ORIGINAL ARTICLE

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A methionine synthase homolog is associated with secretory vesicles in tobacco pollen tubes

Abstract Seven isoforms of 85 kDa polypeptides (p85) were identified as methionine synthase (MetE) homologs by partial aminoacid sequencing in tobacco pollen tube extracts. Immunocytochemistry data showed a localization of the antigen on the surface of tip-focussed post-Golgi secretory vesicles (SVs), that appear to be partially associated with microtubules (Mts). The chemical dissection of pollen tube high speed supernatant (HSS) showed that two distinct pools of MetE are present in pollen tubes, one being the more acidic isoforms sedimenting at 15S and the remaining at 4S after zonal centrifugation through a sucrose density gradient. The identification of the MetE within the pollen tube and its possible participation as methyl donor in a wide range of metabolic reactions, makes it a good subject for studies on pollen tube growth regulation.

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Introduction

Organelle movement and positioning are involved in cell morphogenesis and function. Polarized cells have evolved mechanisms to deliver specific organelles to different portions of the cytoplasm by translocating organelles and protein complexes along the cytoskeletal tracks (Brown 1999). Angiosperm pollen tubes represent attractive models to investigate mechanisms involved in asymmetrical patterns of growth. Pollen tubes follow a precise, regulated tip growth pattern based on the transport and accumulation of post-Golgi SVs at the extreme tip where, upon the fusion with a restricted region of the plasma membrane, they secrete cell-wall material and provide new segments of plasma membrane (reviewed by Hepler et al. 2001). Among the cytoplasmic factors that regulate cell polarity and the direction of pollen tube growth both in vitro and in vivo, are the cytoskeletal apparatus (Cai et al. 1997) and Ca^{2+}-mediated signal transduction pathways (Hepler 1997). As the pollen tube grows, an acto-myosin dependent (Tang et al. 1989; Yokota and Shimmen 1994; Miller et al. 1995) fountain-like cytoplasmic streaming drives SVs anterogradely to the apex where cytoplasmic streaming is inactive. In most eukaryotic systems, cytoplasmic movements also involve

events regulating membrane organization and membrane trafficking (Brown 1999). In pollen tubes these kinds of movements could act synergically, with the actin filament (Af)-based transport, in maintaining the organization of the cytoplasm and consequently, growth efficiency. Afs-based and Mts-based motility systems cooperate to support organelle transport both in animals (Fath et al. 1994; Gross et al. 2002) and in lower plants (Sato et al. 2001). Studies employing anti-Mts drugs suggested that Mts could be involved in organelle positioning (Joos et al. 1994, 1995) and in regulating the entrance of the vegetative nucleus (VN) and the generative cell (GC) within the tube (Åström et al. 1995). Recently, the contribution of Mt-based movements has been supported by the identification of new Mt-based motors: a 90 kDa polypeptide associated with membranous organelles (Cai et al. 2000) and a 105 kDa kinesin-related polypeptide, able to promote organelle movement along Mts in the absence of Afs, have been isolated from tobacco pollen tubes (Romagnoli et al. 2003).

Other than kinesins, Mt-motor proteins comprise a second protein family known as dyneins (King 2000). Although the motor activity of dyneins reside on the heavy chains (HCs), carrying both the Mt- binding and the ATP-binding domains, specific sets of intermediate and light chains (IC, LC) with molecular masses ranging from 8 kDa to 80 kDa are located at the ATP-insensitive binding site of the protein where they regulate dynein activity in different steps of the cell cycle and differentiation, and mediate the binding with different cytoplasmic targets (Susalka et al. 2000). A 28 kDa dynein LC (p28), identified as the gene product of the *IDA4* locus in *Chlamydomonas* axonemes was shown to be specifically associated with a subset of inner dynein arms containing HCs 2′ and 2 and actin (LeDizet and Piperno 1995a, b) and later, p28 homologues were identified in a wide range of organisms from yeast to humans, suggesting that p28-like proteins could be involved in basic biological processes (Gingras et al. 1996; Kastury et al. 1997). Based on biochemical and immunological approaches two dynein HC-related polypeptides were identified in pollen tubes of tobacco as part of protein complexes with sedimentation coefficients of 22 and 12 S (Moscatelli et al. 1995). Since the identification of dyneins in angiosperms has been controversial (Lawrence et al. 2001; King 2002), our goal in the present study was first to identify eventual IC/LC-related polypeptides in order to support the presence of dynein-related proteins in tobacco pollen tubes. The ubiquity of *Chlamydomonas* p-28 homologues in evolutionary distant organisms suggested that the anti-p28 antiserum could be a probe to investigate the presence of IC/LC in tobacco pollen tubes. Surprisingly, the anti-p28 antiserum identified an 85 kDa polypeptide (p85) sharing high homology with the MetE already sequenced in higher plants (Eichel et al. 1995; Eckerman et al. 2000; Zeh et al. 2002) but not previously identified in pollen tubes. Recently, MetE gene expression detected by cDNA microarray, was specifically identified in mature and immature anthers of *Lotus japonicus* (Endo et al. 2002). MetE belong to the class of zinc-binding enzymes that catalyzes the *de novo* synthesis of methionine by the transfer of a methyl group from 5-methyl tetrahydrofolate to homocysteine and serves to regenerate the methyl group of the SAM, a cofactor required as methyl donor in the biological methylation reactions (Matthews and Goulding 1997; Ranocha et al. 2001).

Therefore, the anti-p28 antiserum was then used in all our experiments as a tool to detect MetE. In the present study, the immunostaining data has shown that the pollen tube MetE homolog (MetT) associates with SVs that appear to be partially associated with cortical Mts in the sub-apical regions of the tubes. Procedures of 2D electrophoresis and biochemical dissection identified seven MetT isoforms that are found in two distinct pools of tobacco pollen tubes. To our knowledge, this is the first report on MetT associated with SVs in tobacco pollen tubes, supporting a functional role of the MetT during pollen tube growth.

Materials and methods

Chemicals

Chemicals were purchased from Sigma Chemicals (Milano, I) except as otherwise indicated.

Leaf and stigma-style crude extract

Young leaves and mature stigma-styles were homogenized in liquid nitrogen and resuspended in extraction buffer (12% SDS, 200 mM Tris, 100 mM DTT). Samples were boiled for 2 min and then centrifuged at maximum speed in the microfuge for 5 min. The supernatant was analyzed by SDS-PAGE and western blotting by using the anti-p28 antiserum.

Pollen culture and pollen tube crude extract

Nicotiana tabacum pollen was collected from plants grown in the Botanical Garden of Siena University, dehydrated by incubation for 12 h in a box containing silica gel and then stored at −20°C. Before germination pollen grains were incubated for 15 min in ice, 15 min at 4°C, 15 min at room temperature and finally hydrated in a humid chamber overnight. Pollen grains were then germinated in BK medium (Brewbaker and Kwack 1963) containing 15% sucrose for 45 min, 90 min and 2 h at 25 ± 1°C respectively. Pollen tubes grown for 2 h were homogenized on ice in 2 v of PEM buffer (100 mM Pipes pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, 0.5 mM DTT, 10 μg/ml TAME, 10 μg/ml leupeptin, 1 μM pepstatin A, 1 μM antipain, 1 μM aprotinin) using a Potter homogenizer (20–25 strokes).

Laemmli sample buffer (LSB) was added to the homogenate and the sample boiled for 5 min. It was subsequently centrifuged at 4°C, 20,800 g for 30 min and the resulting supernatant (except the floating material) collected as the crude extract.

Cell fractionation

Tobacco pollen tubes grown for 2 h were homogenized on ice in 2 v of PEM buffer using a Potter homogenizer (20–25 strokes). The homogenate was centrifuged at 4°C, 3,300 g for 4 min and the pellet discarded. The S1 sample was loaded onto a 0.5 M sucrose cushion (2 ml) in PEM buffer and centrifuged at 4°C, 49,200 g for 30 min. The resulting membrane pellet (P2) was resuspended in PEM buffer. Aliquots of S1, P2 and S2 were subjected to protein assay (Bradford) using BSA as the standard protein and then denatured by LSB for electrophoretic analysis. 25 µg of proteins were loaded in each lane.

Pollen tube HSS

Pollen tubes were homogenized in 2 v of PEM buffer (100 mM Pipes pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml TAME, 1 mM DTT) on ice, using a potter homogenizer (20–25 strokes). After centrifugation at 4°C, 20,800 g for 30 min, the resulting supernatant was further spun at 4°C, 118,000 g for 75 min. The pellet was discarded whereas the HSS was assayed for protein concentration (Bradford), using BSA as the standard.

Zonal centrifugation

For the zonal centrifugation, HSS (300 µl of 3 mg/ml) from *Nicotiana tabacum* pollen tubes were loaded on 4.5 ml 5–25% linear sucrose gradients, prepared in PEM buffer by three cycles of freezing/thawing (Baxter-Gabbard 1972). As control, one gradient was used to sediment proteins with known sedimentation constant: thyroglobulin (19.1 S), catalase (11.3 S) and BSA (4.4 S). Gradients were centrifuged at 4°C, 83,700 g for 15 h and then each gradient was separated into 21 fractions. An equal volume of each fraction was denatured and analyzed by SDS-PAGE and western blotting by using the anti-p28 antibody.

Preparation of *Chlamydomonas* axonemes

Cell cultures of *Chlamydomonas reinhardii* wild type strain 137 (kindly provided by Prof. Gianni Piperno, Mount Sinai School of Medicine, NY, USA) were done in solid medium as reported by Luck et al. (1977). Demembranated axonemes were prepared by the dibucain method according to Witman (1986).

1D gel electrophoresis, western blotting and competition experiments

Proteins were denatured with LSB and separated by means of 10% polyacrylamide gels following the method of Laemmli (1970). Electrophoresis reagents were obtained from Bio-Rad Laboratories (CA, USA). In order to precisely define the molecular mass of pollen polypeptides Precision Plus Protein Standards (Bio-Rad) were used both for 1D gels and western blotting. Gels were comassie blue stained. Western blotting was performed according to Towbin et al. (1979), by using a TE22 Transfer Unit (Amersham Pharmacia). The rabbit anti-p28 polyclonal antibody (1:5000 final dilution) (LeDizet and Piperno 1995b) was kindly provided by Prof. Gianni Piperno (Mount Sinai School of Medicine, NY, USA), while the antisera against MetE of *C. roseus* (1:5,000 final dilution) and *S. tuberosum* (1:10,000 final dilution) were provided by Prof. Schroeder (Institute of Biology II, University of Freiburg Germany) and Prof. Hesse (Max Planck Institute of Molecular Plant Physiology, Golm, Germany) respectively. As secondary antibody the goat anti-rabbit peroxidase conjugate (1:4,000, final dilution) were purchased from Cappel Laboratories (Durham, NC, USA). Detection of the antibodies was performed as outlined in the Amersham ECL kit booklet. For the competition experiment, aliquots of the anti-p28 antiserum (diluted 1:500 in TBS) were pre-incubated with 12 and 25 µg of *Chlamydomonas* axonemes respectively, prepared as described above, for 2 h at room temperature. As control, an aliquot containing only the anti-p28 antiserum was incubated in the same conditions. All samples were centrifuged at 10°C 20,800 g for 30 min. The supernatants were collected and diluted to 1:5,000 with TBS. For the western blotting competition experiment 25 µg of HSS proteins was loaded in each lane. The binding of the antibody was allowed following the procedure reported above.

2D gel electrophoresis and 2D western blotting

1.5 ml of pollen tube HSS (6 mg/ml) were precipitated with 5 v of ice-cold ethanol at −20°C for at least 30 min. The mixture was centrifuged at 4°C for 30 min at maximum speed in the microfuge and the pellet was air dried in order to remove the residual ethanol. The pellet was resuspended with 8M Urea, 2% CHAPS, 0.05M DTE, 0.5 ml of 0.4% resolytes 3.5–10 (BDH) and a trace of bromophenol blue (Bini et al. 1997). For the first dimension, linear pH gradient strips eleven or eighteen centimetres long (purchased from Amersham-Pharmacia) were used for analytical and preparative 2D gels

respectively. Strips were incubated overnight with 500 μl of rehydration solution containing 300 μg or 2 mg of pollen tube proteins at room temperature. First-dimension running conditions were as follows: the voltage was linearly increased from 500 to 3,500 V for 3 h, followed by 5 h at 3,500 V and by 24 h at 3,500 V. For the second dimension, the strips were equilibrated for 12 min using 3 ml of buffer 1 (0.5 M Tris-HCl pH 6.8, 6M Urea, 30% v/v glycerol, 2% w/v SDS, 2% w/v DTE) and subsequently blocked for 5 min using 3 ml of buffer 2 (0.5 M Tris-HCl pH 6.8, 6M Urea, 30% v/v glycerol, 2% w/v SDS, 2.5% w/v iodoacetamide and trace of bromophenol blue). In the second dimension a vertical gradient 4–16% SDS-PAGE was used. Both analytical and preparative 2D gels were coomassie blue stained. 2D western blotting was performed in 10 mM CAPS, 10% v/v methanol, pH 11 by using the Pharmacia-Amersham semi-dry apparatus at 150 mA for 2 h and 15 min at room temperature onto PVDF membrane (purchased by Pharmacia-Amersham). P85 Isoelectric Point (PI) was calculated by the PD Quest 2-D Analysis Software (BioRad). Binding of the anti-p28 and anti-MetE of *C. roseus* antisera (1:5,000 final dilution) were carried out by following the procedure reported by Olmsted (1981).

For the N-terminal sequencing the PVDF membrane was stained in a solution containing Coomassie Brilliant Blue R-250 (0.1% w/v) and methanol (50% v/v) for 15 min. Destaining was done in a solution containing methanol (40% v/v) and acetic acid (10% v/v). Gels and western blotting images were performed as reported in the previous session.

Partial amino acid sequencing of p85 by tandem mass spectrometry (MS/MS)

Preparative 2D gels (2 mg total loading) were performed as reported above in order to have enough material for peptide sequencing. The protein spot was excised from the Coomassie blue stained gel and washed with 50% acetonitrile. Gel pieces were dried in a vacuum centrifuge and reswollen in 20 μl of 25 mM NH_4HCO_3 containing 0.5 μg of trypsin (Promega, sequencing grade). After in gel tryptic digestion, the gel pieces were extracted with 5% formic acid solution and then with acetonitrile. The extracts were combined with the original digest and the sample was evaporated to dryness in a vacuum centrifuge. The residues were dissolved in 0.1% formic acid and desalted using a Zip Tip (Millipore). Elution of the peptides was performed with 5–10 μl of 50% acetonitrile, 0.1% formic acid solution. The peptide solution was introduced onto a glass capillary (Protana) for nanoelectrospray ionisation. Tandem mass spectrometry experiments were carried out on a Q-TOF hybrid mass spectrometer (Micromass, Altrincham, UK) in order to obtain sequence information. Collision-induced dissociation (CID) of selected precursor ions was performed using argon as the collision gas and with collision energies of 40–60 eV. MS/MS sequence information was used for database search using the programs MS-Edman located at the University of California San Fransisco (http://prospector.ucsf.edu/) and BLAST located at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The alignment of p85 fragments with MetE and p28 was carried out using CLUSTAL W (http://www.ebi.ac.uk/clustalw/). Analysis of peptide domains were done by using the PROSITE program located at the NCBI.

Immunofluorescence microscopy

Pollen tubes grown in BK medium for 45 min, 90 min and 3 h were processed for immunofluorescence microscopy observation following the protocol reported in Del Casino et al. (1993). For the 85 kDa polypeptide localization, samples were incubated overnight with the anti-p28 antibody diluted 1:1,000 for 3 h at room temperature. After several washes in TBS pH 7.5, samples were incubated overnight with Alexa conjugated goat anti-rabbit IgG secondary antibody purchased from Molecular Probes (1:400 final dilution) at 4°C and then rinsed three times in TBS, for 10 min. Both primary and secondary antibodies were diluted in TBS. For double immunofluorescence, specimens were first incubated with the Mab anti-α tubulin (from Amersham) diluted 1:100 for 2 h at room temperature. After three rinses with TBS pollen tubes were exposed to the anti-p28 antibody as reported above. The incubation with both secondary antibodies: (Alexa-goat anti-rabbit, from Molecular Probes, and the FITC goat anti-mouse diluted 1:80 from Cappel) was done simultaneously overnight at 4°C.

For Mts depolymerization, pollen tubes grown for 2 h in BK medium were incubated with 10 μM nocodazole and samples were taken after 15 min, 30 min, and 1 h in order to verify the time-depending depolymerization effect. In order to avoid an excess of damage to the cells, the double staining procedure was performed on samples incubated with nocodazole for 15 min. A parallel experiment without nocodazole incubation was performed.

Optical sections (1 μm) and three-dimensional projections of specimens were obtained with a Confocal Laser Scanning Microscope (CLSM) (Microradiance, Bio-Rad Instruments, Hemel, UK) mounted on a Nikon Optiphot microscope and equipped with an argon laser. A 60×objective and the BHS filterset were used for imaging. All images were collected using a stepper motor to make Z-series. Images were also obtained, after DAPI staining of the nuclei, with a Zeiss Axiophot fluorescence microscope using a 100×objective.

All photographs were taken from the monitor on Kodak Tmax films (100 ASA). The pictures presented in this paper were chosen from panels consisting on average of 30 images obtained during seven independent staining experiments.

Immunoelectron microscopy

Freshly collected pollen grains from *Nicotiana tabacum* plants grown in a greenhouse were germinated in BK medium for 2 h. Cells were collected and fixed with 2% formaldehyde and 0.2% glutaraldehyde for 25 min and then rinsed three times with Hepes 50 mM pH 7.2. Cells were incubated with 0.5% NaIO$_4$ for 30 min, washed again as reported above and then treated with 50 mM NH$_4$Cl for 30 min. Samples were rinsed three times with Hepes 50 mM pH 7.2 and then dehydrated on ice: 50% methanol, 70% methanol, 90% methanol in Hepes 50 mM pH 7.2. Infiltration and polymerization were done at −25°C according to the protocol furnished with the LR GOLD resin (London Resin, London, England). Anti-p28 (1:1,000 final dilution) was incubated for 1 h at room temperature and an anti-rabbit (10 nm gold conjugated) was used as secondary antibody.

Results

The anti-p28 antibody identifies a cluster of seven 85 kDa polypeptides

A polyclonal antibody against the ubiquitous *Chlamydomonas* inner dynein arm LC p28 was used in pollen tube extracts. The anti-p28 antiserum, probed on tobacco pollen tube crude extracts, specifically identified an 85 kDa polypeptide (p85) (Fig. 1a), showing a molecular mass significantly distant from that of *Chlamydomonas* antigen (28 kDa) (Fig. 1b). In control experiments, the rabbit preimmune antiserum did not show any positive reaction in pollen tube crude extracts (not shown).

To confirm that the positive reaction detected in the pollen tube crude extract was due to the cross-reactivity of anti-p28 antibodies, competition experiments were performed using demembranated *Chlamydomonas* axonemes where the antiserum specifically recognized the p28 polypeptide (Fig. 1d). Equal aliquots of the anti-p28 antibody preassorbed with 12 μg or 25 μg of demembranated *Chlamydomonas* axonemes, were probed on pollen tube HSS. As control, one aliquot of the anti-p28 antiserum was preincubated in the same conditions but without axonemes. The positive reaction with the pollen tube p85 polypeptide, observed in the control (Fig. 1d, Co) was reduced considerably in the presence of 12 μg of axonemes (Fig. 1d, 12 μg Ax) and almost disappeared when the antiserum was preassorbed with 25 μg of axonemes (Fig. 1d, 25 μg Ax). SDS-PAGE and western blotting analysis using the anti-p28 antiserum after cell fractionation experiments of tobacco pollen tubes showed that most of the p85 polypeptide, which is present in the low speed supernatant S1 (Fig. 1e, lane S1), was recovered in the soluble fraction of the cytoplasm S2, after high speed centrifugation of the S1 sample (Fig. 1e, lane S2). Only
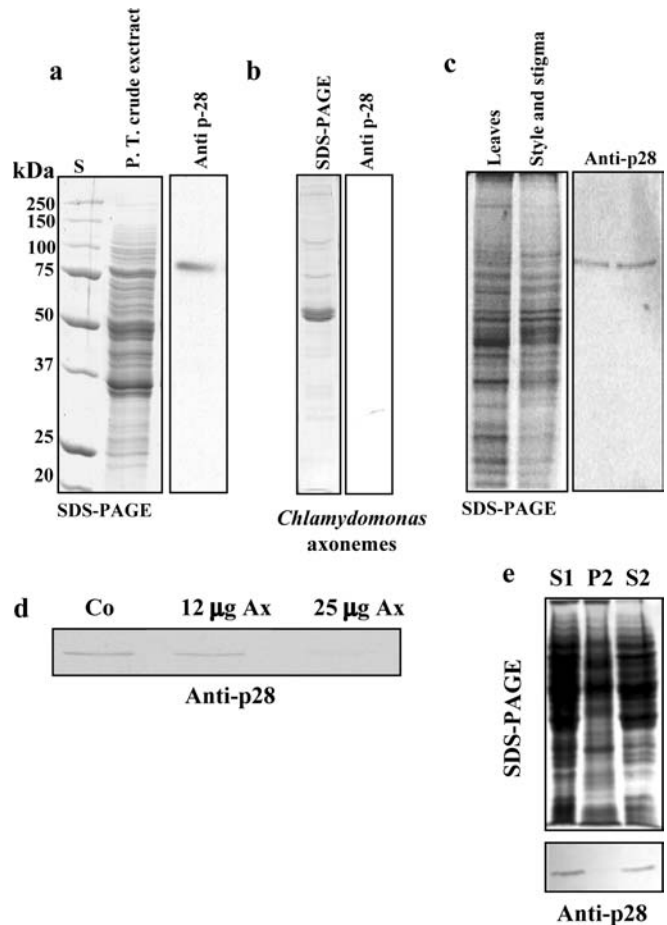

Fig. 1 Reactivity of the anti-p28 antibody on crude extracts of pollen tubes, leaves and stigma-styles of tobacco plants. **a** Crude extracts of pollen tube were probed by the anti-p28 antiserum (P.T. crude extract). The anti-p28 recognized an 85 kDa band in the pollen tube extract whereas in *Chlamydomonas* axonemes **b** it stained the proper 28 kDa polypeptide (Anti-p28). **c** The anti p-28 antiserum probed on other tissues of the tobacco plants recognized a polypeptide showing a molecular mass of 85 kDa both in leaf and stigma-style crude extracts. **d** In competition experiments, equal aliquots of the anti-p28 antibody preassorbed with 12μg or 25μg of demembranated *Chlamydomonas* axonemes, were probed on pollen tube HSS (25μg/lane). The intensity of the reaction decreased progressively depending on the axonemes concentration, with respect to the control (Co) where the anti-p28 antiserum was not preassorbed. (e) The SDS-PAGE and western blotting analysis of pollen tube low speed supernatant (S1), microsomal (P2) and soluble fraction (S2) are reported. The p85 polypeptide is mostly recovered in the soluble fraction (S2) whereas only a weak reaction is seen in P2 (microsomal fraction)

a faint reaction was observed in the microsomal fraction of the cytoplasm P2 (Fig. 1e, lane P2).

As a control the anti-p28 antiserum was probed also on crude extracts of leaves and stigma-styles of tobacco plants. In both samples the antiserum recognized a polypeptide that co-migrated with the p85 already identified in pollen tube extracts (Fig. 1c), confirming that methionine biosynthesis occurs in all plant tissue (Zeh et al. 2002).

In order to understand the polypeptidic composition of the p85 band, pollen tube HSS was analyzed by 2D gel electrophoresis that showed the p85 polypeptide comprising a cluster of seven spots, with PI ranging from 5.7 to 6.1 (Fig. 2, upper panel). All the spots were recognized by the anti-p28 antiserum by western blotting analysis (Fig. 2, lower panel), suggesting that they could represent different isoforms of the same protein.

P85 peptide fragments are highly homologous
to the MetE

Although the immunological crossreactivity between the *Chlamydomonas* p28 and the pollen tube p85 led us to hypothesize a sequence similarity, the difference in the molecular mass between those polypeptides induced us to investigate the identity of pollen tube p85 polypeptides. Although all the attempts to sequence the NH$_2$-terminus by Edman sequencing were not successful, partial aminoacid sequence analysis of proteolytic fragments of p85 2D spots by MS/MS resulted in the identification of 14 protein fragments reported in Fig. 3a (MetT). Protein databases were searched using the partial or complete sequence of 14 peptides. Database searching results revealed a strong homology between the MS/MS sequence information and the sequence of MetE from *Catharanthus roseus* (Eichel et al. 1995) (accession number CAA58474), *Solenostemon scutellarioides* (accession number CAA89019), *Solanum tuberosum* (accession number AAF74983), *Arabidopsis thaliana* (accession number BAB11226), *Sorghum bicolor*

Fig. 2 The tobacco p85 comprises seven isoforms by 2D gel analysis. The anti-p28 antiserum identified seven p85 spots (PI ranging form 5.7 and 6.1) when probed on the HSS polypeptides separated by 2D gel. The electrophoretic pattern is reported in the upper part of the figure; arabian numbers are used to indicate p85 isoforms. The western blotting, reported in the lower part of the figure, shows that all isoforms are recognized by the anti-p28 antibody

(accession number AAL73979) and *Zea mays* (accession number AAL33589), which shows the unambiguous identity of this protein (see Fig. 3a). PIs of MetE calculated by ExPASy (Swiss Institute of Bioinformatics) were 6.1, which is coherent with the p85 PI (5.7–6.1) estimated after 2D gel by the PDQuest 2-D Analysis Software (BioRad). The presence of several p85 isoforms, revealed by 2D gels, suggested that they could represent the expression products of several p85 genes and/or they could result by post-traslational modifications. Domain analysis of p85 fragments by the PROSITE program evidenced a PKC and a CK2 phosphorylation site corresponding to AA 2–4 (SPR, Fig. 3a, in red) and AA 7–10 (STEE, Fig. 3a, in red) of the fragment 14. This suggests that p85 could be the target of protein kinases already identified during pollen tube growth (Moutinho et al. 1998). The crossreactivity of the anti-p28 antiserum with the p85 raised the question about the degree of sequence homology between those polypeptides. The alignment with p28 aminoacid sequence (accession number CAA88139.1) showed that p85 fragments (F1 to F13–14 in Fig. 3b) have up to 50% sequence homology with short stretches of the *Chlamydomonas* p28 (Fig. 3b), which explains the anti-p28 antibody cross-reactivity.

The MetT is found on particle-like structures
aligned on cortical Mt bundles

To observe the localization of the p85 antigen in pollen tubes, indirect immunofluorescence technique was performed using the anti-p28 antiserum. The antibody stained punctate structures that appeared to be more concentrated in the apical region of tubes grown for 45 min and 2 h (Fig. 4a, b), suggesting that the p85 polypeptides could be associated with SVs accumulating in the pollen tube tip. However, spots were observed along the whole cytoplasm of the tube even with a lower density (Fig. 4b). Single longitudinal tangential sections of pollen tubes evidenced fluorescent spots often aligned along cortical filaments in regions behind the apex (Fig. 4c). In pollen tubes grown for 7 h in which GC and VN have already started to move along the tube, the antibody decorated the GC shape (Fig. 4e, f). Control experiments in which the primary antibody was omitted did not show any staining within the pollen tube cytoplasm (Fig. 4d) as well as the use of the preimmune antiserum (not shown). Competition experiments in which the anti-p28 antiserum was preassorbed to 25 μg of *Chlamydomonas* axonemes and then used as primary antibody in tobacco pollen tubes showed a decrease of the staining intensity (Fig. 4g) compared to the control (Fig. 4h). The observation of fluorescent spots aligned in the cortical regions of the tube allowed us to hypothesize that they could bind to cytoskeletal filaments. Although both Afs and Mts have been shown in the cortical region of pollen tubes, Afs seem to be more abundant in the inner cytoplasmic region. The possible presence of
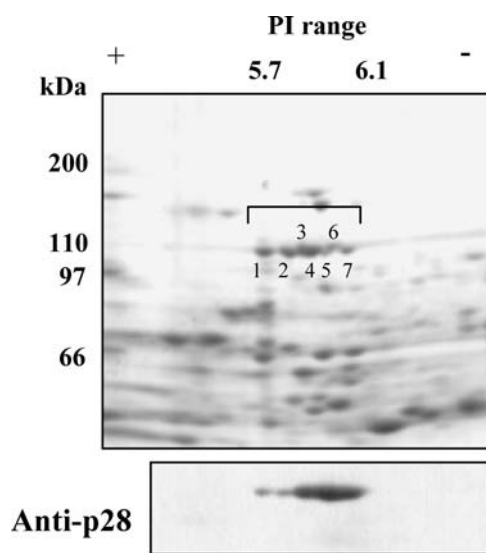
782

Fig. 3 P85 peptide fragments are highly homologous to the MetE. **a** The sequence information obtained by MS/MS from the p85 polypeptides is identical or homologous to the MetE, which shows the unambiguous identity of this protein. The sequence of the MetE from several species of angiosperms *Catharanthus roseus* (Met *Cath*) (accession number CAA58474), *Solenstemon scutellarioides* (Met *Sol*) (accession number CAA89019), *Solanum tuberosum* (Met *Solanum*) (accession number AAF74983), *Arabidopsis thaliana* (Met *Arab*) (accession number BAB11226), *Sorghum bicolour* (Met *Sorgum*) (accession number AAL73979) and *Zea mays* (Met *Zea mais*) (accession number AAL33589), and the sequence information obtained by tandem mass spectrometry experiments from tobacco pollen tube p85 polypeptides (MetT) are shown. Domain analysis of p85 fragments by using the PROSITE program gave evidence of a PKC and a CK2 phosphorylation sites corresponding to AA 2–4 (SPR, in *red*) and AA 7–10 (STEE, in *red*) of the fragment 14. **b** The alignment of the p85 fragments (F1 to F13–14) with the p28 aminoacid sequence (reported in the first line) (accession number CAA88139.1) show that p85 fragments have 15–50% sequence homology with short stretches of the *Chlamydomonas* p28. Green V: amino acid identical to its counterpart in the p28 sequence; yellow V: amino acid homolog to its counterpart in the p28 sequence; blue V: semiconservative substitution

a

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MetCath      --------------------MASHIVGYPRMGPKRELKFALESFWDKKSSAEDLQKVAAD 40
MetSol       MALSFFLDTSVSVPLRCQGKMASHIVGYPRMGPKRELKFALESFWDGKSSAEDLEKCQLI 60
MetSolanum   --------------------MASHVVGYPRMGPKRELKFALESFWDGKSSAEDLKKVSAD 40
MetArab      --------------------MASHIVGYPRMGPKRELKFALESFWDGKSTAEDLQKVSAD 40
MetSorghum   --------------------MASHIVGYPRMGPKRELKFALESFWDGKSSAEDLEKVATD 40
MetZea mays  --------------------MASHIVGYPRMGPKRELKFALESFWDGKSTAEDLEKVATD 40
                                 ****:.*.******.*****.** **:****.:*
MetT         --------------------LSHIVGYPR--------FALESFWXXK------------
                                 (F1)                (F2)

MetCath      LRSSIWKQMADAGIKYIPSNTFSYYDQVLDTATMLGAVPPRYNFAGGEIGFDTYFSMARG 100
MetSol       LG-IHLKQMSDAGIKYIPSNTFSYYDQVLDTTAMLGAVPPRYNWTGGEIGFSTYFSMARG 119
MetSolanum   LRSSIWKQMSDAGIKYIPSNTFSYYDQVLDTTAMLGAVPSRYNWTGGEIEFGTYFSMARG 100
MetArab      LRSSIWKQMSAAGTKFIPSNTFAHYDQVLDTTAMLGAVPPRYGYTGGEIGLDVYFSMARG 100
MetSorghum   LRSSIWKQMSEAGIKYIPSNTFSYYDQVLDTTAMLGAVPERYSWTGGEIGLSTYFSMARG 100
MetZea mays  LRASIWKQMADAGIKYIPSNTFSYYDQVLDTTAMLGAVPERYSWTGGEIGFDTYFSMARG 100
             *     ***: ** *:.*****::.*******:.****** **.::.**** :...*******

MetCath      NASVPAMEMTKWFDTNYHYIVPELGPEVNFSYASHKAVNEYKEAKELGVDTVPVLVGPVT 160
MetSol       NASVPAMEMTKWFDTNYHFIVPELGPDVKFSYASHKAVNEYKEAKALGVDTVPVLVGPVS 179
MetSolanum   NASVPAMEMTKWFDTNYHFIVPELGPDVNFSYASHKAVNEYKEAKAQGVDTVPVLVGPVS 160
MetArab      NASVPAMEMTKWFDTNYHYIVPELGPEVNFSYASHKAVNEYKEAKALGVDTVPVLVGPVS 160
MetSorghum   NATVPAMEMTK-----CHFIVPELGPSTKFTYASHKAVSEYKEAKALGIDTVPVLVGPVS 155
MetZea mays  NATVPAMEMTKWFDTNYHFIVPELGPNTKFSYASHKAVNEYKEAKALGVDTVPVLVGPVS 160
             **:.********     *:*******..:*:*******.****** *:.**********:

MetCath      FLLLSKPAKGVEKTFPLLSLLDKILPVYKEVIGELKAAGASWIQFDEPTLVLDLESHQLE 220
MetSol       YLILSKPAKGVEKTFPLLSLLDKILPIYKEVIAELKAAGASWIQFDEPTLVLDLESHQLD 239
MetSolanum   YLLLSKPAKGVEKSFPLLSLLDKILPIYKEVIAELKAAGASWIQLDEPTLVLDLESHKLE 220
MetArab      YLLLSKAAKGVDKSFELLSLLPKILPIYKEVITELKAAGATWIQLDEPVLVMDLEGQKLQ 220
MetSorghum   YLLLSKPAKGVEKSFSLLSLLGSILPIYKEVVAELKAAGASWIQFDEPTLVKDLDAHELA 215
MetZea mays  YLLLSKPAKGVEKGFPLLSLLSSILPVYKEVIAELKAAGASWIQFDEPTLVLDLDSDKLA 220
             :*:.***.****:* * ***** .***:****: *******:***.***.** **:..:*
MetT         ---------------PLLTLVSR-------------------------------------
                            (F3)

MetCath      AFTKAYSELESTLSGLNVIVETYFADIPAETYKILTALKGVTGFGFDLVRGAKTLDLIKG 280
MetSol       AFTKAYAELESSLSGLSTLIETYFADVPAPAYKTLTSLSGISGFGFDLVRGAQTIELIKG 299
MetSolanum   AFTKAYADLESSLSGLNVLVETYFADVPAEAFKTLTALKGVTAFGFDLVRGTQTLELIKS 280
MetArab      AFTGAYANLESTLSGLNVLVETYFADIPAEAYKTLTSLKGVTAFGFDLVRGTKTLDLVKA 280
MetSorghum   AFSSAYAELESAFSGLNVLIETYFADIPAESYKTLTSLSGVTAYGFDLIRGAKTLDLIRS 275
MetZea mays  AFSSAYAELESVLSGLNVLVETYFADVPAESYKTLTSLSSVTAYGFDLVRGTQTLGLVTS 280
             **:.**::*** :****..::*******:** ::* **:*..:::****:**::*: *: .
MetT         ----------------------------------------TGFGFDLVR---TLDLIK-
                                                     (F4)        (F5)

MetCath      G-FPSGKYLFAGVVDGRNIWANDLAASLSTLQSLEGIVGKDKLVVSTSCSLLHTAVDLVN 339
MetSol       G-FPSGKYLFAGVVDGRNIWANDLASSITTLQALEGIVGKDKLVVSTSSSLLHTAVDLVN 358
MetSolanum   S-FPSGKYLFAGVVDGRNIWANDLAASLALLQSLEGVVGKDKLVASTSCSLLHTAVDLIN 339
MetArab      G-FPEGKYLFAGVVDGRNIWANDFAASLSTLQALEGIVGKDKLVVSTSCSLLHTAVDLIN 339
MetSorghum   S-FPSGKYLFAGVVDGRNIWADDLAASLTLQSLEAVAGKDKLVVSTSCSLMHTAVDLVN 334
MetZea mays  AGFPAGKYLFAGVVDGRNIWADDLATSLSTLQSLEAVVGKDKLVVSTSCSLMHTAVDLVN 340
             . ** ****************:*.*.*:: **.**.:. ***** .***.**.**:*****:*
MetT         -------YLFAGVVDGR-------------------------------------------
                    (F6)

MetCath      EPKLDKEIKSWLAFAAQKVVEVNALAKALAGEKDEAFFSENAAAQASRKSSPRVTNQAVQ 399
MetSol       EPKLDQEIKSWLAFAAQKIVEVNALAKALTGHKDEAFFSPNAAAQASRKSSPRVNNEAVQ 418
MetSolanum   ETKLDDEIKSWLAFAAQKVVEVNALAKALSGAKDEAFFSENAAAQASRKSSPRVTNEAVQ 399
MetArab      ETKLDDEIKSWLAFAAQKVVEVNALAKALAGQKDEALFSANAAALASRRSSPRVTNEGVQ 399
MetSorghum   ETKLDDEIKSWLAFAAQKVVEVNALAKALAGQKDEAYFAANAAAQASRRSSPRVTNEEVQ 394
MetZea mays  ETKLDSEIKSWLAFAAQKVVEVDALAKALAGQKDEAYFAANAAAQASRKSSPRVTNEEVQ 400
             *.***.*************:***.******:* **** *: **** ***:*****.*: **
MetT         ------------------VVEVNALAK--------AFFSANAAAQASR------------
                              (F7)        (F8)

MetCath      KAAAALRGSDHRRATTVSARLDAQQKKLNLPVLPTTTIGSFPQTLELRRVRREYKAKKIS 459
MetSol       KAAAALQGSEHRRVTNVSARLDAQQKKLNLPILPTTTIGSFPQTVELRRVRREFKPTRIS 478
MetSolanum   KASAALQGSDHRRATNVSARLDAQQKKLNLPILPTTTIGSFPQTVELRRVRREYKAKKIS 459
MetArab      KAAAALKGSDHRRATNVSARLDAQQKKLNLPILPTTTIGSFPQTVELRRVRREYKAKKVS 459
MetSorghum   KAAAALKGSDHRRATTVSSRLDAQQKKLNLPVLPTTTIGSFPQTPELRRVRREYKAKKIS 454
MetZea mays  KAAAALKGSDHRRGTNVSARLDAQQKKLNLPILPTTTIGSFPQTVELRRVRREYKAKKIS 460
             **:***:**:*** *.**:***********:*********** ******:*..::*

MetCath      EDDYVKAIKEEISKVVKLQEELDIDVLVHGEPERNDMVEYFGEQLSGFAFTANGWVQSYG 519
MetSol       EEEYVKAIKEEINKVVKLQEELDIDVLVHGEPERNDMVEYFGEQLSGFAFTANGWVQSYG 538
MetSolanum   EEEYVKAITEEIKKVVDLQEELDIDVLVHGEPERNDMVEYFGEQLSGFAFTANGWVQSYG 519
MetArab      EEDYVKAIKEEIKKVVDLQEELDIDVLVHGEPERNDMVEYFGEQLSGFAFTANGWVQSYG 519
MetSorghum   EEEYISAIKEEISKVVKIQEELDIDVLVHGEPERNDMVEYFGEQLSGFAFTANGWVQSYG 514
MetZea mays  EEEYVTAIKEEINKVVKLQEELDIDVLVHGEPERNDMVEYFGEQLSGFAFTANGWVQSYG 520
             *::*:.**.**.***.***.:*******************************************
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Fig. 3 (Contd.)

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MetCath     SRCVKPPIIYGDVSRPNPMTVFWSQTAQSMTKRPMKGMLTGPVTILNWSFVRNDQPRFET 579
MetSol      SRCVKPPIIYGDVSRPKPMTVFWSTAAQSMTQRPMKGMLTGPVTILNWSFVRNDQPRFET 598
MetSolanum  SRCVKPPIIYGDVSRPKPMTVFWSSKAQEMTKRPMKGMLTGPVTILNWSFVRNDQPRFET 579
MetArab     SRCVKPPVIYGDVSRPKAMTVFWSAMAQSMTSRPMKGMLTGPVTILNWSFVRNDQPRHET 579
MetSorghum  SRCVKPPIIYGDVSRPNPMTVFWSKTAQSMTARPMKGMLTGPVTILNWSFVRNDQPRFET 574
MetZea mays SRCVKPPIIYGDVSRPNPMTVFWSKTAQSMTSRPMKGMLTGPVTILNWSFVRNDQPRFET 580
            *******:*********:.******  **.** *********************** .**
MetT        ----KPPIIYGDVSR----------------------LTGPVTIL-----------FET
                (F9)                              (F10)

MetCath     CYQIALAIKDEVEDLEKAGINVIQIDEAALREGLPLRKAEHAFYLDWAVHSFRITNLPLQ 639
MetSol      CYQIALAIKDEVEDLEKAGITVIQIDEAALREGLPLRKAEHAFYLDWAVHSFRITNVGVQ 658
MetSolanum  CYQIALAIKDEVEDLEKAGITVIQIDEAALREGLPLRKAEHAFYLNWAVHSFRITNVGIE 639
MetArab     CYQIALAIKDEVEDLEKGGIGVIQIDEAALREGLPLRKSEHAFYLDWAVHSFRITNCGVQ 639
MetSorghum  CYQIALAIKKEVEDLEAAGIQVIQIDEAALREGLPLRKSEHAFYLDWAVHSFRITNCGVQ 634
MetZea mays CYQIALAIKKEVGDLKAGGIQVIQIDEAALREGLPLRKAEHAFYLDWAVHSFRITNCEIQ 640
            *********.** **:.** ********************:******:*********  ::
MetT        CYQIALAIK-----------VIQIDEAALR----------------------------
                (F11)                (F12)

MetCath     DTTQIHTHMCYSNFNDIIHSIIDMDADVMTIENSRSSEKLLSVFREGVKYGAGIGPGVYD 699
MetSol      DTTQIHTHMCYSNFNDIIHSIINMDADVITIENSRSDEKLLSVFREGVKYGAGIGPGVYD 718
MetSolanum  DTTQIHTHMCYSNFNDIIHSIIDMDADVITIENSRSDEKLLSVFREGVKYGAGIGPGVYD 699
MetArab     DSTQIHTHMCYSHFNDIIHSIIDMDADVITIENSRSDEKLLSVFREGVKYGAGIGPGVYD 699
MetSorghum  DTTQIHTHMCYSNFNDIIHSIIDMDADVITIENSRSDEKLLSVFREGVKYGAGIGPGVYD 694
MetZea mays DTTQIHTHMCYSNFNDIIHSIIDMDADVITIENSRSDEKLLSVFREGVKYGAGIGPGVYD 700
            *:.***********:*********:***** *******.*********************
MetT        --------------------------------------------------YGAGLGPGVYD
                                                              (F13)

MetCath     IHSPRIPSTEEIADRINKMLAVLDTNILWVNPDCGLKTRKYAEVKPALENMVSAAKLIRT 759
MetSol      IHSPRIPSTEEIADRINKMLAVLETNILWVNPDCGLKTRKYAEVKPALENMVAAAKLLRT 778
MetSolanum  IHSPRIPSTEEIADRVNKMLAVLDTNILWVNPDCGLKTRKYTEVKPALQNMVSAAKTIRT 759
MetArab     IHSPRIPSSEEIADRVNKMLAVLEQNILWVNPDCGLKTRKYTEVKPALKNMVDAAKLIRS 759
MetSorghum  IHSPRIPTTEEIADRVNKMLAVLDTNILWVNPDCGLKTRKYTEVKPALTNMVSATKLIRT 754
MetZea mays IHSPRIPSAEEIADRIDKMLAVLDTNILWVNPDCGLKTRKYTEVKPALTNMVSAAKLIRT 760
            *******::******::******: ****************:****** *** *:* :*:
MetT        IHSPRIPSTEELADR---------------------------------------------
                (F14)

MetCath     QLASAK 765
MetSol      QLASAK 784
MetSolanum  QLASAK 765
MetArab     QLASAK 765
MetSorghum  QLASAK 760
MetZea mays QFASAK 766
            *:****

P28         MIPPLSSLVRYDNPVLVSTSKDKGKGAKGTPGKKGALPPVEQKPGLTQTEDILNSILPPR 60
F1  (44%)   ----LSHIVGY--------------------------------------------PR
F3  (50%)   ---PLLTLV----------SR----
F7  (22%)   -VVEVNALAK--
F9  (18%)   -KPP---II-YGD---VSR-
F10 (25%)                                      ---LTG------PVT----------IL--
F12 (20%)       --VIQIDEAALR----
F13-14 (15%)     -Y-----------GAG-LG-PG----VYDIHS-PRIPSTEEIADR-----

P28         EWTEDGQLWVQYVSSTPATRLDVINLQEKLDQQLQQRQARETGICPIREELYAQTFDELI 120
F2  (20%)   -FALES-FWXX--------K--
F4  (33%)                                        --TG--------FG--FD-LV
F5  (50%)                                                  --TLD-LI

P28         RQVTINCAERGLLLLRVRDEMRMTIAAYQTLYESAVAFGIRKALQTEQGKSEMESRITQL 180
            R--
            K--
F6  (30%)                             -Y--LFA-----GV------VDGR-
F11 (33%)                               --FETCYQ--IALAIK-----

P28         EGDVKDLERQVQEWKFKCEAIEKRESERREVEAKKHKDEVAYLENYAKQLKQQLETFLVP 240
F8  (15%)               -AF-FSANAAAQ- ASR-

P28         AKKGAPGAPAAAT 253
```

b

spots-like structures on the surface of cortical Mts was tested by double immunofluorescence experiments using the anti-α tubulin and anti-p28 primary antibodies. Specimens were observed by the CLSM during three independent experiments of double labelling. Optical reconstruction of whole cells showing a merging of both stainings revealed regions of co-localization indicated in yellow (Fig. 5a). In order to observe this aspect in greater detail, longitudinal tangential and longitudinal axial sections of the same pollen tube were observed separately. Tangential sections showed the presence of extensive Mt bundles longitudinally oriented along the tube (Fig. 5b), which appeared to overlap with spot-like structures (Fig. 5c) when the two staining patterns are merged (Fig. 5d). An analogous situation was observed when axial sections of the tube were observed: the anti-α tubulin (Fig. 5e) and the anti-p28 antibody staining (Fig. 5f) coincided in the cortical region of the tube (Fig. 5g), whereas the spots distribution within the cytoplasmic area of the tube seemed to be unrelated to

Mts, which are not shown in this region (Fig. 5e). The eventual co-localization in the tip region could not be observed because of the high fluorescence due to the anti-p28 staining. Control experiments in which the anti-rabbit and the anti-mouse secondary antibodies were used on the anti-α tubulin and the anti-p28 antibodies respectively, did not give any positive reaction (data not shown).

Experiments of Mts depolymerization using 10 μM nocodazole were performed in order to support the data showing a co-localization of anti-p28 stained membranous organelles with cortical Mts. Pollen tubes grown in BK medium, and incubated with nocodazole for different times; after 15 min fragments of Mts were still observed (Fig. 6a), while after 30 min the anti-α tubulin Mab showed only fluorescent spots within the cytoplasm (Fig. 6d). Double immunofluorescence experiments were performed after 15 min incubation and showed that Mts inhibition caused two kind of effects: particles recognized by the anti-p28 antibody aggregate into clusters

Fig. 4 The anti-p28 antiserum recognized particle-like structures by immunofluorescence microscopy. Immunofluorescence microscopy in tobacco pollen tubes at different lengths shows that the anti-p28 antibody recognizes particle-like structures that are more concentrated in the tip region of the tube (a, b) suggesting that it stains epitopes on the surface of post-Golgi SVs. Observing 1μm single sections by the CLSM it was seen that particles aligned along hypothetical filaments **c**. Control experiments in which the preimmune antiserum was used instead of the anti-p28 antibody do not give any positive reaction **d**. Fluorescent spots are also observed to decorate the GC shape **e** whose nucleus was localized by using DAPI (f). In competition experiments performed by using anti-p28 antibody preassorbed with 25 μg of demembranated *Chlamydomonas* axonemes, fluorescent spots were not visible anymore and only a diffuse background is shown **g**, compared with the control **h**. Bars 10 μm
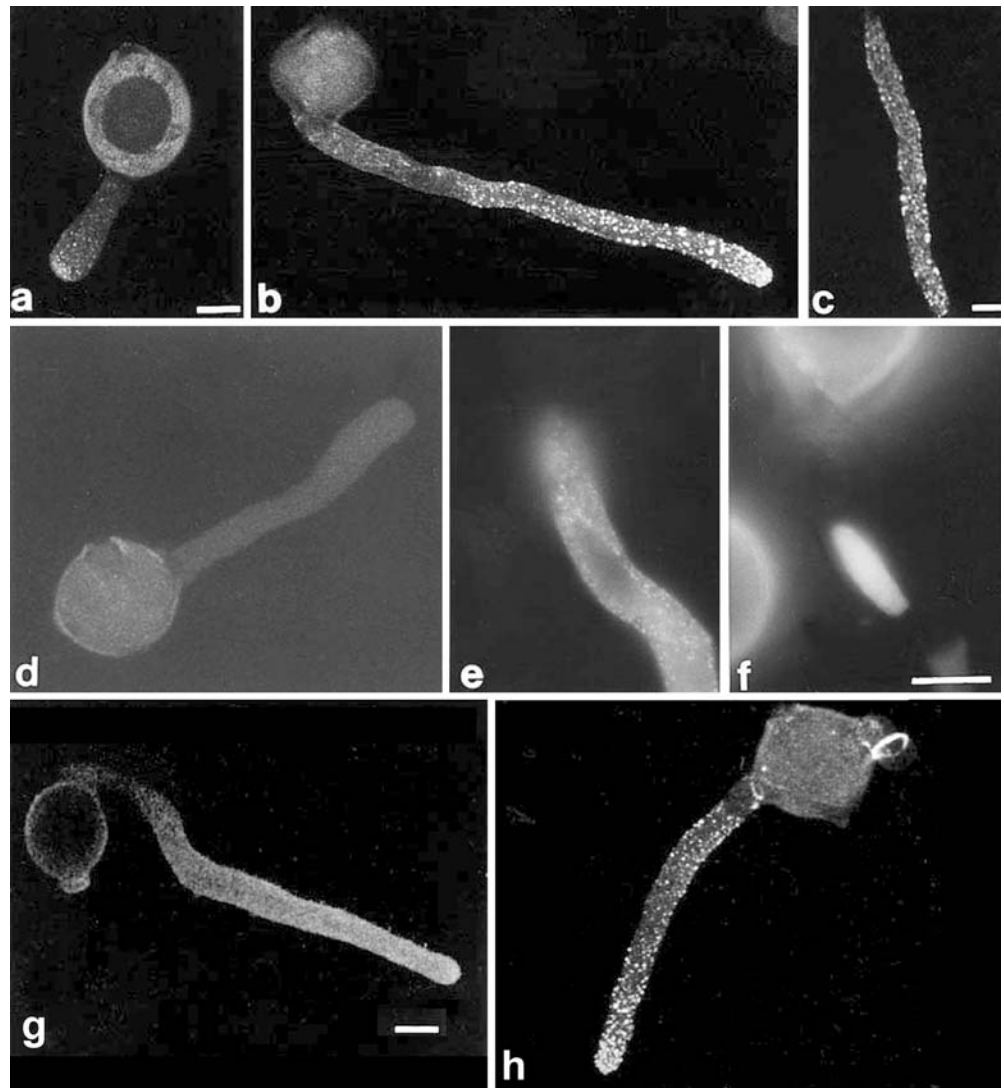
that are mislocalized toward the central part of the pollen tube cytoplasm (Fig. 6b) and the co-localization with cortical Mts is lost (Fig. 6c, see arrows) with respect to a parallel experiment in which nocodazole was not used (data not shown).

Fig. 5 Fluorescent spots recognized by the anti-p28 antiserum colocalize with cortical Mt bundles. Double immunofluorescence experiments, performed using the anti-p28 antiserum and the anti-α tubulin Mab, showed that fluorescent spots co-localize with cortical microtubular bundles. Specimens were observed by the CLSM. A merge project of a pollen tube shows areas of co-localization (*yellow zones*) between anti-p28 fluorescent spots and Mts **a**. Observing in more detail cortical (b, c) and medial (e, f) areas of the tube it was seen that particle-like structures co-localize with cortical microtubular bundles (d, g)

SVs bind antigens recognized by the anti-p28 antibodies

To identify the cytoplasmic targets of p85 polypeptides, immunogold labelling was performed on pollen tube sections using the anti-p28 antiserum. Electron Microscopy (EM) observations revealed a specific staining of 200–300 nm SVs which typically accumulate in the final 10 μm of the tip region (Fig. 7a). Gold particles were seen associated with the plasma membrane only occasionally, suggesting that the binding of p85 with SVs could be removed before their fusion with the plasma membrane, or that these polypeptides could be associated with the excess of SVs that do not fuse with the apical plasma membrane but are transported back by the reverse cytoplasmic streaming. Labelled vesicles with similar sizes were also found in fact, in areas progressively distant from the apex where SVs are present at a lower density (Fig. 7b). Smaller vesicles, of about 50 nm in diameter, which were also present in the tip region were not stained by the anti-p28 antiserum (Fig. 7b) as well as any other organelle in the tube cytoplasm. Since SVs are produced by dictyosomes, we looked for the eventual staining of the Golgi apparatus (G); gold particles were not observed either in association with the cisternae or with newly originating vesicles (Fig. 7c). This suggests that the binding of p85 polypeptides to
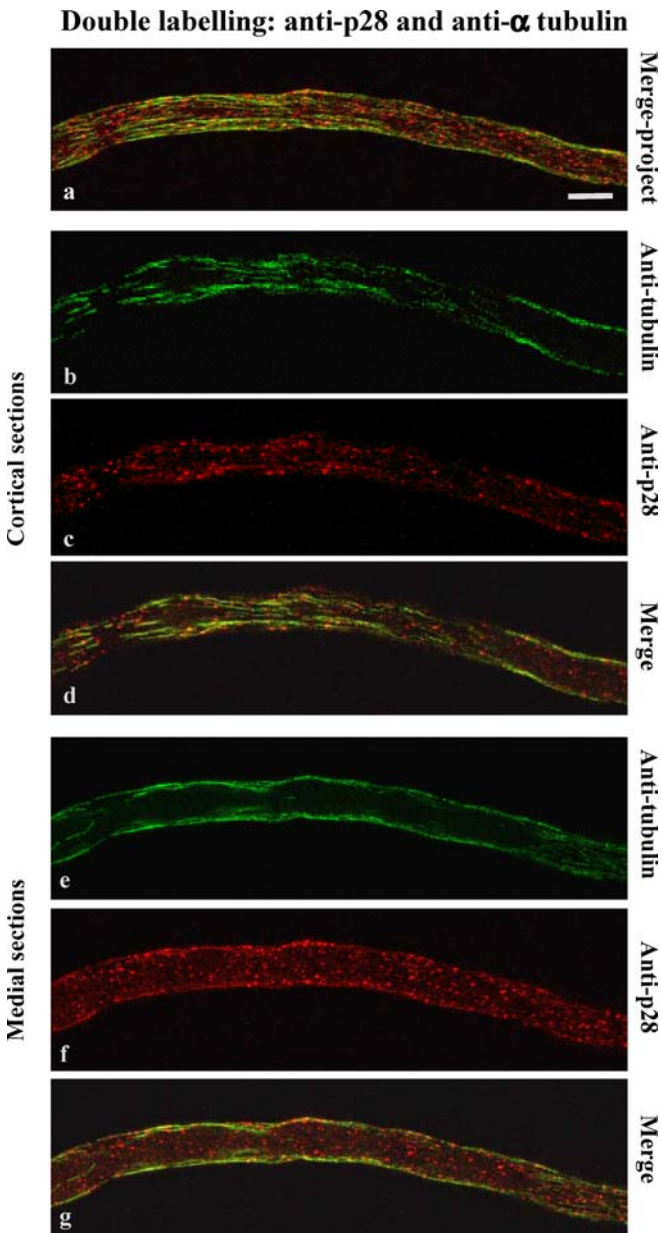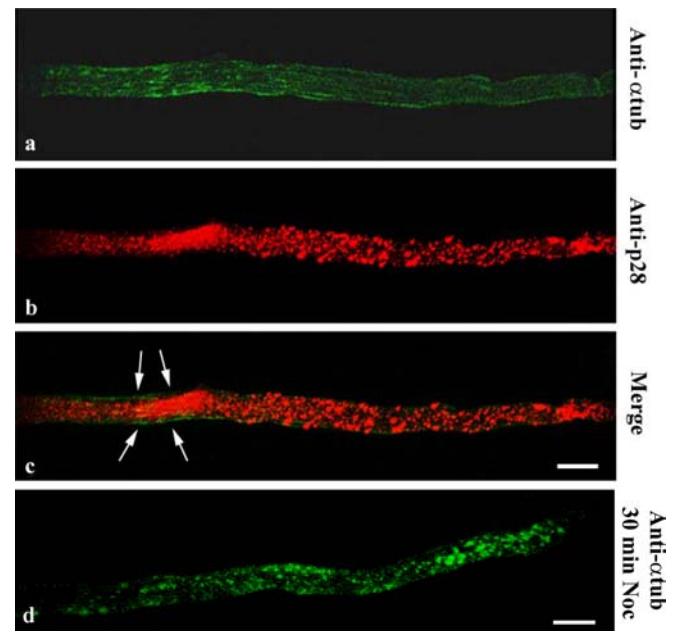

Fig. 6 Mt depolymerization by nocodazole. In double immunofluorescence experiments after 15 min nocodazole treatement, Mts fragments are still observed in some areas of the tube cytoplasm **a**, particles recognized by the anti-p28 antibody tend to aggregate and to form clusters that move toward the center of the tube **b**. The merging between the two images clearly shows that the co-localization between SVs and cortical Mts is lost after nocodazole treatment **c**. In the pollen tubes after 30 min nocodazole treatement the anti-α tubulin *Mab* shows only fluorescent spots within the cytoplasm **d**. Bars 10 μm

SVs could occur during a putative maturation process before or during their movement toward the tip. Gold particles, occasionally seen on the pollen tube cell wall (Fig. 7a), were considered as aspecific cross-reactions since a similar staining of the cell wall was observed using the preimmune antiserum, and they almost disappeared once the anti-p28 antiserum was pre-incubated with intact pollen tubes (data not shown).
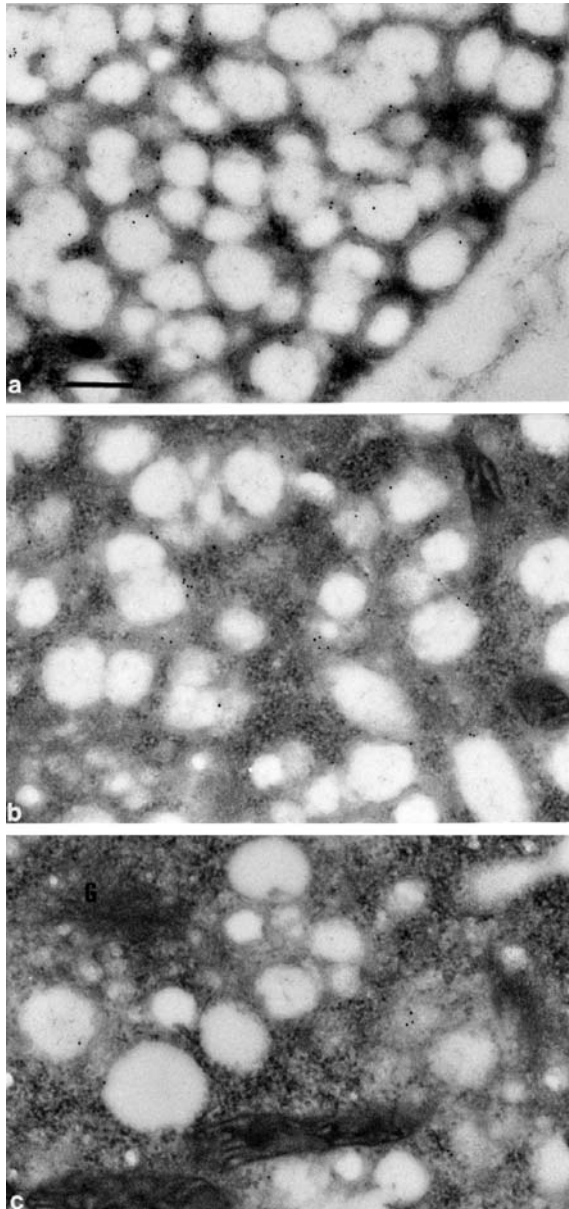

Fig. 7 The MetT is shown on the surface of Golgi-derived SVs by immunoelectron microscopy. The anti-p28 antiserum stained epitopes on the surface of Golgi-derived SVs that accumulate in the tip region during the pollen tube growth **a**. A similar staining is also observed in regions behind the tip **b** where SVs are transported by the retrograde cytoplasmic streaming. Observing the Golgi bodies (G), it can be seen that SVs are not decorated when they are produced **c** and the association with p85 polypeptide occurs later. Bar 0.25 μm

Two pools of the MetT are present in tobacco pollen tubes

In order to explore the MetT heterogeneity, procedures of biochemical dissections involving the zonal centrifugation through a sucrose density gradient, were perormed. Results derived from three independent experiments showed that the p85 polypeptides sedimented in two sets of fractions corresponding to sedimentation coefficients of 15 S and 4 S, suggesting that p85 polypeptides are present in two different pools in pollen tube extracts. The Fig. 8a reports the electrophoretic pattern of the sedimentation fractions 8–21 with a 10% polyacrylamide gel and the western blotting analysis using the anti-p28 antiserum, which positively reacts with 85 kDa polypeptides (see arrow) in fractions 9 and 10 (15 S) and in fractions 17 and 18 (4 S). To study in more detail the specific association of some of the p85 isoforms with the 15 S and 4 S protein complexes, sedimentation fractions 10 and 17 were analyzed by 2D gel and western blotting by using the anti-p28 antiserum. The results revealed a specific association of the two more acidic p85 isoforms in fractions at 15 S (Fig. 8b, 15 S), whereas the remaining p85 isoforms are found in the 4 S fraction (Fig. 8b, 4 S). The presence of p85 isoforms in fractions sedimenting at 15 S and 4 S
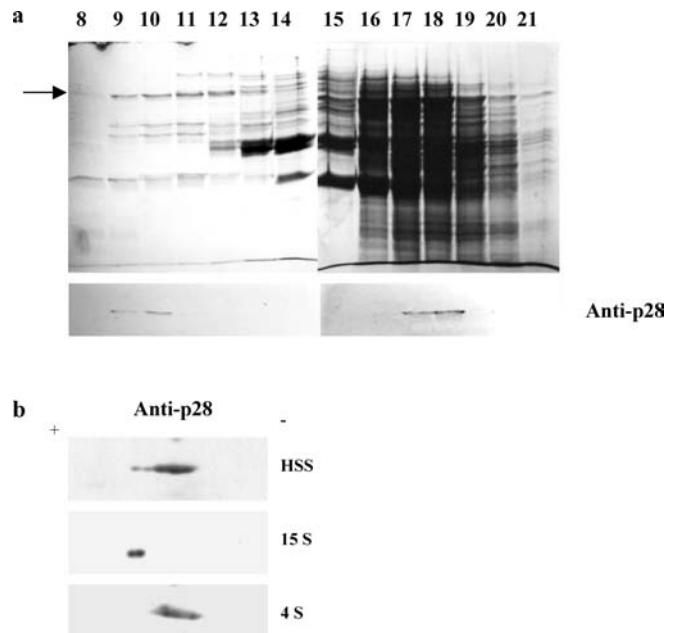

Fig. 8 Two pools of the MetT are present in tobacco pollen tubes. **a** Two distinct pools of p85 polypeptides are present in the tobacco pollen tube HSS. The electrophoretic pattern (10% SDS-PAGE) of fractions 8–21 after sedimentation through a linear sucrose gradient shown in the upper part, were probed with the anti-p28 antibody. The antibody recognized a p85 polypeptide in fraction 9–10 (15 S) and 17–18 (4 S) (see *arrow*). **b** 2D gel and western blotting analysis using the anti-p28 antibody of sedimentation fractions at 15 S and 4 S show that the two more acidic p85 isoforms are present at 15 S, whereas the remaining are found in the 4 S fractions

suggested that MetT could be associated with functionally different protein complexes in tobacco pollen tubes.

Discussion

Pollen tube morphology requires the integrity of the cytoskeletal apparatus (Cai et al. 1997). Molecular motor proteins are known to be involved in generating cytoplasmic movements of organelles and vesicles along Afs and Mts during the pollen tube growth. In an effort to investigate the presence of dynein-related proteins in higher plants, a polyclonal antibody against the *Chlamydomonas* dynein LC p28 was used on tobacco pollen tube extracts by western blotting analysis.

Surprisingly, the anti-p28 antiserum recognized an 85 kDa polypeptide comprising seven different isoforms identified as the pollen tube MetE homolog (MetT) by partial aminoacid sequencing. Immunofluorescence microscopy and immunogold labelling data showed that p85 polypeptides were associated with the surface of SVs that partially interact with cortical Mts, in the subapical regions of the tube, suggesting that the microtubule apparatus could actively cooperate with the Afs in pollen tube growth. The biochemical dissection of the HSS by sedimentation through a sucrose gradient showed the p85 polypeptides sediment in two different sets of fractions: one at 4 S and the other at 15 S, supporting the evidence that two distinct pools of p85 are present in pollen tubes. 2D gel analysis of 15 and 4 S fractions revealed the specific association of the two more acidic p85 isoforms with the 15 S fractions.

MetT within the pollen tube of Nicotiana tabacum

Unexpectedly, partial aminoacid sequencing of p85 spots by MS/MS experiments revealed that all the fourteen peptide fragments share a high-sequence homology with the plant MetE. As a control, two antisera specifically raised against MetE of *Catharantus roseus* (Eichel et al. 1995) and *Solanum tuberosum* (Zeh et al. 2002) were probed on pollen tube crude extracts. Both antisera recognized a polypeptide showing the same molecular mass of the pollen tube p85 identified by the anti-p28, after separation by 1D gel (Fig. 9a). Western blotting analysis of pollen tube polypeptides separated by 2D gel using the anti-MetE of *C. roseus* revealed that it recognized a cluster of spots co-migrating with those previously identified by the anti-p28 antiserum (Fig. 9b).

Domain analysis, by the PROSITE program, of the p85 peptides revealed the presence of two PKC and CK2 phosphorylation sites (SPR and STEE, in red in Fig. 3a). Although polarized growth of pollen tubes is maintained by Ca^{2+} -mediated signal transduction pathways that involve kinase-dependent protein phosphorylation cascade (Trewavas and Malho 1997), we do not have at the present direct evidence of the real phosphorylation state of the p85 polypeptides within the pollen tube. However, the presence of two kinds of phosphorylation sites in F14 (Fig. 3a) makes p85 polypeptides a target candidate for both PKC and CK2 kinases.

Cell fractionation experiments in *Catharanthus* (Eichel et al. 1995) and *Chlamydomonas* (Kurvari et al. 1995) suggested that the MetE is a soluble protein, as also p85 polypeptides from *Nicotiana tabacum* pollen
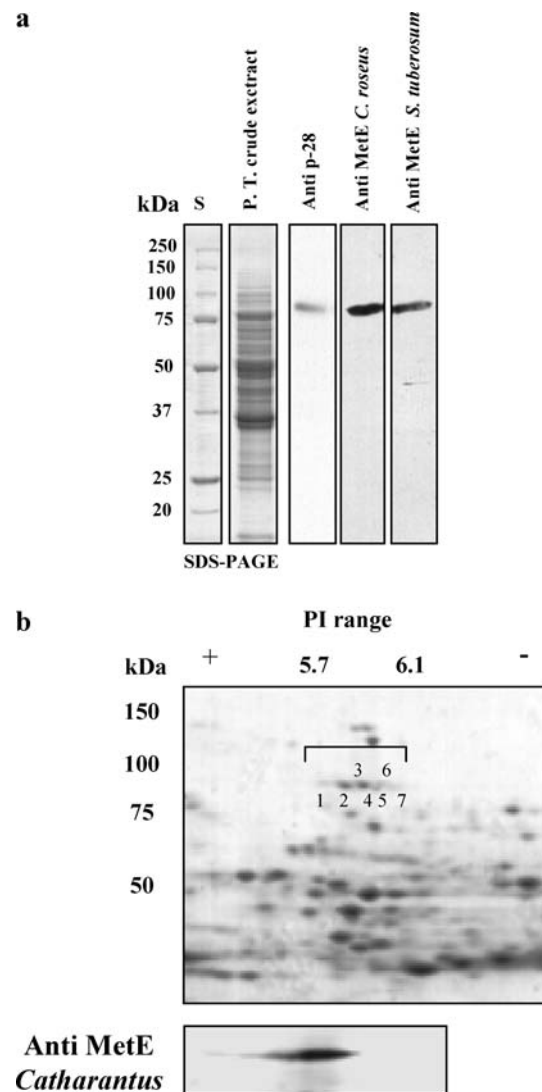

Fig. 9 Anti-MetE of *C. roseus* and *S. tuberosum* antisera recognized a polypeptide comigrating with the p85 polypeptide identified by the anti-p28. **a** Crude extract polypeptides were separated by a 10% polyacrylamide linear gel and then probed by using the anti MetE of the *C. roseus* and the *S. tuberosum*. Both antisera recognized a polypeptide having a molecular mass of 85 kDa when compared to the molecular weight standards (Precision Plus Protein Standards, Bio-Rad). **b** Pollen tube polypeptides separated by 2D gel (*upper part*) were probed by the antiserum against the MetE of *C. roseus*. This antiserum recognized a cluster of spots which comigrated with the p85 polypeptides stained by the anti-p28 (*lower part*)

tubes. However, immunofluorescence microscopy and immunogold labelling by using the anti-p28 antiserum clearly showed that p85 polypeptide is associated with particle-like structures, resembling membranous organelles, within the vegetative cell cytoplasm and decorated the GC shape. Immunofluorescence experiments carried out on pollen tubes grown for different periods, showed a punctate staining, which accumulated in the tube tip and was present in a lesser amount in the regions behind the apex where fluorescent particles seem to be aligned along putative filaments. To test this hypothesis we performed double immunofluorescence experiments using the anti-p28 antiserum and the anti-α tubulin antibody. Mts in pollen tube cytoplasm are preferentially organized as cortical bundles in the regions behind the tip; in the apex, only short, randomly oriented Mts were seen (Del Casino et al. 1993). The double labelling and Mts depolymerization experiments revealed localization of fluorescent spots along cortical Mts bundles, whereas particles in the cytoplasmic area of the tube seemed to be unrelated by the presence of Mts and could be aligned on Afs that are present in higher amounts in the cytoplasmic area of the tube (Cai et al. 1997). Recent studies suggested that Mts could play a role in organelle translocation along the tube (Cai et al. 2000; Romagnoli et al. 2003). Our data further support the idea that Mts could play a synergic role with Afs in regulating SVs movement and/or positioning, so that SVs could shift from the Afs to Mts in the regions far from the tip, according to physiological requirements. In pollen tubes grown for 7h, when GC and VN have already entered the tube, the antibody recognized antigens probably located on the GC surface, which could be related with the regulation of the GC movement.

Immunoelectron microscopy showed that p85 polypeptides were associated with pollen tubes SVs which accumulate in the tip region and are less abundant in areas far from the apex. The association of p85 polypeptides with SVs seemed to occur during a putative maturation process of SVs, since no staining was observed when they originated from the Golgi apparatus.

The MetE are zinc-binding methyl transferases that catalyze the de novo synthesis of methionine (Matthews and Goulding 1997). The presence of the MetE has not been previously shown in pollen tubes, where the occurrence of intensive protein synthesis is strongly related to cell growth. However, since the methionine biosynthesis is part of the complex S-methylmethionine cycle (Ranocha et al. 2000), it can also enter several other metabolic pathways. MetE serves other than the de novo biosynthesis of methionine, for the regeneration of the methyl group of SAM a cofactor required as methyl donor in a wide number of methylation reactions (Ranocha et al. 2001). In *Chlamydomonas* a role of the MetE in the adhesion-induced events during fertilization, was hypothesized by Kurvari et al. (1995) since the MetE transcript level increased dramatically during gamete activation, suggesting that it could be involved in the prolonged signal transduction following cell–cell inter-

action, that occur between gametes during fertilization in sexually reproducing organisms. A similar effect could be supposed in pollen tube growth where signal transduction events involving external stimuli are translated into the typical asymmetrical growth pattern (Hepler et al. 2001).

The involvement of MetE in a wide range of methylation reactions as DNA and cell-wall components and its identification in pollen tube opens new perspectives for studying mechanisms regulating the tube elongations.

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