated during pollen germination [4,5], and that the MAP kinase kinase NtMEK2, which can activate these MAP kinases, led to an inhibition of pollen germination when transiently expressed as a loss-of-function mutant in pollen [4]. The amino acid features of profilin described above together with the possibility that profilin might regulate changes in actin dynamics during pollen germination prompted us to test whether profilin could be a MAP kinase substrate.

In vitro kinase assays were performed using purified GST-tagged p45^{Ntf4}, SIPK, and NtMEK2 with purified untagged tobacco profilin as a substrate (see Materials and methods). Little or no phosphorylation of profilin was observed when it was incubated with any of the kinases alone (Fig. 2). Co-incubation of NtMEK2 with either p45^{Ntf4} or SIPK in the presence of profilin led to strong phosphorylation of profilin (Fig. 2). This again

A

KIM domain

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          74                                          120
TOBAC     KYMV IQGEPGAV IRGKKGSGGITIKKTNQALIFGIYEE PVTPGQCNM
LYCES     KYMV IQGEPGAV IRGKKGPGGITIKKTAQALIFGVYEE PVTPGQCNM
BRANA     KYMV IQGEPGAV IRGKKGAGGITIKKTGQSCVFGIYEE PVTPGQCNM
BETVE     KYMV IQGEAGAV IRGKKGSGGITIKKTGQALVFGIYEE PVTPGQCNM
MAIZE     KYMV IQGEPGAV IRGKKGSGGITVKKTGQALVVGIYDE PMTPGQCNM
WHEAT     KYMV IQGEPGVV IRGKKGTGGITIKKTGMALILGIYDE PMTPGQCNL
HORVU     KYMV IQGEPGVV IRGKKGTGGITIKKTGMPLILGIYDE PMTPGQCNL
ARATH3    KYMV IQGEPGAV IRGKKGAGGITIKKTGQSCVFGIYEE PVTPGQCNM
ARATH4    KYMV IQGEPNAV IRGKKGAGGITIKKTGQSMVFGLYEE PVTPGQCNM
ARATH5    KYMV IQGEPNAV IRGKKGAGGVTIKKTTLALVFGIYDE PMTPGQCNM
ARATH1    KYMV IQGEQGAV IRGKKGPGGVTIKKTNQALVFGFYDE PMTGGQCNL
ARATH2    KYMV VQGEAGAV IRGKKGPGGVTIKKTTQALVFGIYDE PMTGGQCNL
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B

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I QGEAGAV IRGKKGSGGI ITIK Prof2
L LADLKTM VETKKVTSSG VLVL PDE4
I N-VAIPG IMLRRLQKGN LPVR MKP-3
F K-MKPIG LQERRGSNVS LTLD PTP-SL
```

Fig. 1. (A) The MAP kinase phosphorylation site P–X–T–P of a number of plant profilins is marked above the sequences with a thick line. The putative kinase interaction motif (KIM) is indicated. The numbers refer to the amino acid sequence of NtProf2. The Swiss-Prot accession numbers are: *Nicotiana tabacum* Q9ST99; *Lycopersicon esculentum* Q41344; *Brassica napus* Q9FUB8; *Betula verrucosa* P25816; *Zea mays* P35082; *Triticum aestivum* P49234; *Hordeum vulgare* P52184; and *A. thaliana* Prof 3, 4, 5, 1, 2: Q38904; Q38905; Q9FE63; Q42449; Q42418, respectively. (B) Comparison of the putative profilin kinase interaction motif (KIM) to some known MAP kinase interacting proteins; PDE4 [37], MKP-3 [38], PTP-SL [39]. The conserved residues of the KIM domain are shown in bold.

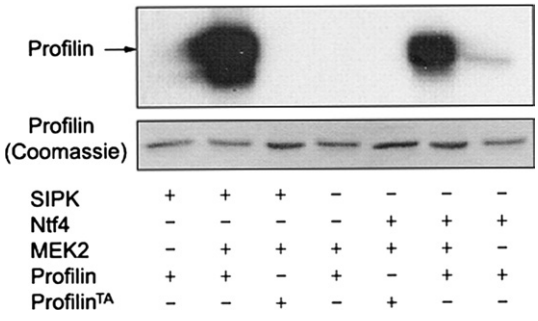

Fig. 2. Phosphorylation of profilin by MAP kinases. In vitro kinase assays with the combination of proteins indicated. Profilin^{TA} refers to the mutated profilin with a threonine to alanine substitution. Top panel, phosphorylation of profilin; bottom panel, Coomassie stain of profilin.

illustrates that NtMEK2 can activate both p45^{Ntf4} and SIPK, and that activation is required for profilin phosphorylation. To determine whether the MAP kinase phosphorylation occurred in the P–X–T–P motif, in vitro mutagenesis was used to change the threonine amino acid of the motif to alanine. The threonine residue is located at position 114 in the tobacco profilin NtProf2 (see Fig. 1A). No phosphorylation of the mutated profilin protein occurred, identifying threonine 114 of NtProf2 as the MAP kinase phosphorylation site.

SIPK may phosphorylate profilin in vivo

The maximal activation of the MAP kinases p45^{Ntf4} and SIPK occurs 5 min after pollen rehydration [4,5]. Pollen germination can be performed in vitro by incubating mature dry pollen grains in a defined germination medium [5]. This leads to almost immediate rehydration of the pollen and subsequently the growth of pollen tubes. Protein extracts were prepared from dry pollen grains or from pollen grains that had been incubated for 5 min in pollen germination medium. Total protein extracts and extracts subjected to high-speed centrifugation to separate cytosolic and non-cytosolic proteins (see Materials and methods) were tested for the ability to phosphorylate profilin after immunoprecipitation with anti-p45^{Ntf4} or anti-SIPK antibodies, and for the presence of the p45^{Ntf4}, SIPK, and profilin proteins. Western analysis showed similar levels of expression of all three proteins in total extracts from dry pollen and after 5 min in germination medium (Fig. 3). Fairly high levels of p45^{Ntf4} activity seem to be already present in dry pollen while SIPK appears to be activated only after pollen hydration. Following high-speed centrifugation, the p45^{Ntf4} protein and activity was found only in the high-speed pellet, while the majority of the SIPK protein and activity was cytosolic (Fig. 3). SIPK kinase activity was found only in the 5-min fraction after centrifugation. Western analysis with an anti-profilin antibody showed that profilin is almost exclusively present in the cytosolic fraction (Fig. 3), as has been described previously [15,24]. This indicates that the kinase responsible for profilin phosphorylation in vivo would be SIPK rather than p45^{Ntf4}. It is interesting that SIPK and p45^{Ntf4} show such a remarkable distribution pattern, as they are over 90% identical at the amino acid level. This suggests that they associate with different molecules in pollen that regulate their differential localization in the cell and thus indicate a different functional role for these highly similar paralogues.

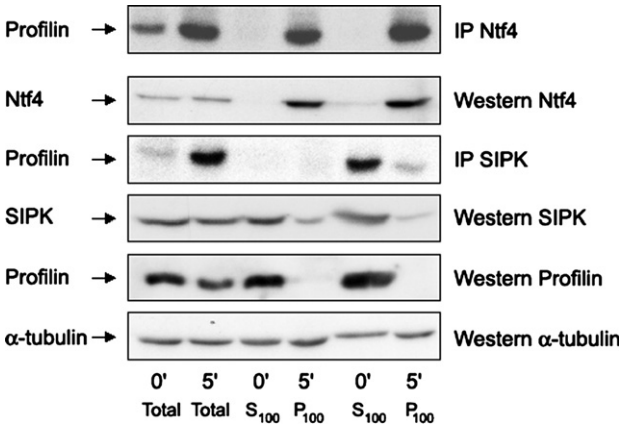

Fig. 3. MAP kinases are located in different pollen fractions. Immunoprecipitates from pollen protein extracts with anti-p45^{Ntf4} antibody (IP-Ntf4) or anti-SIPK antibody (IP-SIPK) were tested in kinase assays with profilin as a substrate. The protein extracts were also tested for p45^{Ntf4}, SIPK, and profilin expression by Western analysis. The tests performed are indicated on the right of the panels, on the left the specific molecule detected. Total protein extracts or fractionated extracts (S_{100}, cytosolic proteins; P_{100}, non-cytosolic proteins) were prepared from dry pollen (0′) or 5 min after germination (5′). An anti-α-tubulin antibody (bottom panel) was used as a loading control.

Discussion

Profilin has been extensively characterized as an actin monomer-binding protein that regulates actin dynamics [11]. Part of this regulation operates through profilin-binding to proline-rich proteins that alter actin filaments [25,26]. A role for profilin in signaling pathways may occur through association with polyphosphoinositides. Profilin interaction with actin in mammalian cells appears to be mediated in part through polyphosphoinositides that can compete with actin for profilin binding [27,28]. Plant profilins, however, have a low affinity for polyphosphoinositides [29,30], so an alternative, or additional, mechanism for signaling to the cytoskeleton might be through the MAP kinase phosphorylation of profilin. Threonine 114 of NtProf2, which is phosphorylated by MAP kinases, is in the actin-binding domain, in the turn VIa just before α helix 3 [29].

The P–X–T–P MAP kinase phosphorylation motif is not present in profilins from mammals or yeast. Therefore, it may represent a plant-specific mechanism for regulating actin dynamics. The restricted expression in pollen of members of the actin [31], profilin [17], ADF/cofilin [10], and Rho-like GTPase [32] families of proteins shows that pollen has a cytoskeletal regulatory machinery specifically designed to control pollen germination and tube growth. The dynamic interaction of profilin with other proteins by MAP kinase phosphorylation may be an adaptation in pollen that explains the presence of the P–X–T–P motif in profilins from a wide variety of plant species. As mentioned above, the constitutively expressed *Arabidopsis* profilins AtProf1 and AtProf2 contain a glycine residue instead of proline after the phosphorylated threonine, although glycine in the P + 1 position can also act as an acceptor site for MAP kinase phosphorylation. Further studies will be required to clarify this point.

MAP kinases are represented by a large number of family members. Some of these are highly related, as in the case of p45^{Ntf4} and SIPK. Specificity of signaling may be achieved at multiple levels, by differential binding to scaffolding proteins and association with different proteins or substrate selection. Evolutionary selection of specific motifs within proteins has been reported to result in a high degree of specificity in MAP kinase signaling in yeast [33]. It has been suggested that such protein–protein interactions may be more promiscuous in multicellular organisms due to the higher number of protein–protein interactions and greater complexity, but that spatial and temporal segregation adds another level of specificity control [34]. This may be the case for p45^{Ntf4} and SIPK, which are over 90% identical and show a similar expression and activation profile in pollen, but appear to be localized in different cellular compartments.

As shown here and in previous reports, NtMEK2 can activate p45^{Ntf4} and SIPK [4,35], and a loss-of-function mutant of NtMEK2 caused an inhibition of pollen germination [4]. Activation of SIPK and p45^{Ntf4} by NtMEK2 leads to the phosphorylation of profilin, but only SIPK and profilin spatially segregate in pollen. A working model is that NtMEK2 is an activator of SIPK following pollen hydration, and that this activation is responsible for the phosphorylation and regulation of the interaction of profilin with actin during the reorganization of the cytoskeleton during pollen germination and tube growth.

While numerous MAP kinase substrates have been described in other organisms [3], almost nothing is known about MAP kinase substrates in plants. A recent report described a transcription factor as a MAP kinase substrate in rice [36]. To our knowledge, profilin is the only other direct MAP kinase substrate that has been described in plants to date.

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