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A Potential Signaling Role for Profilin in Pollen of *Papaver rhoeas*

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Regulation of pollen tube growth is known to involve alterations in intracellular calcium levels and phosphoinositide signaling, although the mechanisms involved are unclear. However, it appears likely that pollination events involve a complex interplay between signaling pathways and components of the actin cytoskeleton in pollen. In many eukaryotic cells, actin binding proteins function as stimulus–response modulators, translating signals into alterations in the cytoplasmic architecture. In this study, we examined whether profilin, which is a member of this class of signaling intermediate, might play a similar role in pollen. We have analyzed the functional properties of native profilin from pollen of *Papaver rhoeas* and have investigated the effects of profilin on the phosphorylation of pollen proteins in vitro by adding a slight excess of profilin to cytosolic pollen extracts. We present clear evidence that profilin interacts with soluble pollen components, resulting in dramatic alterations in the phosphorylation of several proteins. We also show, albeit in vitro, the involvement of profilin in modulating the activity of a signaling component(s) affecting protein phosphorylation. Our data, which suggest that pollen profilin can regulate actin-based cytoskeletal protein assembly and protein kinase or phosphatase activity, indicate a possible role for the involvement of profilin in signaling pathways that may regulate pollen tube growth.

INTRODUCTION

Pollination and control of sexual reproduction in flowering plants not only are important to plant breeders but also serve as a model system for a fundamental understanding of cell–cell recognition, cellular morphogenesis, and signaling involved in these processes (Wilhelmi and Preuss, 1997). It is well established that intracellular free calcium plays a key role in the regulation of pollen tube growth (Derksen et al., 1995; Taylor and Hepler, 1997). Self-incompatibility (SI) is a genetically controlled mechanism used by many higher plants to prevent self-fertilization (reviewed in Williams et al., 1994; Franklin et al., 1995). We have been studying pollen tube growth and the SI response in *Papaver rhoeas.* Regulation of *P. rhoeas* pollen tube growth has been shown to involve inositol 1,4,5-trisphosphate (Ins[1,4,5]P_3)–induced Ca^{2+} release (Franklin-Tong et al., 1996). We also have shown that the SI response is mediated by a Ca^{2+}-mediated signal transduction pathway (Franklin-Tong et al., 1993, 1997), resulting in the increased phosphorylation of at least two soluble phosphoproteins, p26.1 (Rudd et al., 1996) and p68 (Rudd et al., 1997).

Pollen germination and tube growth also depend on a functional actin cytoskeleton (reviewed in Cai et al., 1997; Taylor and Hepler, 1997). In many eukaryotic systems, actin binding proteins can act as stimulus–response modulators of cytosolic calcium and polyphosphoinositide levels (reviewed in Moon and Drubin, 1995; Sun et al., 1995), translating signaling events into dynamic changes in the cytoplasmic architecture. Profilin is a good example of this class of signaling intermediate and is known to be an abundant component of the pollen from many angiosperms (Valenta et al., 1991; Staiger et al., 1993; Huang et al., 1996). However, its exact role in plant cells is as yet poorly understood. Profilin is a small (12 to 15 kD), ubiquitous protein originally discovered through its ability to form a 1:1 complex with actin monomers (reviewed in Sun et al., 1995). Profilin is known to regulate the organization of the actin cytoskeleton in a complex fashion; it can either promote or inhibit actin filament formation, depending on cellular conditions and the presence of other actin binding proteins (Pantaloni and Carlier, 1993; Sun et al., 1995; Staiger et al., 1997). The molecular basis for this dual activity is poorly understood, but the fact that profilin binds to several ligands in addition to actin could explain some of the observed complexity.

Profilin binds to contiguous stretches of proline residues, such as those found in the vasodilator-stimulated phosphoprotein from human platelets (Reinhard et al., 1995) and those found in the FH1 domains of Bni1p and related proteins (Chang et al., 1997; Evangelista et al., 1997; Imamura

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et al., 1997; Watanabe et al., 1997). Profilin also interacts with a complex of seven proteins from Acanthamoeba, which contains two actin-related proteins (Machesky et al., 1994). Whether these protein–protein interactions serve to modulate the function of profilin or vice versa is currently unclear. Profilin also is known to bind phosphatidylinositol 4,5-bisphosphate (PtdIns[4,5]P_2). Lassing and Lindberg (1985) showed that the profilin–actin complex could be disrupted by PtdIns(4,5)P_2, thereby releasing monomeric actin for polymerization. A further consequence of the profilin–PtdIns(4,5)P_2 association is that soluble and membrane-associated phosphoinositidase activities are reduced substantially (Goldschmidt-Clermont et al., 1990; Drøbak et al., 1994). These observations have led to models in which profilin acts at the interface between polyphosphoinositide signaling pathways and reorganization of the actin cytoskeleton (reviewed in Machesky and Pollard, 1993). Further complexity is envisaged, because it has been demonstrated that vertebrate profilins can be phosphorylated in vitro by protein kinase C (Hansson et al., 1988; Singh et al., 1996b) and pp60$^{c\text{-}src}$ (De Corte et al., 1997). However, the functional consequences of profilin phosphorylation are not known.

Because profilin is an abundant protein in pollen from many plant species (reviewed in Staiger et al., 1997), we decided to begin to address the role that it might play in regulating actin organization, tip growth, and response to extracellular signals in pollen. Control of both pollen germination and tube growth seems likely to require a complex interplay between elements of the cytoskeleton and signaling cascades. In this study, we investigated the effects of the addition of a slight excess of profilin to pollen extracts on the phosphorylation of pollen proteins in vitro. Our data indicate that profilin interacts with a soluble pollen component(s), resulting in dramatic alterations in the phosphorylation of a number of phosphoproteins. This implies a signaling role for profilin in angiosperm pollen.

RESULTS

Purified *P. rhoeas* Profilin Is Functional in Vitro and in Living Cells

To demonstrate that native profilin purified from *P. rhoeas* pollen is fully functional, we characterized its ability to bind two known ligands for eukaryotic profilins, G-actin and poly-L-proline (PLP). Equilibrium dissociation constants (K_d) were determined by quantitating changes in intrinsic (tryptophan) fluorescence upon profilin binding to these ligands. Profilin binding to PLP results in an enhancement of intrinsic fluorescence (Perelroizen et al., 1994; Petrella et al., 1996; Gibbon et al., 1997). When the relative fluorescence change was plotted against the concentration of PLP and the data were fit to a hyperbolic function, as shown in Figure 1A, the average K_d derived by nonlinear least squares regression was 130 ± 21 μM proline residues (mean ±SD; $n = 4$). This value was significantly lower ($P < 0.003$) than the K_d values for native and most of the recombinant maize pollen profilins, which ranged from 249 to 305 μM (Gibbon et al., 1997). Indeed, *P. rhoeas* profilin was significantly better ($P = 0.008$) at binding PLP than ZmPRO4, which had the lowest K_d (173 μM) of the maize profilin isoforms (Gibbon et al., 1998, this issue).

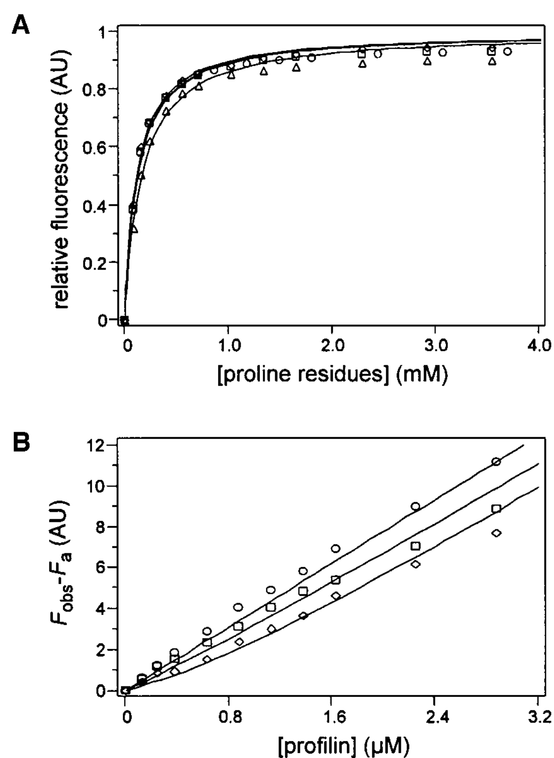

Figure 1. Binding of Purified *P. rhoeas* Pollen Profilin to PLP and G-Actin.

(A) Determination of K_d for PLP binding. The interaction between native pollen profilin and PLP was analyzed by enhancement of intrinsic fluorescence, as described in Methods. The data from four independent experiments are plotted (the symbols represent data obtained from four separate titration experiments), and nonlinear least squares lines of regression for each experiment are overlaid on the graph. The average K_d for these data was 130 ± 21 μM proline residues (mean ±SD). AU, arbitrary fluorescence units.

(B) Determination of K_d for binding to Mg–ATP–G-actin from maize pollen. The association of Mg–ATP–G-actin and native pollen profilin was analyzed by the quenching of intrinsic tryptophan fluorescence. Titration of 0 (circles), 0.15 (squares), and 0.3 (diamonds) μM pollen actin with *P. rhoeas* profilin is shown. The K_d, derived by using the equation defined in Methods, for this representative experiment was 2.4 μM. The average K_d for four experiments was 1.9 ± 0.5 μM (mean ±SD).

The binding of profilin to G-actin was measured by titrating solutions of Mg–ATP–G-actin from maize pollen with native poppy profilin and monitoring the quenching of tryptophan fluorescence. A representative experiment is shown in Figure 1B. When the change in fluorescence was plotted against profilin concentration, the fluorescence quenching due to actin addition relative to profilin alone (upper line) was quite obvious. The lines are best fits of the data, using the equation stated in Methods, and the K_d determined for this experiment was 2.4 μM. From four determinations, using two independent batches of profilin, an average K_d of 1.9 ± 0.5 μM (mean ±SD) was determined. The average K_d for native maize pollen profilin binding to Mg–ATP–actin was 1.1 ± 0.7 μM ($n = 3$) (Gibbon et al., 1998, this issue). These values were not significantly different ($P > 0.05$) by the two-tailed *t* test.

We have recently developed an assay to make quantitative comparisons of the effects of different profilins on actin organization and cytoarchitecture in living plant cells (Gibbon et al., 1997; Ren et al., 1997). Microinjection of doses of profilin that increased the cellular profilin concentration in *Tradescantia virginiana* stamen hair cells resulted in a rapid disruption of cellular architecture, cessation of streaming, and depolymerization of F-actin. A dose of 100 μM needle concentration was used to add ~10 to 15 μM profilin to the cytoplasm of stamen hair cells, which contain ~5 μM endogenous profilin (Staiger et al., 1994). These injections therefore resulted in a two- to threefold increase in the intracellular profilin concentration. In cells with the nucleus located in a central position, recombinant and native maize profilins caused nuclear displacement in an average time of 4.9 to 8.2 min (Gibbon et al., 1997). Injection of *P. rhoeas* profilin resulted in an average nuclear displacement time of 5.7 ± 0.5 min (mean ±SE; $n = 32$). This is not significantly different ($P > 0.05$) from native maize pollen profilin, which previously had been shown to cause nuclear displacement with an average time of 6.3 ± 0.7 min (Gibbon et al., 1997).

Figures 2A and 2B illustrate the purity of the *P. rhoeas* profilin obtained from cytosolic pollen extracts by PLP affinity chromatography, visualized by using Coomassie blue and silver staining. PLP is used extensively for purification of profilin from animal and plant species, because it makes use of the affinity of profilin for long stretches of proline residues (Tanaka and Shibata, 1985; Janmey, 1991; Rozycki et al., 1991). Poppy profilin migrates as a doublet (Figure 2A, lane 2), which is likely to result from different isoforms of profilin. Both bands cross-react with an antiserum raised against maize profilin ZmPRO3 (see later). Silver staining of the same loading confirmed that profilin was the only protein detected in these samples (Figure 2B); the smear at the top was present in all lanes, including those without samples loaded (data not shown). Profilin of this quality was used for all of the experiments described in this study.

All three of the studies described above demonstrate that the native profilin purified from *P. rhoeas* pollen is fully functional: it binds two known ligands for eukaryotic profilins, G-actin and PLP, with equilibrium dissociation constants in the expected range or even better than those published for other plants' profilins (Giehl et al., 1994; Perelroizen et al., 1996; Domke et al., 1997; Gibbon et al., 1997, 1998, this issue); it also has the expected effect on the cytoskeleton of live cells at concentrations similar to those exhibited by maize profilins (Gibbon et al., 1997, 1998, this issue; Ren et al., 1997). These studies therefore firmly establish that the poppy profilin has the properties expected of a pure, native profilin.

Addition of *P. rhoeas* Profilin to Pollen Extracts Alters the Phosphorylation of Several Proteins

We examined the effects of adding an excess of native *P. rhoeas* profilin on the in vitro phosphorylation of *P. rhoeas* pollen extracts (cytosol, microsomes, and a combination of cytosol and microsomes). Phosphoproteins were detected by autoradiography, and all blots were probed with anti-profilin antibodies to demonstrate independently which samples had detectable increases in profilin. In *P. rhoeas* pollen, endogenous profilin is present at an intracellular concentration of 20.3 ± 7.0 μM (mean ±SD; $n = 4$), as determined by PLP purification and protein quantification (see Methods for details). This is somewhat lower than the level of profilin in maize pollen (40 to 80 μM; Staiger et al., 1994) but similar to the level found in lily pollen (25 μM; Vidali and Hepler, 1997). We supplemented soluble pollen extracts with 7 and 14 μM purified pollen profilin, representing an increase in profilin concentration of 34 and 69%, respectively. As mentioned earlier, Figures 2A and 2B illustrate the purity of *P. rhoeas* profilin used in these experiments. The loading on these gels is equivalent to the addition of 14 μM profilin to the cytosol in the labeling reactions.

Figures 2C and 2D illustrate typical examples of the effect of adding native pollen profilin to *P. rhoeas* pollen protein extracts. The addition of profilin to pollen cytosolic proteins consistently resulted in large alterations in the phosphorylation of several soluble pollen phosphoproteins (Figure 2C, lane 1). We have identified five cytosolic phosphoproteins, p26, p30, p50, p68, and p150, which we have studied in detail with respect to alterations in phosphorylation caused by the addition of profilin. There are also alterations in the phosphorylation of proteins in the microsomal fraction, but these are less clear-cut (see Figure 2C, lanes 2 and 5). Because they potentially could be caused by some cross-contamination with cytosolic material, we have not analyzed these further.

It is apparent that a 14-kD protein (p14), which comigrates with profilin, is phosphorylated in pollen cytosolic extracts (Figure 2C, lane 4). Evidence that profilin from pollen is phosphorylated will be presented in a subsequent study (S.R. Clarke, J.J. Rudd, C.J. Staiger, and V.E. Franklin-Tong, unpublished data). Analysis of the data from several experiments ($n = 3$) allowed us to determine average changes in phosphorylation levels in cytosolic extracts relative to the

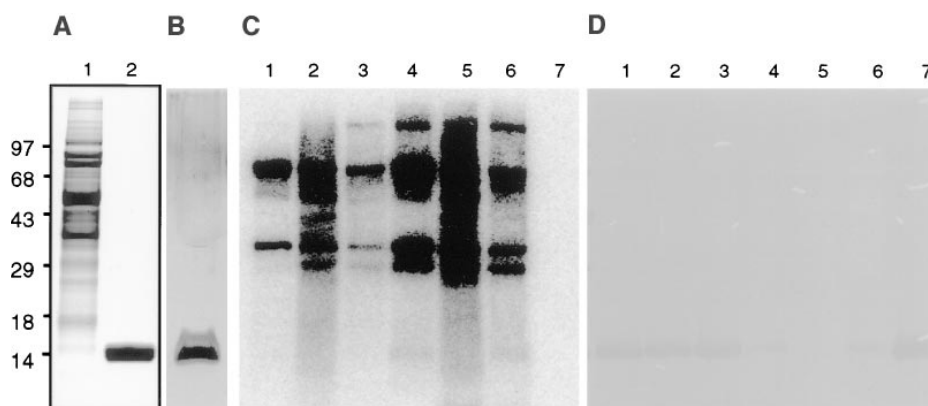

Figure 2. Effect of Excess Profilin on the Phosphorylation of Cytosolic Proteins from Pollen.

(A) Coomassie blue–stained gel of the cytosolic pollen fraction from *P. rhoeas* pollen (lane 1) and the profilin obtained from the pollen cytosol after PLP affinity chromatography (lane 2).

(B) Silver staining of the same *P. rhoeas* pollen profilin as shown in **(A)**. The loading of profilin is the same as in most of the labeling reactions (equivalent to 14 μM, which is a loading of 5 μg of profilin per lane). *P. rhoeas* profilin appears to comprise a doublet, which likely results from different isoforms of profilin. The smear at the top of the gel is caused by contamination in the sample buffer (also detected in lanes with no protein but with sample buffer only added).

(C) Autoradiography showing phosphorylation of *P. rhoeas* pollen proteins after labeling with ^{32}P-γ-ATP in vitro by pollen cytosolic kinases and inhibition of some of this phosphorylation by profilin. The results show that the addition of excess *P. rhoeas* profilin to pollen extracts inhibited the phosphorylation of several proteins. Lanes 1 to 3 show the effect of the addition of 14 μM profilin to cytosol, microsomal, and cytosol plus microsomal fractions, respectively. Lanes 4 to 6, which contain the same fractions as lanes 1 to 3 without the addition of profilin but with the addition of buffer to the same volume as profilin as a control, provide a comparison. Lane 7 shows that profilin alone is not phosphorylated in the absence of pollen cytosolic protein kinases.

(D) The blot shown in **(C)** probed with the recombinant maize pollen profilin anti-ZmPRO3 antibody. The immunoblot shows the addition of profilin in lanes 1 to 3; endogenous levels of profilin are just detectable in lanes 4 and 6 (which contain cytosol), whereas lane 5 (which contains microsomal proteins) does not contain detectable levels of profilin. Lane 7 contains purified *P. rhoeas* profilin, which is shown to cross-react with the maize profilin antibody.

The numbers at the left indicate molecular masses in kilodaltons.

untreated controls after addition of 14 μM profilin. The most striking alterations detected were a mean decrease of 69 and 60% phosphorylation for the cytosolic proteins p150 and p26, respectively. The cytosolic proteins p68, p50, and p30 also showed marked decreases in phosphorylation, ranging from 32 to 52%, after the addition of excess pollen profilin. Probing the blot with antibody raised against recombinant maize profilin ZmPRO3, as shown in Figure 2D, demonstrated the addition of profilin to each sample (Figure 2D, lanes 1 to 3) and the recognition of endogenous *P. rhoeas* profilin in the soluble extracts by profilin antiserum (Figure 2D, lanes 4 and 6) but not in the microsomal fraction (Figure 2D, lane 5). A sample containing only purified *P. rhoeas* pollen profilin confirmed that the native profilin is recognized by maize profilin antiserum (Figure 2D, lane 7).

Addition of Maize Pollen Profilins to *P. rhoeas* Pollen Extracts

Because the ligand binding properties for maize pollen profilins have been well characterized (Gibbon et al., 1997), we also determined the effects of excess recombinant ZmPRO1 and ZmPRO3, in addition to that of native *P. rhoeas* profilin, on the in vitro phosphorylation of cytosolic proteins from *P. rhoeas* pollen, as shown in Figure 3A (lanes 2 to 4). The immunoblot shown in Figure 3B demonstrates the addition of profilin-reactive proteins in all the samples with profilin added (lanes 2 to 4). Endogenous levels of profilin in the pollen extracts were not strongly detected because of the comparatively lower cross-reactivity of the *P. rhoeas* profilin with the ZmPRO3 antibody (Figure 3B, lanes 1 and 5). The addition of maize profilin gave a ladder of protein bands, recognized by the profilin antiserum, that is likely to result from multimerization of profilin (Figure 3B, lanes 3 and 4; see also Babich et al., 1996). Thus, in addition to the monomeric form of profilin at 14 kD, we detected profilin-reactive polypeptides at ∼28, ∼42, and ∼60 kD, which presumably are dimer, trimer, and tetramer forms of profilin, respectively. Formation of multimers of profilin, which are stable during SDS-PAGE, is probably due to these samples not being fully denatured. It is also worth noting that ZmPRO3 (Figure 3B, lane 4) is more strongly cross-reactive than is ZmPRO1 (Figure 3B, lane 3), because the antibody was raised against re-

combinant ZmPRO3 (Karakesisoglou et al., 1996). Not surprisingly, *P. rhoeas* profilin (Figure 3B, lane 2) is less cross-reactive than either of the maize profilins. Figure 3C illustrates the purity of the recombinant maize profilins added to the reactions (see also Karakesisoglou et al., 1996; Gibbon et al., 1997); the faint band at 28 kD is assumed to be dimerized profilin; Figures 2A and 2B illustrate the purity of *P. rhoeas* profilin.

In addition to buffer controls, in which no effects on phosphorylation were detected, we also tested the effect of addition of different proteins to the pollen cytosolic extracts. Figures 3D and 3E illustrate the effect of the addition of lactalbumin, which at 14.2 kD has a molecular mass similar to that of profilin. Figure 3D clearly shows that 14 μM lactalbumin has no significant effect on protein phosphorylation (lane 3), whereas 14 μM ZmPRO1 has a marked effect (lane 2). Quantification of the effects of lactalbumin on pollen protein phosphorylation revealed that the maximum alterations in phosphorylation detected were only 1.5%. This lends credence to the idea that the effect on phosphorylation is likely to be due to a relatively specific interaction of profilin with cytosolic components.

Comparisons were made between the effects of *P. rhoeas* and recombinant maize profilins on the phosphorylation of cytosolic *P. rhoeas* pollen proteins, as illustrated in Figure 3A. The two maize pollen profilins both had a similar effect on pollen protein phosphorylation when added to cytosolic pollen extracts at 7 μM (Figure 3A) to 14 μM (data not shown). This represents a 34 and 69% increase in profilin concentration over endogenous levels. There is a distinct difference between the relative effects of *P. rhoeas* and maize profilins on pollen cytosolic protein phosphorylation, with the maize profilins generally having a much stronger overall effect on phosphorylation (Figure 3A). However, there are also apparent qualitative differences between the effects of maize and *P. rhoeas* profilin on pollen protein phosphorylation.

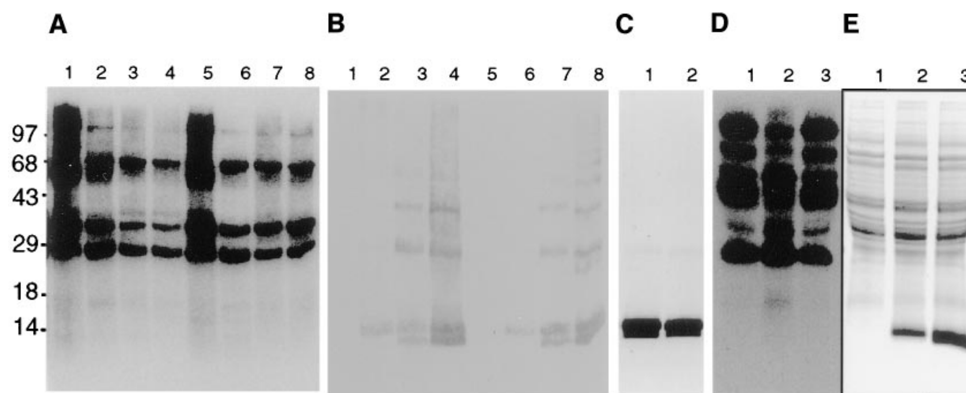

Figure 3. Effect of the Addition and Depletion of Maize Profilin on Cytosolic Pollen Protein Phosphorylation.

(A) Autoradiography showing the addition of 7 μM *P. rhoeas* profilin and maize recombinant profilins ZmPRO1 and ZmPRO3 (lanes 2 to 4, respectively). Lanes 1 and 5 are cytosolic extracts with no additions. They show endogenous protein kinase activity. Lanes 6 to 8 have the same additions as lanes 2 to 4, except they were also treated with PLP–Sepharose to deplete the extracts of profilin. Decreased levels of phosphorylation in lanes 2 to 4 compared with lanes 1 and 5 are attributable to the addition of profilin. Depletion of added profilin dramatically restored the phosphorylation levels of the affected proteins, although not completely. All reactions were performed at the same time, run on the same gel, and examined under identical autoradiography conditions.

(B) The gel shown in **(A)** probed with the anti-ZmPRO3 antiserum. The immunoblot demonstrates the addition of profilin in lanes 2 to 4 and the partial depletion of added profilin by the addition of PLP–Sepharose in lanes 6 to 8. Lanes 1 and 5 are control lanes, as given in **(A)**.

(C) Coomassie blue staining of purified, recombinant maize profilins ZmPRO1 (lane 1) and ZmPRO3 (lane 2). The loading of ZmPRO1 is double that in most of the labeling reactions (10 μg of profilin per lane). This illustrates the purity of the recombinant maize profilin samples; the faint doublet at 28 kD is assumed to be dimerized profilin, because it comigrates with the profilin dimer on the immunoblot.

(D) Autoradiography showing endogenous protein kinase activity in a cytosolic pollen extract with no additions (lane 1), the addition of 14 μM ZmPRO1 (lane 2), and the addition of 14 μM lactalbumin (lane 3) to the same cytosolic extract. Lactalbumin, which has a molecular mass similar to that of profilin, had no significant effect on protein phosphorylation, whereas ZmPRO1 had a marked effect on phosphorylation. This provides a control showing that the effect on phosphorylation attributed to profilin is likely to be specific and is not due to an alteration in the reaction conditions caused by the addition of a protein or buffer.

(E) Coomassie blue staining of the gel in **(D)**, demonstrating that there is equal loading of pollen cytosol in each lane and showing addition of 14 μM ZmPRO1 (lane 2) and lactalbumin (lane 3).

Numbers at left in **(A)** indicate molecular masses in kilodaltons.

Quantification of these effects showed that an excess of 7 μM poppy profilin, ZmPRO1, and ZmPRO3 reduced the phosphorylation of p30 by 45, 82, and 87%, respectively. Similarly, their effects on p26 phosphorylation were reductions of 29, 62, and 70%, respectively. However, the effects of the three proteins on the phosphorylation of p150 were very similar, with a reduction in phosphorylation of 87, 94, and 91%, respectively. Comparing this with the mean data ($n = 3$) for addition of 14 μM of these profilins, some differences were apparent (see also Figure 3). ZmPRO1 and ZmPRO3 reduced the phosphorylation of p30 by 83 and 89%, whereas *P. rhoeas* profilin led to a 52% reduction. The maize profilins also reduced the phosphorylation of p150 by an average of 91 and 85%, compared with 69% for *P. rhoeas* profilin. A reversed trend was observed when p26 phosphorylation was investigated. *P. rhoeas* profilin consistently resulted in reduced phosphorylation of p26 (60% reduction), whereas addition of maize profilin resulted in either far less reduced phosphorylation (13% reduction by ZmPRO3) or an increase (49% stimulation by ZmPRO1). These data indicate that the interaction between the profilins and the proteins may involve a concentration-dependent effect, but it may also involve a more specific interaction, which might be species dependent.

Depletion of Profilin Results in Some Alleviation of the Effects on Pollen Protein Phosphorylation

We added profilin to pollen cytosolic extracts, incubated them for >30 min to allow interaction, and then subsequently attempted to remove at least some of the profilin by extracting the reactions with PLP–Sepharose. These "profilin-reacted, partially depleted" extracts subsequently were labeled, and their phosphorylation was examined. The results of these experiments are shown in Figures 3A and 3B. The effectiveness of PLP at specifically extracting profilin from cytosolic pollen samples is illustrated in Figures 2A and 2B. Depletion of profilin from these reactions was not complete (most likely because of the small volumes treated), as confirmed by the antibody probing of the blots for profilin, which indicated that there were reduced but still detectable levels of profilin in these samples. Nevertheless, comparisons between the samples with no additions (Figure 3A, lanes 1 and 5), those with profilin additions (Figure 3A, lanes 2 to 4), and those with profilins added and subsequently depleted (Figure 3A, lanes 6 to 8) reveal that there is alleviation of the inhibition of phosphorylation detected for some of the pollen proteins treated with the maize profilins.

Phosphorylation of p68 in cytosolic pollen extracts depleted of *P. rhoeas* profilin, ZmPRO1, and ZmPRO3 was affected in a similar manner for all three profilins, with 13, 10, and 67% increases, respectively, in phosphorylation compared with the undepleted, profilin-added samples. However, differences between the profilins were again apparent. For example, phosphorylation of p150 in extracts depleted of ZmPRO1 and ZmPRO3 was 70 and 27% increased compared with the undepleted, profilin-added samples, respectively; the same comparison made for *P. rhoeas* profilin revealed a 66% reduction of phosphorylation in the depleted sample compared with the profilin-added, undepleted sample. Phosphorylation of p30 was affected strikingly by ZmPRO1 and ZmPRO3. Its phosphorylation was increased 168 and 399%, respectively, in the depleted sample compared with the undepleted, profilin-added sample, whereas its phosphorylation was only reduced by 33% by *P. rhoeas* profilin.

Despite these apparent species-specific differences, in all of these depleted extracts, phosphorylation was generally substantially reduced compared with the untreated cytosolic pollen proteins. The less-than-complete alleviation of the effects of profilin is most likely due to the less-than-complete extraction of profilin from the extracts. We have demonstrated the purity of the profilin added, the specificity of the extraction of profilin by PLP, and the lack of effect from the addition of lactalbumin; therefore, we are confident that these effects on phosphorylation may be attributed specifically to the effect of profilin interacting with pollen cytosolic components.

Concentration-Dependent Effects of Profilin on Alterations in Phosphorylation

We tested whether the effects of profilin on pollen protein phosphorylation in vitro were concentration dependent. The results are illustrated in Figure 4. Our data indicate that the effect of profilin on the phosphorylation of pollen proteins is highly concentration dependent. The autoradiograph illustrated in Figure 4B shows a general trend of decreasing phosphorylation of p30, p50, and p150 after the addition of 0.3 to 28 μM ZmPRO1 to pollen cytosol extracts. Because pollen has 20 to 25 μM endogenous profilin, these additions represent a 1.5 to 138% increase in profilin concentration over the endogenous levels. We believe these are physiologically relevant levels of profilin, because it has been demonstrated previously that much larger increases in intracellular profilin concentration than this are required to detect a marked physiological response with respect to the actin cytoskeleton in living cells (Staiger et al., 1994; Gibbon et al., 1997; Ren et al., 1997; Valster et al., 1997).

Comparison of protein phosphorylation (Figure 4B) with the immunoblot in Figure 4A, which clearly shows the increasing amounts of profilin added to the samples, reveals a good inverse relationship between profilin concentration and phosphorylation levels of p30, p50, and p150. Quantification of these results shows that these reductions in phosphorylation levels are quite unambiguous and suggests that these phosphoproteins are highly sensitive to excess profilin in a concentration-dependent manner.

We observed that phosphorylation of p150 is inhibited by 44% at 0.3 μM ZmPRO1, and the effect is saturated at 7 μM

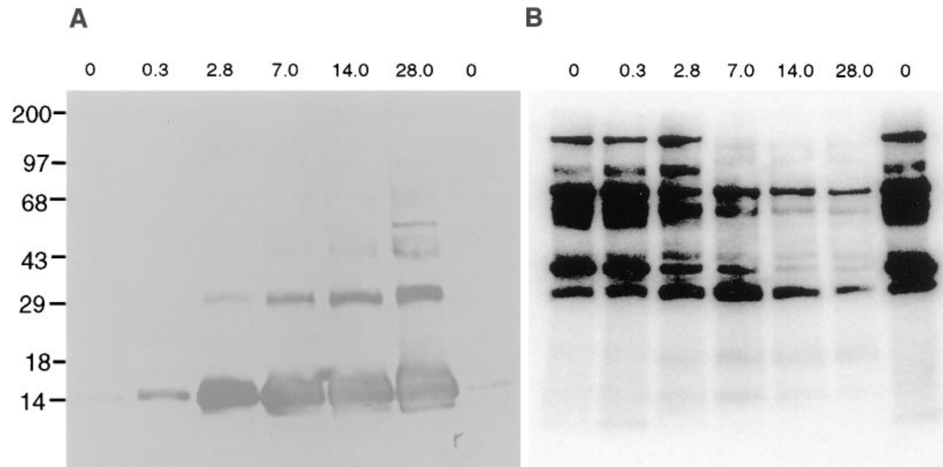

Figure 4. Concentration-Dependent Alteration of Protein Phosphorylation by the Addition of ZmPRO1.

(A) The immunoblot shows increasing amounts of recombinant maize profilin ZmPRO1 added to the cytosolic pollen extracts. Lanes 2 to 6 have additions of 0.3, 2.8, 7, 14, and 28 μM ZmPRO1, respectively. All lanes contain only pollen cytosolic proteins, with no ZmPRO1 added.
(B) Autoradiography of the blot shown in **(A)** shows the effect of the addition of profilin on the phosphorylation of the pollen cytosolic proteins. There is a good inverse relationship between increasing profilin and decreasing phosphorylation of p30, p50, and p150. In contrast, phosphorylation of p26 was barely affected by the addition of ZmPRO1 at low concentrations (0.3 μM) but was dramatically stimulated by intermediate concentrations (2.8 to 7 μM), whereas its phosphorylation was inhibited at higher concentrations (28 μM).
Numbers at left in **(A)** indicate molecular masses in kilodaltons.

ZmPRO1, with complete abolishment of any detectable phosphorylation at this concentration. The phosphorylation of p30 is slightly less affected by profilin, with only 24% inhibition of phosphorylation at 0.3 μM ZmPRO1, >75% inhibition at 2.8 μM ZmPRO1, and virtually complete inhibition (95 to 97%) in the presence of 14 and 28 μM ZmPRO1. Phosphorylation of p50 was slightly less affected, being very slightly stimulated (by 12%) at 0.3 μM ZmPRO1 and only 18% inhibited at 2.8 μM ZmPRO1. Inhibition of phosphorylation appeared to be saturated at ~14 μM ZmPRO1, with ~85% reduction of phosphorylation of p50. The phosphorylation of p68 is strongly inhibited by ZmPRO1 but apparently in a less concentration-dependent manner. The inhibition of p68 phosphorylation reaches ~60% at 2.8 μM ZmPRO1. However, a 2.5-fold further increase in profilin did not result in significantly lower phosphorylation, whereas increasing profilin to 14 μM resulted in an apparent maximum inhibition of p68 phosphorylation of ~85%. This may indicate that p68 is more sensitive to profilin than some of the other phosphoproteins.

In contrast, phosphorylation of p26 is barely affected by addition of ZmPRO1 at low concentrations (0.3 μM) but is dramatically stimulated by intermediate concentrations (2.8 to 7 μM), and its phosphorylation is inhibited at higher concentrations of these maize profilins. The level of phosphorylation of p26 in the presence of 0.3 μM ZmPRO1 is virtually identical to those levels normally detected without the addition of profilin. p26 phosphorylation is stimulated by 85% in the presence of 2.8 μM ZmPRO1, and phosphorylation is increased by 200% at 7 μM profilin. However, in the presence of 28 μM ZmPRO1, phosphorylation of p26 is inhibited by 45%.

DISCUSSION

Profilin is well characterized as a modulator of the actin cytoskeleton in both plant and animal cells (Sun et al., 1995; Staiger et al., 1997). The cloning and sequencing of profilin, and other actin binding proteins such as actin depolymerizing factors, from a number of plant species have provided a wealth of sequence information on these multigene families (Valenta et al., 1991; Huang et al., 1996; Lopez et al., 1996; Staiger et al., 1997). Functional studies of the plant profilins strongly suggest that the roles played by these actin binding proteins in the reorganization of the actin cytoskeleton in plant cells are likely to be similar to animal profilins. However, although there is good evidence for the involvement of profilin in intracellular signaling in animal cells, there is relatively little evidence to support the idea that profilin also plays this role in plant cells. In this study, we have provided good evidence that plant profilins have the ability to alter the phosphorylation state of other pollen cytosolic proteins. This strongly implicates a signaling role for profilin in pollen and therefore opens up the possibility that it may do so in other higher plant tissues.

The Concentration of Profilin Required to Effect Alterations in Phosphorylation

We have demonstrated that the effects of profilin on pollen protein phosphorylation are highly concentration dependent. It is worth noting that the effect of profilin on actin polymerization is known to be highly concentration dependent and that this characteristic is considered to suit the role of profilin as a regulator of the highly dynamic actin cytoskeleton particularly well, because conditions in different regions of the same cell can vary dramatically (Sohn and Goldschmidt-Clermont, 1994). We have shown that relatively small alterations in profilin concentration are sufficient to modulate phosphorylation of pollen proteins. The levels of change in profilin concentration are within a physiologically relevant concentration range. An excess of 2.8 μM profilin, which represents an increase of ~14% over the total intracellular concentration of profilin, was sufficient to cause large alterations in the phosphorylation of several pollen proteins, and a 7-μM (34%) increase in profilin resulted in marked stimulation of p26 phosphorylation. Dramatic inhibition of pollen protein phosphorylation was detected at concentrations up to 28 μM, which represents a greater than twofold increase in profilin. Functional studies, involving the microinjection of profilin that resulted in the gross disruption of cellular architecture and depolymerization of F-actin in *T. virginiana* stamen hair cells, required at least a doubling of the intracellular profilin concentration (Gibbon et al., 1997; Ren et al., 1997; Valster et al., 1997). Thus, the magnitude of the increases in profilin that modulate some of the protein phosphorylation is far lower than that required to have a measurable effect on the actin cytoskeleton. This strongly supports the idea that under these conditions, profilin is likely to be acting in a signaling capacity rather than as a structural protein through direct binding to actin.

Species-Specific Differences in Profilin Activity

Our data indicate that profilins from different species vary in their ability to alter cytosolic pollen protein kinase activity. Although we have demonstrated that native *P. rhoeas* profilin binds to pollen G-actin with similar affinity to maize profilins, there appear to be clear differences between the effects of the recombinant maize profilins ZmPRO1 and ZmPRO3 and the *P. rhoeas* profilins on the phosphorylation of pollen proteins. This could reflect differences between the species, or more likely, it may reflect underlying differences in the functional and structural properties of these profilins. Differences between the in vitro properties of native and recombinant profilins from maize have been documented previously (Gibbon et al., 1997, 1998, this issue). However, the molecular basis for these differences is not known. Future work will include studies of the effects of other profilins on pollen protein phosphorylation because information about these differences may shed some light on the binding sites involved in this interaction. Because plant and vertebrate profilins, despite being well conserved with respect to actin binding activity (Valenta et al., 1993; Giehl et al., 1994; Ruhlandt et al., 1994; Perelroizen et al., 1996), share only 32 to 40% amino acid sequence identity (Staiger et al., 1993; Thorn et al., 1997), kingdom-specific differences in phosphorylation may be expected and may help analysis of the other potential functions of this protein.

Although profilin is known to bind to contiguous stretches of prolines and proline-rich proteins (Reinhard et al., 1995; Chang et al., 1997; Evangelista et al., 1997; Imamura et al., 1997; Watanabe et al., 1997), whether these interactions serve to modulate the activity or function of profilin or vice versa is presently unclear. Current models suggest that these factors function to target profilin to regions of active actin polymerization. Although plant homologs for vasodilator-stimulated phosphoprotein and formin-homology proteins have not yet been identified, it will not be surprising if they are isolated from angiosperms. We have established that native *P. rhoeas* pollen profilin has a K_d of 130 μM proline residues, which makes it a significantly better binder of PLP than the native or recombinant maize pollen profilins, which have K_d values in the range of 250 to 300 μM (Gibbon et al., 1997, 1998, this issue). Our data on the effects of different profilins on pollen protein kinase activities, taken together with information on the relative affinities of these profilins for PLP, suggest that the direct interaction of profilin with PLP is not likely to be implicated in the effect of profilin on protein phosphorylation. Further investigation of the differences between profilins from different species should help elucidate how they achieve their biological effects. Future work will focus on identifying which binding site(s) on profilin might be involved in the alteration of phosphorylation of pollen proteins.

A Possible Role for Profilin in Signal Transduction in Pollen

We have presented clear evidence that profilin interacts with pollen proteins, resulting in the differential dephosphorylation/phosphorylation of several soluble pollen proteins. We also provide data, albeit in vitro, that suggests the involvement of profilin in modulating the activity of the signaling component(s) affecting protein phosphorylation. Relatively little is known about the roles of protein kinases and phosphorylation in pollen, although several calcium-dependent and -independent protein kinase activities have been detected in pollen extracts (Polya et al., 1986). Evidence from a variety of sources indicates the involvement of protein kinase activity in the modulation of pollen tube growth. There is relatively good evidence for serine/threonine protein kinases playing a role in modulating pollen tube growth in *P. rhoeas* (Rudd, 1997; J.J. Rudd, S. Whittaker, A. Furmston, S.R. Clarke, and V.E. Franklin-Tong, manuscript in preparation). The gene for a maize pollen-specific calcium-depen-

dent calmodulin-independent protein kinase (CDPK) has been cloned (Estruch et al., 1994), and CDPK has been shown to be functionally important for pollen germination and pollen tube growth. Also, the stylar S RNases involved in the self-incompatibility response in *Nicotiana alata* have been identified as a substrate for CDPK-like pollen protein kinases from *N. alata* (Kunz et al., 1996). Data from work on *P. rhoeas* pollen also indicate a role for protein kinases in the self-incompatibility response (Rudd et al., 1996).

Our preliminary observation that profilin apparently can alter the phosphorylation of p26 is potentially of interest, because this pollen phosphoprotein is implicated in an early signaling event in the SI response (Franklin-Tong et al., 1993, 1995; Rudd et al., 1996). Further evidence in support of signaling at the cytoskeleton interface in pollen tubes comes from the identification of a Rho GTPase localized at the pollen tube tip (Lin et al., 1996). Because Rho GTPases play a central role in regulating actin-associated cellular responses, this implies a role for them in controlling tip growth in pollen.

The data presented here provide evidence that pollen profilin not only plays a role in regulating the actin cytoskeleton but also that it interacts with cytosolic components affecting protein phosphorylation. The evidence that several pollen protein kinases are implicated in controlling pollen tube growth, taken together with the evidence that profilin modulates protein kinase or phosphatase activity as well as regulating the actin cytoskeleton, suggests that it is not inconceivable that profilin may act in a signaling capacity to regulate pollen tube growth through its modulation of protein kinase activity.

On the question of how profilin acts upon protein phosphorylation, three models should be considered. The simplest explanation is that excess profilin titrates out the kinase or phosphatase activities for these phosphoproteins. Profilin potentially could interact directly with the phosphoproteins and alter their ability to act as substrates for specific kinases or phosphatases. Alternatively, profilin could interact, either directly or indirectly, with pollen protein kinases and/or phosphatases to modulate their activity. It is worth noting that vertebrate PI-3 kinase can bind to bovine spleen profilin in vitro and that profilin stimulates PI-3 kinase activity (Singh et al., 1996a). Profilin could indirectly affect protein phosphorylation by altering the amounts of polymeric actin in cytosolic extracts, which potentially could affect protein kinase activity; CDPKs have been shown to interact with F-actin in plant cells (Putnam-Evans et al., 1990). A third possibility is that profilin acts through binding a shared ligand, competing with the kinase or phosphatase for these cofactors. In addition to the three well-known profilin ligands—G-actin, PtdIns(4,5)P_2, and contiguous stretches of PLP—there is evidence that other cellular factors may interact with profilin (Machesky et al., 1994; Alvarez-Martinez et al., 1996, 1997). Identification of profilin-interacting proteins in pollen would be valuable in advancing our knowledge about regulation of the cytoskeleton in plant cells.

Although actin and PLP may be implicated indirectly in signaling through their interaction with profilin, PtdIns(4,5)P_2 and other polyphosphoinositides that are involved directly in signal transduction also interact in a highly specific manner with profilin. Both animal and plant profilin can control PtdIns(4,5)P_2 turnover by inhibiting phosphoinositide-specific phospholipase C (PIC) activity (Goldschmidt-Clermont et al., 1990; Drøbak et al., 1994; Lu et al., 1996). Because profilin can reduce substantially plant PIC-II activity at low micromolar concentrations (Drøbak et al., 1994) and the effects on pollen protein phosphorylation that we have observed are in this range, it is feasible that this pathway may be implicated in these interactions. If this is the case, it provides a direct link between signaling events and modulation of the cytoskeleton in pollen. Although Vidali and Hepler (1997) suggest that pollen profilin is unlikely to interact with PtdIns(4,5)P_2 because of its observed cytosolic localization, this possibility should not be ruled out. Future work will investigate the potential role of profilin in interacting with inositol lipids and actin to modulate actin polymerization and PIC signaling.

METHODS

Plant Material

Plants (*Papaver rhoeas* var Shirley) segregating for known incompatibility genotypes (S_1S_3 and S_2S_4) were used for these experiments, as described by Franklin-Tong et al. (1988). Collected pollen was stored at −20°C.

Extraction of Profilin from *P. rhoeas* Pollen

Native profilin from pollen was isolated essentially according to the methods of Janmey (1991) by using a small-scale batch method. Pollen (100 mg) was resuspended in buffer I (20 mM Tris, 150 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM ATP, 0.2 mM DTT, pH 7.5) and homogenized on ice with a glass homogenizer (Jencons Scientific Ltd., Leighton Buzzard, UK). The extract was centrifuged at 1200*g*, and the supernatant was removed. A 100-μL aliquot of poly-L-proline (PLP)–Sepharose was added to the supernatant and incubated at 4°C for 90 min. The Sepharose with bound profilin was collected by sedimentation and washed three times with buffer I, and the profilin was eluted by treatment with 7 M urea.

For large-scale native profilin isolations, a 1- to 10-g sample of *P. rhoeas* pollen was ground in buffer I supplemented with 0.1% Triton X-100, a small amount of washed silica, and a protease inhibitor cocktail (Ren et al., 1997). The samples were sonicated and centrifuged at 43,700*g* for 30 min. The supernatant was removed carefully and filtered through MiraCloth (Calbiochem Novabiochem Ltd., Nottingham, UK). The pollen cytoplasmic extract was passed over a 6-mL PLP–Sepharose column preequilibrated with buffer I containing 0.1% Triton X-100. The PLP column was washed with 7 bed volumes of buffer I followed by 5 bed volumes of buffer I containing 2 M urea. Profilin was eluted from the column with buffer I containing 7 M urea, dialyzed extensively against buffer I, and concentrated with a

Centrex-10 ultrafiltration device (Schleicher & Schuell). Protein concentrations were determined by the Bradford method (Bio-Rad) by using BSA as standard. Profilin yields were determined from multiple experiments and expressed as a percentage of total extracted protein (w/w) or as an intracellular concentration by using a molecular mass for profilin of 14,200 and assuming that 10 g of pollen had a cytoplasmic volume of 10 mL (see also Vidali and Hepler, 1997).

Expression and Purification of ZmPRO1 and ZmPRO3

The maize pollen profilin isoforms ZmPRO1 and ZmPRO3 were overexpressed in *Escherichia coli* and purified by PLP–Sepharose chromatography, as described previously (Karakesisoglou et al., 1996; Gibbon et al., 1997).

Determination of Binding Constants for PLP and Pollen G-Actin

The equilibrium dissociation constant (K_d) of native pollen profilin for PLP was determined by monitoring the enhancement of intrinsic tryptophan fluorescence observed when profilin binds PLP (Perelroizen et al., 1994; Petrella et al., 1996; Gibbon et al., 1997). Solutions of 2.5 or 5 μM profilin were titrated with PLP to a final concentration of ~2000 μM proline residues. After each PLP addition, tryptophan fluorescence was monitored for ~1 min on a spectrofluorometer (model 8000; SLM Instruments Inc., Urbana, IL) with excitation at 292 nm and emission at 335 nm. The fluorescence maximum (F_{max}) was estimated by extrapolation of the regression line to the ordinate of double reciprocal plots of fluorescence change (ΔF) versus (proline residues). The data were plotted as $\Delta F/F_{max}$ versus (proline residues), and the points were fit to a hyperbolic function by using MacCurveFit (Kevin Raner Software, Mt. Waverly, Australia) to derive a K_d. Values for K_d were determined using two independent batches of native *P. rhoeas* profilin.

The affinity of native *P. rhoeas* profilin for pollen G-actin was determined by measuring the quenching of intrinsic tryptophan fluorescence, as described previously (Perelroizen et al., 1994; Gibbon et al., 1997). Actin from maize pollen was purified according to the method of Ren et al. (1997) and used immediately or stored as G-actin at 4°C in dialysis tubing for several days. Ca^{2+} was exchanged for Mg^{2+} on G-actin by the addition of 200 μM EGTA and 50 μM $MgCl_2$ (Perelroizen et al., 1994). Quartz cuvettes were loaded with 0, 0.3, or 0.45 μM pollen actin in buffer G (5 mM Tris, 0.2 mM $CaCl_2$, 0.01% NaN_3, 0.5 mM DTT, and 0.4 mM ATP, pH 8.0), and profilin was added sequentially from stock solutions of 200 to 300 μM in buffer G. The total increase in volume after profilin addition was <1.5%. Fluorescence was recorded with excitation at 295 nm and emission at 330 nm. After each addition of profilin, fluorescence was recorded for ~1 min. Total fluorescence minus fluorescence of actin alone was plotted against the concentration of profilin at each point. To determine the K_d value at each actin concentration, the resulting plots were fit with the equation $F_{obs} - F_a = F_p + (F_{pa} - F_a - F_p)[PA]$, where F_{obs} is the measured fluorescence; F_a, F_p, and F_{pa} are the intrinsic fluorescence coefficients for actin, profilin, and the profilin–actin complex, respectively; and [PA] is the concentration of the profilin–actin complex (Perelroizen et al., 1994). Data for each series were discarded if one or more curve fits failed to converge on a value for K_d.

Microinjection of Profilin into *Tradescantia virginiana* Stamen Hair Cells

Stamen hair cells from open flowers of *T. virginiana* were microinjected, as described previously (Karakesisoglou et al., 1996; Gibbon et al., 1997; Ren et al., 1997). The final concentration of profilin injection solutions was 100 μM, and at least two separate protein preparations were used. Nuclear displacement was measured by monitoring the time required for the nucleus to move completely outside the starting perimeter, defined by the edge at the beginning of the experiment. Time zero was recorded when the entire contents of the injection needle had entered the cytoplasm, and each cell was monitored for a maximum of 20 min. If the nucleus did not move sufficiently to exit the predefined area, the time recorded was 20 min. Cells in which the nucleus fell toward the bottom of the cell and those in which the nucleus was unable to move out of the perimeter because it reached the cell wall were discarded. Injection of buffer alone had no effect on nuclear position (see Gibbon et al., 1997; Ren et al., 1997).

Preparation of Pollen Cytosolic and Microsomal Fractions

Pollen was homogenized in 50 mM Hepes, pH 7.7, containing 0.5 M sucrose, 5 mM ascorbic acid, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 0.6% (w/v) PVP-10 at 4°C. High-speed centrifugation was used to obtain a microsomal pellet, which was resuspended in Hepes, and a supernatant comprising soluble proteins, as described by Rudd et al. (1996). The fractions were assayed for protein content and stored at −70°C until use in in vitro–labeling procedures.

Labeling of Pollen Extracts with ^{32}P-γ-ATP

Aliquots of soluble or microsomal proteins equivalent to 100 μg of total protein were labeled in a total volume of 50 μL in 50 mM Hepes, pH 7.7, containing 5 mM $MgCl_2$, 10 mM Mn^{2+}, 50 mM NaF, and 1 mM vanadate. Each sample was labeled with 10 μCi of ^{32}P-γ-ATP (specific activity of 5000 Ci $mmol^{-1}$; Amersham International) at 37°C for 10 min. The labeling reaction was terminated by the addition of 2 × SDS-PAGE sample buffer.

Gel Electrophoresis and Protein Blotting

Samples were analyzed with SDS-PAGE Tricine gels (Schägger and von Jagow, 1987) by using the Mini-Protean II system (Bio-Rad). After electrophoresis, the separated proteins were blotted onto a Hybond C membrane (Amersham International). Blots were either air-dried or stained with Ponceau S (Sigma) and then air-dried. Some samples were analyzed by staining the gels with either Coomassie Brilliant Blue R 250 or silver staining.

Autoradiography and Quantification

Autoradiographs were exposed at −70°C by using BioMax MS film (Kodak). Imaging was also performed using a PhosphorImager (Molecular Dynamics, Chesham, UK), and quantitative analyses were

performed on the Image Quant 3.3 image processing package (Molecular Dynamics). For analysis of phosphoproteins, the counts per minute for individual proteins were determined and compared using volume integration with background labeling subtracted.

Probing of Blots with Profilin Antibody

Protein immunoblots were blocked with 3% (w/v) BSA in Tris-buffered saline (TBS; 50 mM Tris, 200 mM NaCl, pH 7.5). The primary antiserum, anti-ZmPRO3 raised in rabbit (Karakesisoglou et al., 1996), was applied at a dilution of 1:1000 in TBS plus 0.05% Tween-20 (TBST) overnight at 4°C. After TBST washes, a horseradish peroxidase–conjugated anti–rabbit antiserum (Sigma) was applied at 1:2000 dilution for 2 hr at room temperature. The color reaction was developed by addition of 0.5 mg/mL 4-chloro-1-naphthol and 0.0025% (v/v) H_2O_2 in TBS.

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