

ORIGINAL PAPER

Monika Grote · Ines Swoboda · Richard B. Meagher
Rudolf Valenta

Localization of profilin- and actin-like immunoreactivity in in vitro-germinated tobacco pollen tubes by electron microscopy after special water-free fixation techniques

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Abstract Double immunogold labeling of profilin and actin was performed on ultrathin sections of in vitro germinated tobacco pollen using different anti-profilin and anti-actin antibodies. Since profilin, besides its role as an actin-binding protein, is known as an allergen, water-free fixation in *p*-formaldehyde vapor was used. Profilin labeling occurs throughout the cytoplasm of the pollen tube. There is no profilin in the pollen tube wall. Actin reactivity is found in the cytoplasm and extracellularly in the pollen tube wall where three out of four different anti-actin antibodies give a positive signal. This labeling of the pollen tube wall may result from a wall-bound actin, an isoform of actin not yet described or from the presence of a molecule immunologically indistinguishable from actin.

Key words Immunogold labeling · Profilin · Actin
Tobacco pollen · In vitro germination

Introduction

Profilin is a low-molecular-weight protein known to bind to actin monomers in the cell. It can thus restrict and regulate actin polymerization. Whereas the presence of profilin in animal cells has been known for a long time, the occurrence of profilins in higher plant cells and the relationship between profilin and actin in profilactin complexes has been biochemically verified in various pollen species by Valenta et al. (1993). The role

of profilin, as a widely distributed and cross-reactive allergen, has been shown by Valenta et al. (1991a, 1992).

Although immunogold electron microscopic localizations of profilin in resting tobacco pollen have been performed by Grote (1993), we wanted to localize profilin in in vitro-germinated tobacco pollen in the pollen tube. Because of the known interaction of profilin with actin, we included some experiments to demonstrate simultaneously the distribution of both proteins by double labeling techniques.

Since profilin had previously been shown to have allergenic properties and is rapidly dissolved upon contact with aqueous solutions (Grote et al. 1993), we could not prepare specimens for electron microscopy using conventional aqueous fixation techniques. Instead, we decided to apply the anhydrous vapor fixation technique developed for the localization of birch (Grote 1991, 1992, 1993) and grass (Grote et al. 1994) pollen allergens to profilin localizations in germinated tobacco pollen.

Materials and methods

Pollen material

Pollen was harvested from a plant of tobacco (*Nicotiana tabacum*, cv Petit Havana SR 1) grown in a climate room under controlled conditions. Fresh pollen grains were germinated at room temperature in a pollen germination medium (Benito Moreno et al. 1988). Pollen tube growth was monitored by light microscopy.

Preparation for immunocytochemistry

Germinated pollen was anhydrously fixed in *para*-formaldehyde vapor for 6 days at room temperature, dehydrated in DMP (dimethoxypropane) and embedded in Lowicryl K4M at -35°C .

Immunocytochemistry

Primary antibodies

For the localization of profilin, affinity purified rabbit antibodies against celery profilin, RP 1 and RP 2, were used. Another anti-

M. Grote (✉)
Institute of Medical Physics and Biophysics,
University of Münster, Robert-Koch-Strasse 31,
D-48149 Münster, Germany;
FAX no: +49-251-83-5125

I. Swoboda · R. Valenta
Institute of General and Experimental Pathology,
University of Vienna, Vienna, Austria

R. B. Meagher
Department of Genetics, University of Georgia,
Athens, Georgia, USA

body, RP 3, was used only in biochemical experiments, not in immunocytochemistry (for details see Valenta et al. 1992). Actin labeling was performed using four different anti-actin antibodies. (1) Monoclonal antibody to chicken gizzard actin (MAB 1501, Chemikon, Temecula, Calif., USA). This antibody shows specificity for the N-terminal two thirds of the actin molecule and cross-reacts with actin from all animal species and plant actins. The antibody reacts with all actin isoforms derived from skeletal, cardiac, gizzard and aorta tissues (Lessard 1988). (2) Monoclonal antibody to rabbit sarcomeric muscle actin (Amersham, N 350, London, UK). This antibody is reported to have a broad actin specificity. Its sequence specificity, however, is not known (McLean et al. 1990). (3) Sac1, a rabbit polyclonal antibody raised against an internal sequence of soybean actin (FGCPGV-LYQPSMVGME). (4) Sac3, a rabbit polyclonal antibody reacting with the 12 N-terminal amino acids of soybean actin. Sac1 and Sac3 are described in detail elsewhere (McLean et al. 1990). To ensure specificity of anti-actin antibodies for plant actin, all antibodies were cross-reacted with recombinant *Arabidopsis* actin expressed in *E. coli* (Valenta et al. 1991b) and with protein extracts prepared from tobacco pollen.

Secondary antibodies

Secondary antibodies conjugated to colloidal gold particles of 10 nm and 20 nm size were obtained from British BioCell (Plano, Marburg, Germany).

Postembedding double labeling protocol

Ultrathin sections of tobacco pollen tubes were successively incubated (at room temperature, in a moist chamber) on drops of the following solutions: (1) 5% BSA (Serva, Heidelberg, Germany, Cat. no. 11924) in Phosphate Buffered Saline (PBS) (Polak and Priestley 1992); (2) anti-actin antibody, diluted in PBS; (3) PBS; (4) secondary antibody conjugated with 10 nm colloidal gold particles, diluted in PBS; (5) PBS; (6) anti-profilin antibody, diluted in PBS; (7) PBS; (8) secondary antibody conjugated with 20 nm colloidal gold particles, diluted in PBS; (9) PBS; (10) distilled water.

Controls

Four control experiments were carried out. (1) Monoclonal primary antibodies were replaced by monoclonal antibodies of different specificity and rabbit polyclonal primary antibodies by rabbit pre-immune antibodies. (2) Double labeling was conducted in reverse order. (3) Single labeling was conducted for actin and profilin. (4) Primary antibodies were omitted.

Staining and microscopy

Labeled sections were stained with uranylacetate (3 min) and lead citrate (5 s). They were analyzed in a transmission electron microscope (Hitachi H500) and photographed using Agfa Gevaert 23D56 films.

Protein extracts and immunoblots

Total pollen proteins were prepared from tobacco (*Nicotiana glauca*) pollen, timothy grass (*Phleum pratense*) pollen, and in vitro-germinated tobacco pollen by homogenizing pollen in SDS-sample buffer with an ultraturrax and subsequent boiling for 10 min. Extracts were centrifuged for 10 min in a bench centrifuge at 10 000 g to separate insoluble material from the supernatants. Supernatants containing total pollen proteins were subjected to SDS-polyacrylamide electrophoresis (Laemmli 1979), stained with Coomassie Brilliant Blue to ensure that comparable amounts of

intact proteins were blotted to nitrocellulose (Schleicher & Schuell, Dassel, Germany) (Towbin et al. 1979). Immunoblotting on nitrocellulose strips was done as described elsewhere (Valenta et al. 1993).

In order to show the reactivity of anti-actin antibodies with tobacco pollen actin, nitrocellulose strips containing tobacco pollen proteins were incubated with 1:50 diluted Sac1 antibody, 1:50 diluted Sac3 antibody and a 1:100 diluted mouse anti-chicken gizzard actin antibody (Chemikon). Controls were run without addition of primary antibody. To demonstrate competitive inhibition, 50 µg recombinant *Arabidopsis* actin or 50 µg recombinant *Bet v 1* (negative control) (Valenta et al. 1991b) were used to preadsorb 1:100 diluted mouse anti-chicken gizzard actin antibodies (Chemikon).

In order to characterize the anti-profilin antibodies, the following controls and competitive inhibition experiments were done: normal rabbit serum, rabbit anti-profilin antibody RP 1, RP 2, and RP 3 were diluted 1:1000 and preincubated with 50 µg of a control peptide (no sequence similarity to profilin) or with 50 µg of a 25-amino-acid peptide representing the C-terminus of birch pollen profilin. The preincubated antibodies were then applied to nitrocellulose blotted pollen proteins of tobacco (dicotyledonous plant) and timothy grass (monocotyledonous plant).

Results

As Fig. 1a–c shows, the general ultrastructural preservation of pollen tube morphology after vapor fixation is satisfactory, as was the case with previous investigations in birch (Grote 1991, 1992), tobacco (Grote 1993), and grass (Grote et al. 1994) pollens. Numerous mitochondria, strands of endoplasmic reticulum, dictyosomes and vesicles can be identified. Due to absence of osmium postfixation, membranes appear in negative contrast. Elements of the cytoskeletal system (microfilaments or microtubules) are not discernible. The wall of the pollen tube appears as a two-layered, comparatively thick cell wall encompassing the protoplast of the pollen tube. The border between the two layers, however, is not sharply drawn. The outer, darker region of the wall is morphologically characterized by the presence of fibrous material, whereas the inner, more electron-transparent part of the pollen tube wall is composed of predominantly amorphous substances with few fine fibers between.

Figure 1a shows the results of double labeling with profilin and actin antibodies using the RP 1 anti-profilin and the chicken gizzard actin antibody (Chemikon). Gold particles labeling profilin (20 nm) are confined to the cytoplasmic part of the pollen tube. Here they are found to be associated with profiles of the endoplasmic reticulum but they also label sites in the cytoplasmic matrix. There is no labeling of profilin in the pollen tube wall. This labeling pattern is also obtained with anti-profilin antibody RP 2 (not shown). Intense labeling for actin (10 nm gold particles) occurs in the cytoplasmic matrix of the pollen tube. Since no microfilaments are discernible, a co-ordination of actin labeling to specific ultrastructural details cannot be made. The labeling pattern of actin in the cytoplasm of the pollen tube is similar regardless of the type of antibody used (Sac1

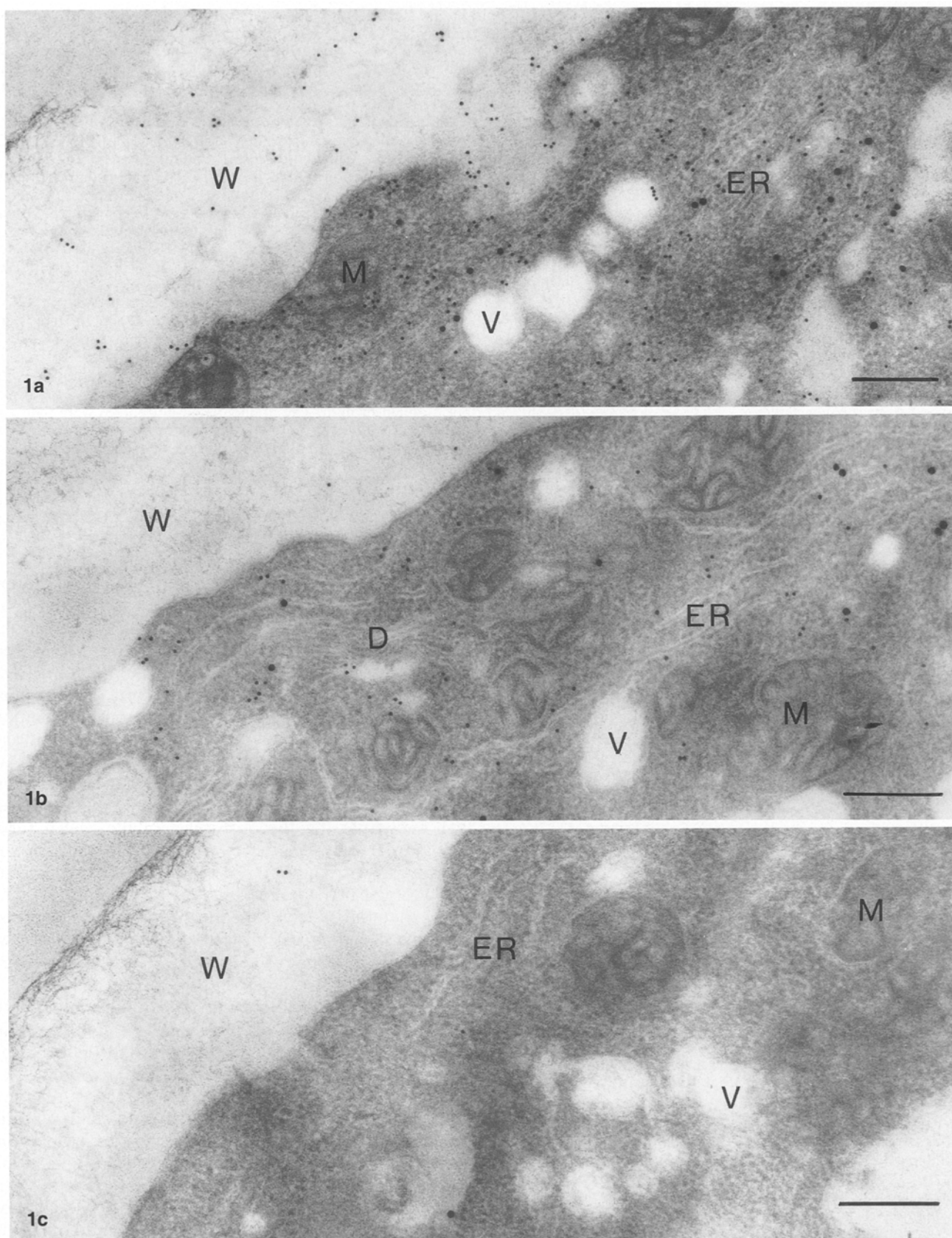


Fig. 1a-c Tobacco pollen tube after anhydrous fixation. Ultra-thin sections of subapical wall and cytoplasm (approximately 500 μm from tip). **a** Immunogold labeling with antibody to chicken gizzard actin (Chemikon) (small gold particles) and RP 1 anti-profilin antibody (large gold particles). Bar 0.2 μm . **b** Immunogold labeling with anti-actin antibody (Amersham) (small gold particles) and RP 1 anti-profilin antibody (large gold particles). Bar 0.2 μm . **c** Immunogold labeling with control monoclonal antibody (small gold particles) and rabbit pre-immune antibody (large gold particles). Bar 0.2 μm . *D* Dictyosome, *ER* endoplasmic reticulum, *M* mitochondrion, *V* vesicle, *W* pollen tube wall

cles) and RP 1 anti-profilin antibody (large gold particles). Bar 0.2 μm . **c** Immunogold labeling with control monoclonal antibody (small gold particles) and rabbit pre-immune antibody (large gold particles). Bar 0.2 μm . *D* Dictyosome, *ER* endoplasmic reticulum, *M* mitochondrion, *V* vesicle, *W* pollen tube wall

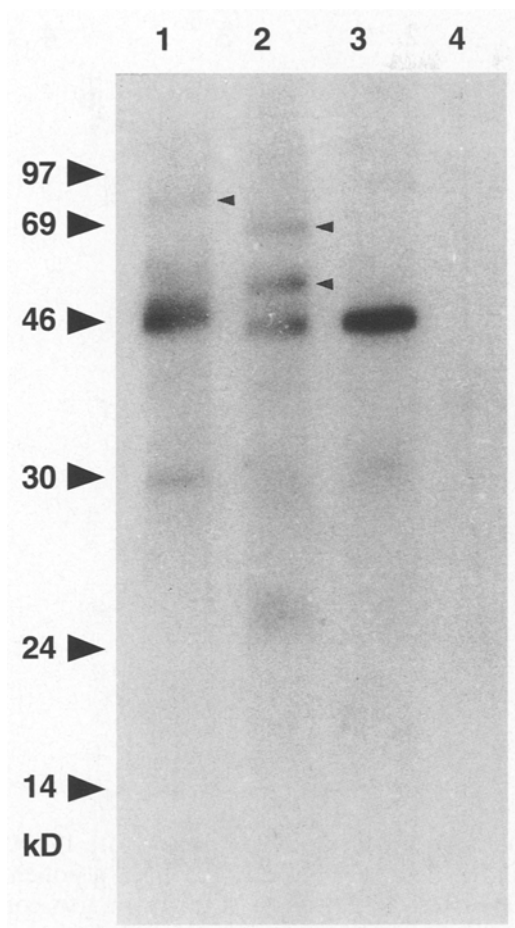


Fig. 2 Immunoblot identification of tobacco pollen proteins by reactivity with different anti-actin antibodies. Lane 1 Sac1, lane 2 Sac3, lane 3 anti-chicken gizzard actin antibody (Chemikon), lane 4 control (additional bands recognized by Sac1 and Sac3 are indicated by small arrowheads)

[not shown], Sac3 [not shown], anti-chicken gizzard actin antibody (Chemikon) [Fig. 1a], Amersham [Fig. 1b]).

This is, however, quite different in the case of the pollen tube wall. After labeling with the antibody to chicken gizzard actin (Chemikon), there are numerous gold particles in the wall, predominantly associated with the inner, electron-transparent region. However, some labeling is also observed in the outer, more fibrous part of the pollen tube wall (Fig. 1a). This distribution pattern is also obtained after use of the anti-actin antibodies Sac1 and Sac3 (not shown). After incubating sections with the anti-actin antibody from Amersham, there is almost no actin labeling in the inner or outer pollen tube wall (Fig. 1b).

Control experiments gave very low background staining. This is shown in Fig. 1c in which the specific primary antibodies were replaced by non-specific and preimmune antibodies, respectively.

Biochemical reactivity of anti-actin antibodies with tobacco pollen actin is demonstrated in Fig. 2. Mouse monoclonal antibody against chicken gizzard actin

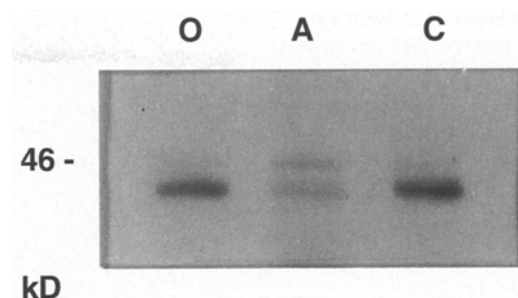


Fig. 3 Immunoblot experiment showing reactivity of anti-chicken gizzard actin antibody to germinated pollen tobacco pollen tube proteins without preadsorption (lane O) and after preincubation with recombinant *Arabidopsis* actin (lane A) or recombinant *Bet v I* (lane C)

(Chemikon, lane 3) binds at around 42 kDa, whereas Sac1 antibody (lane 1) and Sac3 antibody (lane 2) show additional binding to higher-molecular-weight bands (small arrowheads). Similar labeling with these antibodies has already been described (McLean et al. 1990). These authors attributed this binding to chaperonin-like proteins which, though different in sequence and structure to actin, cross-react with anti-actin antibodies. There is no reactivity in lane 4 (control).

Figure 3 shows competitive inhibition of actin antibody binding using recombinant *Arabidopsis* actin. The mouse anti-chicken gizzard actin antibody (Chemikon) binds to a double band migrating at the molecular weight of actin (around 42 kDa, lane O). Fig. 3 demonstrates that this binding could be almost completely inhibited with recombinant *Arabidopsis* actin (lane A), whereas pre-adsorption with recombinant *Bet v I* (lane C) did not affect the binding.

The results of preadsorption of anti-profilin antibodies with a control peptide of irrelevant sequence (lane a) or with a 25-amino-acid peptide derived from birch profilin C-terminus (lane b) are shown in Fig. 4 for tobacco pollen proteins and in Fig. 5 for timothy grass pollen proteins. Binding of RP 2 is not significantly affected by preincubation with the C-terminal peptide whereas binding of RP 1 is weakly reduced (Figs. 4 and 5, blocks 2 and 3). Binding of RP 3 is completely abolished by preincubation (Figs. 4 and 5, block 4). This indicates that RP 2 shows no reactivity, RP 1 shows weak reactivity and RP 3 exclusively reacts with the profilin C-terminus. There is no binding of normal rabbit serum to pollen proteins (Figs. 4 and 5, block 1).

Discussion

The present experiments using various profilin and actin antibodies as subcellular probes in tobacco pollen tubes were designed to confirm the results of our biochemical and immunoelectron microscopic findings

Fig. 4 Inhibition experiment showing reactivity of anti-profilin antibodies to tobacco pollen proteins after preadsorption with a control peptide of irrelevant sequence (lane a) or a 25-amino-acid peptide of specific sequence (lane b). Block 1 control; block 2 RP 2; block 3 RP 1; block 4 RP 3

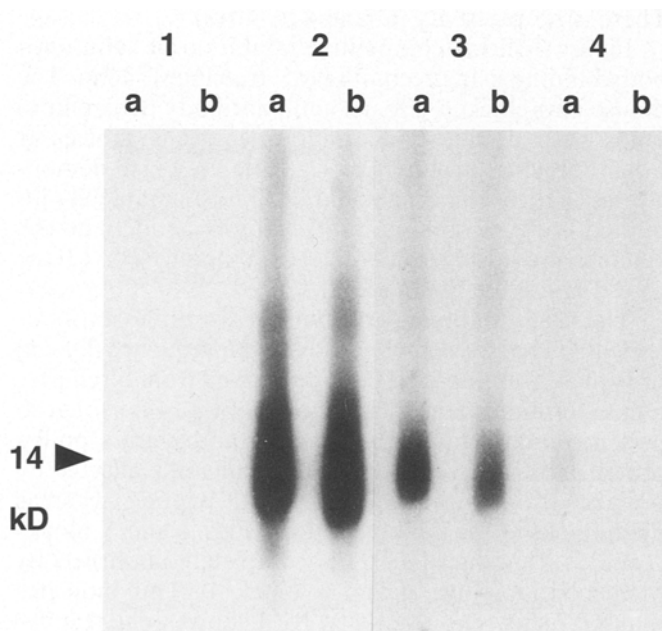
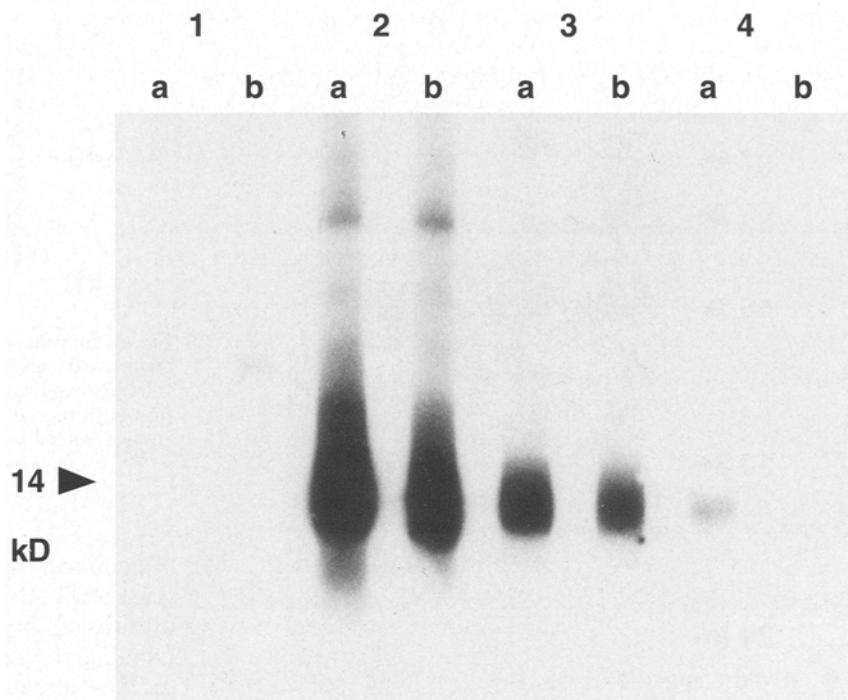


Fig. 5 Inhibition experiment showing reactivity of anti-profilin antibodies to timothy grass pollen proteins after preadsorption. For further explanation see legend of Fig. 4

which showed profilin to be an important constituent of the resting pollen (Valenta et al. 1991a, 1992; Grote 1993). As Fig. 1a–b demonstrate, profilin is found distributed in the pollen tube cytoplasm with a preference for an association with the endoplasmic reticulum. Similar distribution patterns were observed in the dry,

ungerminated tobacco pollen grain (Grote 1993). Considering the described role of profilin as a pollen allergen (Valenta et al. 1991a), it is interesting to compare this localization with the similar labeling pattern of *Bet v* I, a major birch pollen allergen (Grote 1991, 1992) and of allergens in grass pollen (Grote et al. 1994).

Beyond the fact that actin is distinctly present in the pollen tube cytoplasm, its labeling pattern cannot be connected with specific subcellular structures. This is probably due to the difficulties which are generally encountered in the chemical fixation of actin for electron microscopy (Lancelle et al. 1987; Lancelle and Hepler 1991; Tang et al. 1989) and which become even more aggravating when using a mild fixative with little cross-linking effect such as is presented by *para*-formaldehyde.

Nonetheless, in the course of actin labeling, new information was obtained because three out of four anti-actin antibodies used gave a positive signal in the pollen tube wall.

To test whether immunological cross-reactivity of the anti-actin antibodies to portions of actin similar to other proteins might be responsible for the immunolocalization of actin in the pollen tube wall, the peptide sequences of the antibodies Sac1, Sac3 and anti-chicken gizzard actin (Chemikon) were run against the SwissProt Database. No significant homologous proteins other than actin could be found as candidates. Likewise, when comparing the actin binding sequences of the three anti-actin antibodies with the SwissProt Database, no significantly homologous proteins could be found which should be taken into consideration for non-specific reactivity with the three antibodies. From

this result and from the immunoelectron and immunochemical findings of the present study we come to the conclusion that the three antibodies involved in the positive labeling of tobacco pollen tube wall detect either a form of wall-bound actin, or a certain actin isoform, which, in contrast to other isoforms (McLean et al. 1990; Meagher and McLean 1990), has not yet been described, or a molecule which has a molecular weight almost identical to that of actin and which cannot be immunologically discriminated from actin.

Last but not least: since the anti-actin antibodies do not recognize any other band in the immunoblotted protein extracts from resting and germinated tobacco pollen but just the one major actin double band shown, our electron microscopic labeling in the tube wall must very probably originate from the presence of actin (or actin-like) molecules in the wall and not from accidental cross-reactivity with an unknown wall protein synthesized de novo during pollen tube growth.

The fact that the fourth anti-actin antibody (Amersham) did not recognize this molecule does not necessarily negate these explanations. Possibly, the wall-bound molecule is complexed with other proteins or substances of unknown compositions which mask the epitope necessary for binding the Amersham antibody. Actin isoform variability may be another reason why the Amersham antibody does not recognize the wall-bound molecules. In any case, no comparable data have so far been reported on the presence of actin in pollen tube walls (reviewed by Pierson and Cresti 1992).

The presence of proteins in the wall of pollen germination tubes was observed some years ago by, for example, Heslop-Harrison (1987), who found that the inner, callosic layer of the pollen tube contains proteinaceous material. The possible transport of actin or actin-like molecules from the pollen tube cytoplasm into the tube wall is presently a matter of speculation. However, tubular inclusions filled with cytoplasm and ribosomes in the intine of pollen grains have been observed by Hess (1993), who reports that in *Ledebouria* pollen intine matrix substances originate from the Golgi apparatus and leave the cytoplasm via exocytosis. Moreover, extracellular staining of actin or actin-like molecules has been reported before (Spudich et al. 1988). Unfertilized sea urchin eggs contain a discrete cortical shell of actin that is subdivided into organizational states. Our report would reword these findings.

The apparent absence of strong evidence of colocalization of actin and profilin in the pollen tube cytoplasm is noteworthy. This labeling pattern on our micrographs may indicate that in the pollen tube actin and profilin are largely dissociated. It could be by the dissociation of these two proteins that actin microfilament synthesis is initiated. The role of profilin in the resting pollen grain would then be that of a 'microfilament regulator' which, according to certain signals, releases actin molecules from profilactin complexes so that polymerization and formation of filaments in the growing tube can take place.

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