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Stefano del Duca, Donatella Serafini-Fracassini, Philip Bonner, Mauro Cresti, Giampiero Cai. Effects of post-translational modifications catalyzed by pollen transglutaminase on the functional properties of microtubules and actin filaments. *Biochemical Journal*, 2009, 418 (3), pp.651-664. 10.1042/BJ20081781 . hal-00479094

**HAL Id: hal-00479094**

**<https://hal.science/hal-00479094>**

Submitted on 30 Apr 2010

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# Effects of post-translational modifications catalyzed by pollen transglutaminase on the functional properties of microtubules and actin filaments

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## Synopsis

Transglutaminases (TGases) are a class of calcium-dependent enzymes that catalyze the interactions between acyl acceptor glutamyl residues and amine donors, potentially making cross-links between proteins. To assess the activity of apple (*Malus domestica*) pollen TGase on the functional properties of actin and tubulin, TGase was prepared from apple pollen by hydrophobic interaction chromatography and assayed on actin and tubulin purified from the same cell type. The enzyme catalyzed the incorporation of putrescine in the cytoskeleton monomers. When tested on actin filaments, pollen TGase induced the formation of high molecular mass aggregates of actin. Use of fluorescein-cadaverine showed that the labeled polyamine was incorporated in actin by pollen TGase, comparably to guinea pig liver TGase. The pollen TGase also reduced the enzyme activity and the binding of myosin to TGase-treated actin filaments. Polymerization of tubulin in the presence of pollen TGase also yielded the formation of high molecular mass aggregates. Furthermore, the pollen TGase also affected the binding of kinesin to microtubules and reduced the motility of microtubules along kinesin-coated slides. These results indicate that the pollen TGase can control different properties of the pollen tube cytoskeleton (including the ability of actin and tubulin to assemble and their interaction with motor proteins) and consequently regulate the development of pollen tubes.

**Page heading:** Modifications of actin and tubulin by pollen TGase

**Keywords:** plant transglutaminase, microtubule, actin filament, kinesin, myosin, pollen tube

## Introduction

The pollen is the male gametophyte of seed plants and its tube transports the male gametes toward the egg cell. The growth of pollen tubes is a complex process that needs the simultaneous activity of many different molecules working in the apical domain [1]. The movement of cytoplasmic content of pollen tubes is dependent on the pollen tube cytoskeleton [2], which consists essentially of microtubules, actin filaments and related proteins organized to facilitate and promote the unidirectional cell expansion and the transport of sperm cells. Actin filaments are generally widespread in the pollen tube cytoplasm but are absent in the very tip; a consistent “actin fringe” is present immediately behind the tip and is thought to delimit the growth area [3]. On the contrary, microtubules are preferentially organized as bundles in the cortical cytoplasm and are absent in the last 10-20  $\mu\text{m}$  of the tube apex [4]. Actin filaments are mainly involved in organelle movement and in tube growth [5]; on the other hand, microtubules likely play a role in the transport of sperm cells [6], probably participate in the regulation of organelle movement [4] and might also guide pollen tube growth *in vivo* or have a role in directing the elongation activity [7].

Tubulin is post-translationally modified to realize its multitude functions during pollen tube growth [8]. On the other hand, post-translational modifications of actin are poorly known in eukaryotic cells [9]. An important form of post-translational modification is the conjugation of polyamines, which is mediated by calcium-dependent transglutaminases (E.C. 2.3.2.13) (TGases). TGases catalyze the interaction between an acyl acceptor glutamyl residue and amine donors, like lysyl residues, thus forming cross-links within the same or different proteins. Polyamines also act as physiological substrates of TGases: their terminal amino-group binds one or two glutamyl residues giving rise either to mono-( $\gamma$ -glutamyl)-polyamines or bis-( $\gamma$ -glutamyl)-polyamines [10;11]. Consequently, the “bridge” between two proteins has different length: very short for the Lys-Gln binding, gradually longer for polyamines with different backbone length. Moreover, polyamines exhibit positive charge and thus “cationise” the partner protein. TGases form bridges between specific proteins, including cytoskeleton proteins or extracellular matrix proteins, and are probably involved in the regulation of cell growth and differentiation of animal and plant cells [12]. TGases are also defined as “biological glues” for their capacity to join proteins [13]. Previous results from animal cells suggested the involvement of TGase in the modification of cytoskeleton proteins. After early indications that actin is substrate of TGase [14], actin in *Physarum* was also recognized as such [15]. Recently, the cytoskeleton substrates of TGase 2, one of the nine TGases identified in animals, were summarized by Robinson et al. [16]. Polyamines are also involved in the rearrangement of actin and tubulin cytoskeleton as demonstrated by Pohjanpelto et al. [17] in CHO dividing cells.

The crude extract of apple (*Malus domestica*) pollen contained unpurified TGase activity and catalyzed the incorporation of polyamines into proteins with molecular mass of 43 kDa and 52–58 kDa [18]. These bands matched immunolabelled spots identified by mouse monoclonal antibodies to actin and  $\alpha$ -tubulin. The supply of exogenous actin and tubulin in cell-free extracts of pollen enhanced the TGase activity. Moreover, actin of germinating pollen gave rise to cross-linked products of high molecular mass [18].

The present study was undertaken to analyze the involvement of purified pollen TGase in the post-translational modification of pollen actin and tubulin via Gln-Lys or polyamine bridges. Pollen from *Malus domestica* was chosen as the starting material because apple pollen was already shown to catalyze the incorporation of polyamines (therefore, it presumably contains a TGase activity) [18]. This work also assayed the polymerization/depolymerization properties of actin and tubulin after modification by TGase. This possibility was studied by supplying two different amines, the FITC-cadaverine (for labeling the substrates) with only one amino group available for conjugation and the putrescine with both the primary amino groups available for linkages and possibly involved in cross-bridge formation. We also assessed the TGase-induced modifications of actin/tubulin in connection with their related motor proteins, myosin and kinesin. Since the proper dynamic of actin filaments and microtubules, coupled to the activity of motor proteins, is

responsible of organelle movement in pollen tubes [4], TGase may consequently be recognized to take part in the control of many active processes occurring during pollen tube growth.

## Materials and Methods

### Plant material

Mature pollen of *Malus domestica* Borkh. cv Red Chief was collected from experimental plots (Dipartimento di Colture Arboree, Bologna, Italy; Azienda Agricola Rondinini, Faenza, Italy). Handling and storage were performed as described by Bagni et al [19].

### Antibodies and chemicals

Rabbit antibody to plant actin was a generous gift of Dr. Richard Cyr (Pennsylvania State University, Biology Department, PA). Donkey polyclonal antibody to tubulin was purchased from Cytoskeleton Inc. (code number ATN02). Unless otherwise indicated, chemicals used in this work were purchased from Sigma Aldrich (St. Louis, MO). Guinea pig liver TGase (gplTGase) was obtained from Sigma Aldrich. The ID10 anti-TGase monoclonal antibody was raised against purified gplTGase; clones were screened out against gplTGase, ion exchange-purified extracts of 14-days old *Pisum sativum* leaves and against germinated *Vicia faba* cotyledons (used 14 days after imbibitions). ELISA-positive fractions were pooled, concentrated in a dialysis tube against PEG 8000 and stored at -20°C. The specificity of ID10 for pollen TGase was tested against partially purified enzyme fractions and was compared to that of the well-known TGase antibody AbIII (Neomarker, Fremont, CA) already assayed against plant [20] and pollen [21] TGases; the two antibodies showed the same immunorecognition profile.

### Preparation of a pollen TGase-enriched fraction

Pollen was rehydrated at 30°C and 100% relative humidity for 30 min. All purification steps were conducted at 4°C. One gram of hydrated pollen was extracted using a Potter-Elvehjem tissue homogenizer with 100 ml of 25 mM Tris buffer pH 8.0, 2 mM DTT, 0.1 mM PMSF and 1 mM EDTA. The extract was centrifuged at 20400 *g* for 10 min and the supernatant was dialyzed overnight against 5 L of dialysis buffer (5 mM Tris buffer pH 8.0, 1 mM 2-mercaptoethanol) with three changes of buffer. The dialyzed sample was supplemented with 5 mM CaCl<sub>2</sub> and centrifuged at 20400 *g* for 15 min to eliminate the calcium-induced protein precipitation. The supernatant was loaded on a Phenyl Sepharose hydrophobic interaction chromatography (HIC) column (1.6 x 25 cm, Sigma-Aldrich) equilibrated with 20 mM Tris buffer pH 8.0 containing 5 mM CaCl<sub>2</sub> and 1 mM 2-mercaptoethanol. Elution was performed at 2.5 ml/min with an isocratic flow of the same buffer containing EGTA instead of CaCl<sub>2</sub>, followed by two column washings with distilled water and ethylene glycol (50% in water) respectively. Samples were dialyzed overnight against 1000 volumes of 5 mM Tris buffer pH 8.0 and 1 mM 2-mercaptoethanol with three changes of buffer, then freeze-dried and stored at -80°C. Samples were diluted in 50 mM Tris buffer pH 8.0 containing 1.5 mM DTT and incubated 5 min at room temperature before assaying the TGase activity.

### Incorporation assay of biotin-labeled cadaverine by TGase

Dialyzed fractions from the HIC and the pollen extract were tested by the biotin-cadaverine incorporation assay according to Lilley et al [22] with EGTA replacing 10 mM EDTA in negative controls.

### Purification of actin from apple pollen

Purification of actin from apple pollen was performed according to the literature [23]. Hydrated apple pollen (1 g) was germinated for 3 hours in the growth medium (0.2 M sucrose, 0.33 mM H<sub>2</sub>BO<sub>3</sub>, 1.3 mM Ca<sub>2</sub>NO<sub>3</sub>) at the concentration of 1 mg/ml. After germination, pollen was collected



by centrifugation at 1000 *g* for 10 min. The pellet was washed with washing buffer (2 mM  $\beta$ -mercaptoethanol, 0.005%  $\text{NaN}_3$ , 0.2 mM  $\text{CaCl}_2$ , 2 mM Tris pH 8.0) and the final pellet was suspended in 5 ml of the lysis buffer (also called buffer A: 2 mM  $\beta$ -mercaptoethanol, 0.2 mM ATP, 0.005%  $\text{NaN}_3$ , 0.2 mM  $\text{CaCl}_2$ , 2 mM Tris pH 8.0, 1 mM PMSF, plant protease inhibitor cocktail from Sigma code P9599). All purification steps were conducted at 4°C unless differently stated. Cells were lysed with a Potter-Elvehjem Tissue Homogenizer. The homogenate was centrifuged at 10000 *g* for 20 min. The supernatant was centrifuged at 100000 *g* for 30 min. The pellet was discarded while the supernatant was precipitated with 2.2 M  $(\text{NH}_4)_2\text{SO}_4$  under agitation. After 10 min, the sample was centrifuged at 10000 *g* for 10 min. The resulting pellet was dissolved in 2 ml of buffer B (0.1 mM  $\text{CaCl}_2$ , 2 mM  $\beta$ -mercaptoethanol, 10 mM imidazole pH 7.5, 0.5 mM ATP, 0.05%  $\text{NaN}_3$ , 1 mM PMSF) and dialyzed overnight against buffer B. The sample was fractionated using a Mono-Q HR 5/5 column (GE HealthCare) equilibrated in buffer B over 20 column volumes (19.6 ml) with a linear gradient from 0 to 1 M KCl; elution was done at 1 ml/min at room temperature. Fractions were analyzed by spot-test with a plant actin antibody. Cross-reacting fractions were pooled and supplemented with 2 mM  $\text{MgCl}_2$ . After 10-hours incubation at 4°C to stimulate actin polymerization, the sample was centrifuged at 160000 *g* for 1 h 45 min. The pellet was dissolved in 300  $\mu\text{l}$  of buffer C (2 mM  $\beta$ -mercaptoethanol, 0.2 mM ATP, 0.005%  $\text{NaN}_3$ , 0.2 mM  $\text{CaCl}_2$ , 2 mM Tris pH 8.0, 1 mM PMSF) and dialyzed overnight against buffer C using the 8-kDa Cut-Off Mini Dialysis kit (GE HealthCare). The sample was fractionated onto a Superdex 200 HR 10/30 column (GE HealthCare) equilibrated with buffer C at room temperature, flow rate of 0.8 ml/min. Chromatography steps were done using a AKTA Purifier system (GE HealthCare). Fractions were assayed by spot-test with the actin antibody and positive fractions were pooled, frozen in liquid nitrogen and stored at -80°C.

### **Purification of tubulin from apple pollen**

Tubulin from apple pollen was purified according to the literature [24]. Apple pollen (1 g) was germinated as described above and homogenized in 5 ml of lysis buffer containing 50 mM Pipes pH 6.8, 2 mM  $\text{MgCl}_2$ , 2 mM EGTA, 1 mM DTT, plant protease inhibitor cocktail from Sigma, 0.1 mM GTP. All steps were carried out at 4°C (unless otherwise mentioned). After high-speed centrifugation, the supernatant was fractionated onto a Resource Q anion exchange column (1x1 ml, GE HealthCare) equilibrated in buffer A (50 mM Pipes-KOH pH 6.8, 2 mM  $\text{MgCl}_2$ , 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.1 mM GTP). The sample was eluted over 20-column volumes (20 ml) from 0 to 1 M KCl at room temperature, flow rate of 1 ml/min. Fractions were analyzed by spot-test with tubulin antibodies and positive ones pooled. The resulting 4-ml sample was loaded onto a HiPrep 16/60 Sephacryl S-300 column (GE HealthCare) equilibrated with buffer A at room temperature; elution was achieved at 1 ml/min. Fractions were again assayed by tubulin antibody. Cross-reacting fractions were pooled and applied to a Mono-Q HR 5/5 column equilibrated with buffer A. Protein separation was achieved over 20-column volumes (19.6 ml) from 0 to 1 M KCl at room temperature, with a flow rate of 1 ml/min. Fractions positive to the spot-test analysis were dialyzed for several hours in Mini Dialysis kit 8-kDa cut-off against buffer A. Chromatography steps were done using a AKTA Purifier system (GE HealthCare). The sample was frozen under liquid nitrogen and stored at -80°C.

### **TGase radiometric assay**

The *in vitro* reactivity of TGase with cytoskeleton proteins was performed as previously described by Serafini-Fracassini et al. [25] but modified as follows. The incubation mixture included 50  $\mu\text{l}$  of pollen actin or tubulin (0.5 mg/ml), 15  $\mu\text{l}$  of 500 mM Tris buffer pH 8, 2  $\mu\text{l}$  of TGase active fraction (2 mg/ml) and final concentrations of the following: 0.1 mM putrescine, 10 mM DTT and 2.5 mM  $\text{CaCl}_2$ . As radioactive tracer, 444 kBq  $[1,4(n)^3\text{-H}]\text{-putrescine}$  (0.55 TBq  $\text{mmol}^{-1}$ ; GE HealthCare) was added to the assay mixture for a total volume of 150  $\mu\text{l}$ . After 120 min of incubation at 30°C, the reaction was stopped and repeatedly pelleted with 5% (w/v) TCA as described [26] and

radioactivity counted. Another aliquot was used for the  $\gamma$ -glutamyl polyamine derivatives analysis (see below). Enzyme reactions were also carried out in the presence of the commercial TGase inhibitor 1,3-dimethyl-2-[(2-oxopropyl)thio] imidazolium chloride (Zedira).

### HPLC identification of $\gamma$ -glutamyl polyamine derivatives

The incorporation of labeled putrescine into TGase substrate proteins was evaluated in the TCA-insoluble fractions as mono- and bis- $\gamma$ -glutamyl polyamine derivatives. The final pellet was washed at least three times with anhydrous diethyl ether and proteolyzed according to Folk et al [10]. The digestion products were separated by ion exchange chromatography using a Jasco HPLC system (4.5 x 90 mm column, packed with Ultropac 8 resin, Na<sup>+</sup> form; Jasco Europe, Milan, Italy) and the five-buffer system previously described by Folk et al [10]. The identity of the polyamine derivatives was determined by comparison with the corresponding retention times of glutamyl polyamine standards. The identity of conjugated polyamines was determined after release of free polyamines by acid hydrolysis (6 M HCl) of the ion exchange chromatographic fractions corresponding to the predicted retention times [27].

### Assay of pollen TGase activity on actin filaments

In order to determine the effects of pollen TGase on cytoskeleton proteins, actin (Cytoskeleton Inc, cat. # AKL-99) was diluted to 0.4 mg/ml in A-buffer (5 mM Tris-HCl pH 8.0, 0.2 mM ATP, 0.2 mM CaCl<sub>2</sub>) and incubated for 1 hour on ice. Actin was added to the polymerization buffer (0.5 M KCl pH 8.0, 10 mM ATP, 20 mM MgCl<sub>2</sub>) and incubated for 1 hour at room temperature. Actin and pollen TGase were mixed in the ratio of 10:1 in the presence of 1 mM FITC-labeled cadaverine and 7 mM CaCl<sub>2</sub>. All samples were incubated for 3 hours at 37°C. After incubation, 10  $\mu$ l-aliquots were perfused within a perfusion chamber and further incubated for 5 min. After brief staining with rhodamine-phalloidin buffer (10  $\mu$ l of polymerization buffer 10X, 25  $\mu$ l of 6.6  $\mu$ M rhodamine-phalloidin stock from Invitrogen, 65  $\mu$ l of water), samples were observed with a fluorescence microscope. Enzyme reaction of remaining samples was stopped by addition of 7 mM EGTA. Samples were centrifuged at 15000 g for 1 h at 25°C and supernatants and pellets were processed for SDS-PAGE. Similar analysis was performed with rhodamine-labeled actin (Cytoskeleton Inc, code number AR05); in this case, the rhodamine-phalloidin buffer was not applied.

### Optical microscopy analysis of incorporation of FITC-cadaverine in actin filaments

Samples were observed with a Zeiss Axiophot fluorescence microscope, equipped with a 63X objective. Images were captured with an MRc5 AxioCam video camera using AxioVision software. Samples were alternatively analyzed with a Bio-Rad MicroRadian Confocal microscope using a 63X objective.

### Binding and enzyme assay of myosin with TGase-treated actin filaments

Actin filaments were prepared and treated with pollen TGase as described above. Myosin (Cytoskeleton Inc, code MHO1) was made 1 mg/ml in 4 mM Tris-HCl, pH 7.6, 2 mM MgCl<sub>2</sub>, 100 mM KCl, and added to pre-treated actin filaments. After incubation for 30 min at 25°C, samples were centrifuged at 15000 g for 1 h at 25°C. Supernatants and pellets were processed for SDS-PAGE.

For myosin enzyme assay, 10, 20 and 50  $\mu$ l of actin filaments were added to myosin in the ATPase assay buffer (10 mM KCl, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, 5 mM ATP, 20 mM Pipes pH 7.0). After incubation at 25°C for 5, 10, 20, 25 and 30 min, the amount of free inorganic phosphate was measured using the PhosFree kit from Cytoskeleton Inc. Controls were done with gplTGase (same amount of pollen TGase).

### **Polymerization/depolymerization assay of tubulin in the presence of pollen TGase**

Tubulin aliquots (10 mg/ml, from Cytoskeleton Inc., rhodamine-labeled, code TL331M, or unlabelled, code TL238) were diluted with buffer (80 mM Hepes pH 7.5, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ , 2 mM GTP, 20% glycerol) at 5 mg/ml. Tubulin samples were mixed with pollen TGase (10:1 ratio), 5 mM FITC-cadaverine and 7 mM  $\text{CaCl}_2$ . Samples were incubated at 37°C for 3 hours, and then observed at the optical microscope (DIC view, see below). Enzyme reaction was blocked by adding 7 mM EGTA. Samples were centrifuged at 15000 *g* for 1 h at 25°C, and supernatants were prepared for SDS-PAGE. Pellets were suspended in PEM buffer (80 mM Pipes pH 6.8, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ ); part of them was prepared for SDS-PAGE while remaining was incubated for 30 min at 4°C (depolymerization phase). Afterward, samples were centrifuged at 15000 *g* for 1 h at 4°C and supernatants and pellets were prepared for SDS-PAGE.

### **DIC microscopy of TGase-treated microtubules**

Samples of treated and untreated microtubules were perfused into perfusion chambers and incubated for 5 min. Slides were washed with PEM buffer and observed at the optical microscope in DIC view to assess the effects of TGase-mediated incorporation of FITC-cadaverine on the microtubule structure. Samples were also observed in the rhodamine channel to prove that filamentous structures were definitely microtubules and in the FITC channel to visualize the incorporation of cadaverine.

### **Binding assay of kinesin to TGase-treated microtubules**

Kinesin from Cytoskeleton Inc. (code number KR01, 0.1 mg/ml) was added to microtubules (treated and untreated with pollen TGase) prepared as described above. After incubation for 30 min at 25°C, samples were centrifuged at 15000 *g* for 1 h at 25°C. Supernatants were prepared for SDS-PAGE. Pellets were washed with ATP buffer (80 mM Pipes pH 6.8, 7 mM EGTA, 1 mM  $\text{MgCl}_2$ , 1 mM DTT, 20  $\mu\text{M}$  taxol, 10 mM ATP); part of pellets was prepared for electrophoresis while the remaining was incubated for 30 min at room temperature. Samples were centrifuged at 15000 *g* for 1 h at 25°C and then prepared for electrophoresis.

### **In vitro motility assays**

Ten microliters of kinesin solution from Cytoskeleton Inc. (0.1 mg/ml) was incubated for 5 min in perfusion chambers. Microtubules (treated or untreated with pollen TGase) were diluted 1:5 with motility buffer (80 mM Hepes pH 7.5, 7 mM EGTA, 1 mM  $\text{MgCl}_2$ , 1 mM DTT, 20  $\mu\text{M}$  taxol, 5 mM ATP). Then, 10  $\mu\text{l}$  of microtubule solution was incubated for 1 min in the perfusion chamber. Samples were observed at the optical microscope under DIC view. Motility of microtubules was recorded according to the literature [28].

### **Incorporation of putrescine in TGase-treated microtubules**

Tubulin and microtubules were prepared as described above and adjusted at the concentration of 5 mg/ml. Pollen TGase was added to the ratio of 1:10 with tubulin supplemented with 7 mM  $\text{CaCl}_2$  and 0.1 or 1 mM putrescine. Samples were incubated at 37°C for 3 hours and then observed with DIC microscopy. The enzyme reaction was stopped by addition of 7 mM EGTA. Samples were centrifuged at 15000 *g* for 1 h at 25°C. Supernatants and pellets were prepared for SDS-PAGE.

### **Electrophoresis and immunoblot**

Electrophoresis of proteins was done according to Laemmli [29]. Molecular mass standards were from Sigma Aldrich or from Bio-Rad (Precision series). Gels were stained with Bio-Safe Coomassie (Bio-Rad). Immunoblot was performed according to Towbin et al [30]. Secondary antibodies against mouse and rabbit IgG were conjugated with horseradish peroxidase (GE



HealthCare); secondary antibody to donkey IgG was conjugated with horseradish peroxidase (Cytoskeleton Inc.). Immunoreactive bands were detected with the Enhanced ChemiLuminescent (ECL) Plus reagents from GE Healthcare.

### Protein Assay

The protein content was determined using a modified bicinchoninic acid method [31]. BSA was used as the standard protein.

## Results

### Isolation and characterization of a TGase-enriched fraction from apple pollen

A TGase activity-enriched fraction from apple pollen was eluted by HIC with 10 mM EGTA (Fig. 1A) and contained a prominent band at 70 kDa (Fig. 1B, lane 1), which catalysed transamidating reactions (see below). The EGTA-eluted fractions were enriched in TGase activity about tenfold as shown by comparison with HIC-unfractionated sample (Fig. 1A). The 70-kDa polypeptide cross-reacted with two monoclonal TGase antibodies, ID10 and AbIII (Fig. 1B, lane 2-3). The cross-reactivity of ID10 was also evaluated on gplTGase and apple pollen extracts (lanes 4-5), while AbIII was tested on pollen extract (lane 6). Both antibodies cross-reacted with the 70-kDa band in pollen extracts.

### Incorporation of polyamines in apple tubulin and actin

To assess the enzyme activity of pollen TGase on tubulin and actin, the two proteins were first isolated from apple pollen. Tubulin was purified by chromatography (Fig. 2A, top), yielding about 150 µg of homogeneous tubulin (lane 4). After treatment with taxol, the protein was mostly found in the pellet (lane 6) and partly in the supernatant (lane 5), indicating that apple tubulin was competent of forming microtubules. The position of tubulin was monitored using a specific antibody (Fig. 2A, bottom).

Actin was isolated by precipitation with  $(\text{NH}_4)_2\text{SO}_4$  and by chromatography. About 250 µg of actin was purified to homogeneity from apple pollen (Fig. 2B, top). After the Mono-Q step (lane 4), actin was still contaminated by additional proteins, whose level was reduced by the polymerization/depolymerization step (lane 5 and 6). The use of Superdex 200 column greatly enhanced the purity of actin (lane 7). Actin was monitored with the specific antibody (bottom of figure 2B). Figures 2C-D show the elution profiles of the digested TCA-insoluble fractions of apple tubulin (C) and actin (D) after incubation with the pollen TGase activity-enriched fraction and [ $^3\text{H}$ ]-putrescine. Incubation of tubulin and actin with 0.1 mM putrescine led to the recovery of bis-( $\gamma$ -glutamyl)-putrescine and mono-( $\gamma$ -glutamyl) derivatives, the free putrescine being internal standard. Presence of the TGase inhibitor 1,3-dimethyl-2-[(2-oxopropyl)thio] imidazolium chloride in the assay drastically reduced the formation of  $\gamma$ -glutamyl-putrescine, whereas the absence of the TGase substrates (tubulin and actin) did not allow the production of derivatives.

### Effects of pollen TGase on actin filaments

Because mammalian actin/tubulin reacted with pollen TGase comparably to pollen actin/tubulin and because of their availability in larger quantities, rabbit muscle actin and bovine brain tubulin were used in all subsequent assays. The effects of pollen TGase on actin filaments was first investigated by analyzing the sedimentation properties of *in vitro* polymerized actin (Fig. 3). Pollen TGase (Fig. 3A, lane 1, arrow) and FITC-cadaverine were incubated with filamentous actin (lane 3) polymerized from rabbit G-actin (lane 2). As control, F-actin was also examined in the absence of pollen TGase (lane 4) but in presence of FITC-cadaverine. The supernatant obtained in the presence of pollen TGase contained a small amount of actin (lane 5), while the corresponding pellet showed the presence of large amounts of actin in addition to high molecular mass bands (lane 6,

arrowheads) showing a molecular mass of 93, 133 and 155 kDa. The volumetric measure of those bands indicated that the ratio was 6:4:1. In the absence of pollen TGase, the supernatant (lane 7) contained only a small amount of actin, and the corresponding pellet (lane 8) did not reveal the presence of additional bands other than actin. An actin antibody (Fig. 3B) reacted with actin at 43 kDa, but also with the high molecular mass polypeptides (lane 6). When fractions were tested with the ID10 antibody (Fig. 3C), the pollen TGase was found in lane 1 (containing the enzyme only), in lane 3 (enzyme plus actin) and in the sample containing the actin-related high molecular mass aggregates (lane 6). The pollen TGase was not found in the high molecular mass aggregates. As control, the same assay performed with gplTGase and FITC-cadaverine yielded results similar to those obtained with pollen TGase (supplemental figure 1).

To demonstrate that FITC-cadaverine bound to actin filaments, the pellet obtained in the presence/absence of pollen TGase was observed under fluorescence microscopy in the rhodamine channel (for visualizing actin) and in the FITC channel (for visualizing FITC-cadaverine). Sample in lane 6 (gel of figure 3A) showed the presence of a conspicuous network of actin filaments (Fig. 4A), which was also detected in the FITC-channel (Fig. 4B). In the absence of pollen TGase (lane 8 of gel in figure 3A), we still observed the network of fluorescent actin filaments (Fig. 4C), but no signal was detected in the FITC channel (Fig. 4D) apart from random bright spots. This result indicated that FITC-cadaverine did not bind to actin in the absence of pollen TGase. The control in the presence of gplTGase was almost comparable to that of pollen TGase (supplemental figure 2).

We also analyzed the enzyme activity of myosin and its binding to actin filaments. First, the ATPase activity of rabbit myosin was tested in the presence of different concentrations of F-actin (10, 20 and 50 µg/ml), pre-treated (+ TGase) and untreated with either pollen or gplTGase (Fig. 5A). Actin alone showed no significant ATPase activity, as well as actin in combination with either pollen TGase or gplTGase. The ATPase activity of myosin alone was slightly detectable. In the absence of TGase, the ATPase activity of myosin increased following the different concentrations of actin. An increasing trend was also observed with TGase-treated actin; however, the increase of ATPase activity was greatly reduced when compared to untreated actin.

To visualize the binding of myosin to actin, we used a sedimentation assay. In the absence of pollen TGase (Fig. 5B), myosin bound almost completely to actin filaments (lane 2) and was not found in the supernatant (lane 1). When pollen TGase-treated actin was used, part of myosin (around 40%) was recovered in the supernatant (lane 3) and the remaining 60% in the pellet (lane 4). A comparable result was obtained with gplTGase (supplemental figure 3).

### Effects of pollen TGase on microtubules

We also analyzed the effects of pollen TGase on the assembly/disassembly of microtubules at different temperatures. Pollen TGase (Fig. 6A, lane 2) was mixed with rhodamine-labeled or unlabelled tubulin (lane 3), which was polymerized at 37°C in the presence of FITC-cadaverine. In the absence of TGase, tubulin was partly found in the supernatant S1 (around 30%, lane 4) but more consistently in the pellet P1 (around 70%, lane 5). When Ca<sup>2+</sup>-free buffers were used (80 mM Pipes pH 6.8, 1 mM MgCl<sub>2</sub>, 1 mM EGTA), the percentage of tubulin in the pellet increased to 90% (not shown). Around 55% of pollen TGase-treated tubulin was found in the supernatant S1 (lane 6), while the corresponding pellet P1 contained 45% of initial tubulin and additional high molecular mass bands (arrowheads) at 106, 121, 143 and 165 kDa (the latter being the most abundant), plus a very heavy band in the gel top. When the pellets of lane 5 and 7 were depolymerized, the TGase-untreated sample yielded a supernatant (S2, lane 8) enriched in tubulin (around 73% of total tubulin) compared with the pellet P2 (lane 9). When TGase-treated tubulin was depolymerised, the supernatant S2 (lane 10) contained low levels of tubulin (32% of total tubulin) in comparison with the pellet P2 (lane 11); the pellet also contained the high molecular mass aggregates (arrowheads). Presence of tubulin in the high molecular mass aggregates was shown by immunoblot with the tubulin antibody (Fig. 6B). Pollen TGase was detected by immunoblot in the tubulin pellet containing the high molecular mass aggregates (Fig. 6, lane 7). Although the pollen TGase

remained in the P2 pellet after depolymerization (lane 11), it was not found in association with the high molecular mass aggregates.

The microtubule pellets of lane 5 and 7 (Fig. 6A) were observed with DIC microscopy. TGase-treated microtubules were observed as bundles from which single microtubules radiated (Fig. 6D, left panel). Identity of microtubules was confirmed by fluorescence microscopy in the rhodamine channel (Fig. 6D, central panel). The co-distribution of FITC-cadaverine with microtubules was evaluated in the FITC channel (Fig. 6D, right panel). As control, the pellet of lane 5 (TGase-untreated microtubules) was observed as single filaments but not as bundles (Fig. 6E). When unlabelled tubulin was used, fluorescence in the rhodamine channel was not detected but it was in the FITC channel.

We investigated the effects of TGase and FITC-cadaverine on the binding of kinesin to microtubules. Tubulin (Fig. 7A, lane 2) was polymerized into microtubules (lane 4) in the presence of pollen TGase (lane 3) and the mixture (lane 5) was used for the binding assays. In controls, nearly all of kinesin pelleted with untreated microtubules (lane 7) comparing with the corresponding supernatant (lane 6). When the pellet was washed with ATP (release step), kinesin was found essentially in the supernatant (lane 9) but not in the pellet (lane 8). Kinesin bound only partially (around 60%) to TGase-treated-microtubules (lane 11) compared to soluble kinesin (lane 10, around 40%). After the ATP-releasing step, all kinesin was found in the supernatant (lane 13) and not in the microtubule pellet (lane 12). Pollen TGase was immunostained with the ID10 (Fig. 7B).

The effect of pollen TGase on the kinesin-mediated microtubule motility was analyzed by *in vitro* gliding assay. In the control, (Fig. 8A), untreated microtubules (arrows and arrowheads) moved with speed of 0.4 to 0.6  $\mu\text{m}/\text{sec}$  on kinesin-coated glass slides. The mean velocity of TGase-treated microtubules was significantly lower (0.2-0.3  $\mu\text{m}/\text{sec}$ , Fig. 8B) and we frequently observed presumptive microtubule bundles (black arrowhead) moving along kinesin-coated slides.

The ability of pollen TGase to form polyamine-mediated bridges between tubulin molecules was tested by mixing tubulin with pollen TGase and putrescine (Fig. 9). Bovine brain tubulin (Fig. 9A, lane 1) was polymerized in the presence of pollen TGase (lane 2) and either 0.1 or 1 mM putrescine and centrifuged. In the absence of putrescine, the supernatant (lane 3) contained small amounts of tubulin, while the pellet (lane 4) showed high molecular mass aggregates (arrowheads) at 97, 106, 150 (the most abundant) and 258 kDa. In the presence of 1 mM putrescine, the supernatant contained only tubulin monomers (lane 5), while the corresponding pellet showed larger amounts of the high molecular mass aggregates (lane 6). When pollen TGase was omitted, the supernatant (lane 7) showed essentially tubulin monomers, while the corresponding pellet (lane 8) contained tubulin but not additional high molecular mass aggregates. At DIC microscopy, the microtubule pellet of lane 4 (Fig. 9A) showed the previously described bundles of microtubules (Fig. 9B). Microtubules in the presence of pollen TGase and 1 mM putrescine (lane 6 of figure 9A) appeared as amorphous structures without the typical filamentous aspect of microtubules (Fig. 9C). When microtubules were incubated with putrescine but without TGase (lane 8 of figure 9A), they were indistinguishable from untreated microtubules (data not shown). The same experiment was performed in the presence of 0.1 mM putrescine (Fig. 9D). Arrows indicate the presence of lower amounts of high molecular mass aggregates. At DIC microscopy, microtubules treated with TGase in the absence of putrescine (lane 6 in figure 9D) appeared as bundles (Fig. 9E). In contrast, the microtubule pellet after treatment with pollen TGase and 0.1 mM putrescine (lane 4 in figure 9D) showed the presence of aberrant microtubule structures (Fig. 9F).

## Discussion

Using the  $\text{Ca}^{2+}$ -binding property of TGases, a 70 kDa polypeptide was identified in apple pollen extracts (Fig. 1B), which showed molecular and immunological characteristics very similar to those of the well known TGase-2, widely expressed in many animal cells. The pollen protein shared typical functions of TGases, as shown by its capacity to catalyze the formation of glutamyl-polyamine

derivatives, by its inhibition with a site-directed TGase inhibitor and by the comparative analysis of its products (Fig. 2C-D). The pollen TGase recognized actin and tubulin, catalyzing the formation of high molecular mass aggregates (Fig. 3A and 6A). In addition, pollen TGase influenced the binding of myosin and kinesin to actin filaments (Fig. 5B) and microtubules (Fig. 7), respectively. Pollen TGase can also reduce the enzyme activity of myosin (Fig. 5A) and the gliding activity of kinesin (Fig. 8B). Taken together, these findings showed that pollen TGase potentially acts as regulator of the cytoskeleton activity in pollen tubes.

### **Pollen TGase induces the formation of high molecular mass aggregates of actin**

Regulation of actin filament dynamics by post-translational modifications is poorly documented in plant cells. Evidence that actin is substrate of the partially purified 70-kDa pollen TGase (Fig. 2D and 3A) is in agreement with preliminary data showing that pollen actin was modified with polyamines after incubation with apple pollen crude extracts [18] to produce actin aggregates similar to those catalyzed by TGase from bacteria [32], animals and *Physarum polycephalum* [15]. In animals the formation of aggregates were attributed to intermolecular (and intramolecular) linkages by Gln-Lys cross-links. The intermolecular bond between Gln-41 and Lys-113 observed by linking N-(4-azido-2-nitrophenyl)-putrescine (ANP) makes G-actin more resistant towards proteolytic degradation and reduces the depolymerization of F-actin [33]. In addition, the occurrence of intramolecular cross-linking between Gln-41 and Lys-50 in rabbit muscle actin was followed by stabilization of F-actin structure [32]. Reports on animal or bacterial TGases indicated that Gln-41 is the only glutamine residue on actin that reacts with primary derivatized amines, like ANP [33] and dansyl-ethylene-diamine [34] in the TGase-catalyzed reaction [26] forming Gln-mono derivatives. In the present manuscript, the FITC-cadaverine test (Fig. 4) and the formation of radioactive mono-putrescine via unmodified labeled polyamines (Fig. 2C-D) suggested that the pollen TGase has identical activity. When unmodified polyamines were conjugated, a role in addition to cationisation of the free polyamine amino group in F-actin stabilization can be discussed. F-actin-like filaments are reversibly formed when G-actin was incubated with spermine and spermidine at relatively low concentrations in the absence of TGase. This reversible polymerization was attributed to the formation of ionic linkages with polyanionic regions at the N-terminus of actin [35].

The formation of bis-putrescine derivatives by pollen TGase (Fig. 2C-D) is a clear demonstration of cross-links between two Gln. Assuming that Gln-41 is the only residue modified by pollen TGase, the polyamine bis-derivatives should form intermolecular cross-linking between Gln-41 of different monomers or filaments. A single polyamine molecule can thus use its two terminal amino-groups to form either reversible ionic and irreversible covalent linkages with actin, the latter being mediated by TGase. Either excessive concentrations of polyamine or its absence disturbs the correct polymerization of actin in the presence of TGase; moreover, unordered bundles of actin appeared at high concentration suggesting that the actin binding sites are possibly saturated, giving rise to many mono-polyamine derivatives instead of forming the correct site-specific cross-links by bis-derivatives [36]. The natural polyamine concentrations (estimated around 50  $\mu$ M in hydrated pollen) should decrease the competition for the protein binding sites allowing bis-derivatives to form, as it occurs with 100  $\mu$ M putrescine (the optimal concentration used for the *in vitro* TGase assay reported here).

### **Pollen TGase affects the polymerization dynamic of microtubules**

TGases were shown to catalyze the conjugation of putrescine to tubulin in animal embryos, generating high molecular mass cross-linkage products [37] as also occurred in cells committed to apoptosis [38] and in the human placenta [16]. In plants, there are few data on this topic; we previously showed that pollen tubulin was modified by polyamines after incubation with pollen crude extract suggesting the occurrence of a catalyzing TGase activity [18]. Present results showed that pollen TGase changed the polymerization rate of tubulin in the presence of polyamines (Fig. 6).



Given that pollen TGase was never found in the actin and tubulin aggregates, its binding to aggregates should be transient or relatively weak to be broken by SDS. At 37°C, TGase-treated tubulin assembled to a small extent into microtubules and, simultaneously, formed high molecular mass aggregates. Temperature-dependent depolymerization is decreased, suggesting that the conjugation of polyamines generated more stable microtubules (Fig. 6). When the protein aggregates from the cadaverine (one binding site) and putrescine (two binding sites) assays were compared, some bands showed similar or identical molecular mass (i.e. 106 kDa); other aggregates appeared specific for the used polyamine, although the most abundant aggregates always showed a molecular mass around 150 to 160 kDa (Figs. 6 and 9). These aggregates are probably covalent dimers or trimers of tubulin produced by TGase activity. At DIC microscopy, the presence of relatively high concentrations (1 mM) of putrescine generated amorphous structures with no filamentous appearance (Fig. 9). On the contrary, presence of either the same concentration of FITC-cadaverine (having half available binding sites) or TGase only yielded filamentous structures (presumably microtubule bundles). Putrescine at lower concentration (closer to the presumptive natural one) generated either filamentous or amorphous microtubule structures (Fig. 6D). A reasonable hypothesis is that pollen TGase formed monomer aggregates at 1 mM putrescine concentration, which could slow down the polymerization rate of tubulin, thus explaining the low quantity of tubulin in the pellet. Rather than forming true microtubules, monomer aggregates could induce the formation of microtubule bundles, not necessarily formed only by covalent aggregates of tubulin but also by monomeric tubulin “entrapped” into the aggregates.

### **Modification of actin and tubulin by pollen TGase influences the activity of myosin and kinesin**

Actin filaments treated with pollen TGase bound less efficiently to myosin and enhanced the ATPase activity of myosin to a small extent, suggesting that pollen TGase had effects on the overall activity of the acto-myosin system (Fig. 5A-B). Our findings are therefore in agreement with data showing that the cross-linking between Gln-41 and Cys-374 on adjacent monomers of actin filaments inhibits the acto-myosin motility and force generation [39;40]. In addition, the TGase-mediated intramolecular cross-linking between Gln-41 and Lys-50 disturbed the structure of G-actin but apparently had small effects on actin polymerization and on myosin interaction [32] or could even activate the ATPase activity of myosin subfragment 1 [9]. Oriol-Audit [41] also reported that the polyamine-induced F-actin strongly triggered the  $Mg^{2+}$ -ATPase activity of myosin.

In this work, we showed that kinesin activity was reduced after polyamination of microtubules by pollen TGase. Specifically, kinesin bound with low affinity to TGase-modified microtubules but the ATP-releasing step was not affected (Fig. 7A). The low binding affinity also reflected on the motor activity of kinesin; *in vitro* motility assays showed that TGase-modified microtubules moved with lower speed (at least 50% of reduction) compared with unmodified microtubules (Fig. 8A-B). These data confirm that pollen TGase also can act as “biological glue” because of its capacity to cross-link proteins and to reduce their movement ability. These results are in agreement with the evidence that the motor activity of kinesin is also regulated by post-translational modification of tubulin. Members of the kinesin subfamily Kif5c moved predominantly along stable microtubules, which are mainly composed of dephosphorylated tubulin [42]. Other post-translational modifications that affected kinesin-based motility are glycylation, glutamylation and acetylation [43].

### **Hypothesis on the role of TGase during pollen tube growth**

Data in this manuscript suggested that the covalent cross-link of polyamines by pollen TGase generates stable actin aggregates, which alter the dynamic properties of actin filaments and the interaction of myosin with actin. Consequently, pollen TGase may participate in actin regulation during pollen tube growth. Since the proper organization of the actin cytoskeleton is essential for tube growth and organelle movement [2], any disorganization of the motor apparatus generates



critical changes in organelle motility and pollen tube growth. Given that  $\text{Ca}^{2+}$  is an important regulatory factor of TGase activity [18], local variation of  $\text{Ca}^{2+}$  concentration in the pollen tube may regulate the protein conformation and its enzymatic activity. We hypothesize that pollen TGase controls the transition between actin bundles and short filaments at the boundary between the apical and base domains of pollen tubes [44], favoring the assembly of more stable bundles. TGase activity may also be regulated by pH changes. The optimum pH value of TGase is alkaline [45] and the presence of an alkaline band in pollen tubes [46] suggests that local variation in pH may trigger the TGase activity in these restricted areas. The polyamine concentration is also relevant by pushing toward the formation of mono- or bis-derivatives [18]. Consequently, the actin-actin interactions, the actin-mono polyamine linking and the possible ionic bonds between the free amino groups of bound polyamines with other pollen molecules are finely regulated by the *in vivo* conditions of the pollen tube.

Current data also suggested that the TGase-mediated conjugation of polyamines is part of the mechanism that regulates microtubule dynamic and functioning. In eukaryotic cells, the dynamic of microtubules is dependent on microtubule-associated proteins (MAPs), which are poorly known in pollen tubes; the exception is represented by a couple of polypeptides that enhance microtubule polymerization and possibly mediate the interaction of microtubules with the plasma membrane [47]. The functioning of pollen tube microtubules involves the control of vacuole positioning [48], the trafficking velocity of mitochondria [49] and the focusing of secretory vesicles in the tip domain (unpublished data from our laboratories and [49]). Results in this manuscript, coupled to current literature, suggest that TGase-modified microtubules represent a subset of microtubules more resistant to depolymerization and along which the activity of motor proteins is partly inhibited.

In conclusion, these *in vitro* experiments show that pollen TGase affects the organization and functioning of the cytoskeleton; we propose that the activity of TGase might be modulated *in vivo* (under less extreme conditions) to exert a regulatory role on pollen tube growth.

## Funding

This work was supported by the Italian Ministero dell'Università (PRIN 2003 and PRIN 2005, <http://prin.miur.it/>) to the Dipartimento Biologia Evoluzionistica Sperimentale (DSF, University of Bologna) and by the Human Frontier Science Program (<http://www.hfsp.org>) to the Dipartimento Scienze Ambientali, University of Siena.

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## Figure legends

### Figure 1. Isolation of TGase from apple pollen.

(A) Hydrophobic interaction chromatography with Phenyl Sepharose of 50 ml-protein extract (0.5 mg/ml) from 30 min-hydrated apple pollen. The black arrows indicate the starting isocratic flow of buffer containing (a) 5 mM  $\text{CaCl}_2$ , (b) 10 mM EGTA, (c) water and (d) 50% ethylene glycol in water. The left axis reports the protein absorbance ( $A_{280}$  nm, white squares), while the right axis reports the specific TGase activity ( $\text{U mg prot}^{-1}$ , closed circle) determined by the biotin-labeled cadaverine incorporation assay. (B) SDS-PAGE and immunoblot of pollen extract and EGTA-eluted peak. Lane 1, Coomassie stained EGTA-eluted peak blotted on PVDF membrane (20  $\mu\text{g}$ ). Lane 2, immunoblot of the EGTA-eluted peak with the ID10 antibody. Lane 3, immunoblot of the EGTA-eluted peak with AbIII. Lane 4, ID10-probed blotted proteins from guinea pig liver TGase (gplTGase). Lane 5, ID10 antibody probed on germinated pollen extract. Lane 6, AbIII probed on germinated pollen extract. The molecular mass of main bands is indicated on the left in kDa (and in all gels/blots of other figures).

### Figure 2. Purification of tubulin and actin from apple pollen and assay of pollen TGase.

(A) SDS-PAGE illustrating the main steps of tubulin purification. Molecular mass standards are on the left; lane 1, pollen extract (20  $\mu\text{g}$ ); lane 2, pool after Resource-Q column (20  $\mu\text{g}$ ); lane 3, pool after Sephacryl S-300 column; lane 4, pool after Mini-Q column; lane 5, supernatant after taxol polymerization; lane 6, pellet after taxol polymerization (lanes 3-6 contain 5  $\mu\text{g}$  of protein). The gel is Coomassie-stained. Bottom panel: blot with anti-tubulin. (B) Gel showing the main steps of actin purification. Molecular mass standards are on the left; lane 1, pollen extract (20  $\mu\text{g}$ ); lane 2, proteins that do not sediment (15  $\mu\text{g}$ ); lane 3, proteins that precipitate with  $(\text{NH}_4)_2\text{SO}_4$  (15  $\mu\text{g}$ ); lane 4, pool after Mono-Q column (10  $\mu\text{g}$ ); lane 5, proteins that do not polymerize; lane 6, proteins that polymerize and sediment with  $\text{MgCl}_2$ ; lane 7, pool after Superdex 200 column. Lanes 5-7 contain 5  $\mu\text{g}$  of protein. The gel is Coomassie-stained. Bottom panel: blot with anti-actin. (C and D) Distribution of radioactivity in the TGase assay products performed by incubating the pollen TGase active fraction with [ $^3\text{H}$ ]-putrescine, in the presence/absence of TGase inhibitor and pollen tubulin (C) or pollen actin (D). Arrows indicate standard retention times for bis- $\gamma$ -glutamyl-putrescine and mono- $\gamma$ -glutamyl-putrescine.

### Figure 3. Enzyme activity of pollen TGase on F-actin.

(A) Enzyme assay in the presence/absence of pollen TGase and FITC-cadaverine. Lane 1, pollen TGase (1  $\mu\text{g}$ , arrow); lane 2, monomeric actin (10  $\mu\text{g}$ ); lane 3, filamentous actin (10  $\mu\text{g}$ ) plus pollen TGase; lane 4, control of F-actin without pollen TGase (10  $\mu\text{g}$ ). Lane 5, supernatant in the presence of pollen TGase; lane 6, corresponding pellet; lane 7, supernatant in the absence of pollen TGase; lane 8, corresponding pellet. Arrowheads indicate high molecular mass polypeptides appearing in the presence of pollen TGase and cross-reacting with the anti-actin antibody. Protein content of lane 5-8 is three-fold (30  $\mu\text{g}$ ) that of lanes 3-4 to show the high molecular mass aggregates. The gel is Coomassie-stained. (B) Western blot with anti-actin antibody on fractions shown in A. (C) Western blot with ID-10 on fractions shown in A.

### Figure 4. Incorporation of FITC-cadaverine in rabbit actin by pollen TGase.

(A) F-actin after incorporation of labeled cadaverine in the presence of pollen TGase as observed in the rhodamine filter. (B) Same field observed in the FITC filter set. (C) Rhodamine-labeled F-actin obtained in the absence of pollen TGase as observed in the related filter set. (D) Same view field observed in the FITC filter set. Bar: 2  $\mu\text{m}$ .

**Figure 5. Enzyme activity and binding assay of myosin in the presence/absence of pollen TGase and FITC-cadaverine.**

(A) Enzyme activity of myosin in the presence of different concentrations of F-actin after incubation with either pollen TGase or gplTGase. In the absence of TGase, the activity of myosin is always higher than the corresponding activity in the presence of either pollen TGase or gplTGase. For comparison, the enzyme activity of actin (Act), actin and TGase (Act + TGase) and myosin (Myo) alone were also tested. All values are means  $\pm$  s.e.m. for  $n=3$  separate determinations. (B) Binding assay of myosin to actin filaments after incubation with pollen TGase. Lane 1 and 3 are supernatants after binding assay in the absence or presence of pollen TGase, respectively. Lane 2 and 4 are the corresponding pellets. Six micrograms of total proteins were loaded in each lane; the myosin band approximately corresponded to one microgram. Lane 5 is rabbit myosin (1  $\mu$ g). The gel is Coomassie-stained.

**Figure 6. Tubulin polymerization/depolymerization assay in the presence/absence of pollen TGase and FITC-cadaverine.**

(A) Coomassie-stained gel showing the main fractions obtained in the assay. Lane 1, Mr standards. Lane 2, pollen TGase (1  $\mu$ g). Lane 3, bovine brain tubulin. Lane 4, supernatant (S1) after polymerization of tubulin at 37°C in the absence of TGase. Lane 5, corresponding pellet (P1). Lane 6, supernatant (S1) after polymerization of tubulin at 37°C in the presence of TGase. Lane 7, corresponding pellet (P1). Arrowheads indicate the high molecular mass polypeptides in the microtubule pellet after incubation of tubulin with pollen TGase. Lane 8, supernatant (S2) after depolymerization of the microtubule pellet of lane 5. Lane 9, the corresponding pellet (P2). Lane 10, supernatant (S2) after depolymerization of the microtubule pellet of lane 7. Lane 11, the corresponding pellet (P2). Arrowheads indicate the high molecular mass aggregates already found in P1. Lanes 3-11 contain 10  $\mu$ g of protein. (B) Western blot with the anti-tubulin antibody on the same fractions shown in A. (C) Western blot with ID10 antibody on the same samples shown in A. (D) Visualization of rhodamine-microtubules after treatment with pollen TGase. The pellet in lane 7 was observed with DIC microscopy (left panel), with fluorescent microscopy in the rhodamine filter (central panel) and in the FITC filter (right panel). (E) Visualization of the microtubule pellet in lane 5. Bars: 4  $\mu$ m.

**Figure 7. Binding assay of kinesin to microtubules in the presence of pollen TGase and FITC-cadaverine.**

(A) Coomassie-stained gel showing the main fractions obtained during the binding and releasing of kinesin. Lane 1, standard of molecular mass. Lane 2, tubulin (10  $\mu$ g). Lane 3, pollen TGase (1  $\mu$ g). Lane 4, microtubules (10  $\mu$ g). Lane 5, mix of microtubules and pollen TGase (10  $\mu$ g). Lane 6, supernatant (S) after binding of kinesin to TGase-untreated microtubules. Lane 7, corresponding pellet (P). Lane 8, pellet after ATP-release of kinesin. Lane 9, corresponding supernatant. Lane 10, supernatant after binding of kinesin to TGase-treated microtubules. Lane 11, corresponding pellet. Lane 12, pellet after ATP-release of kinesin. Lane 13, corresponding supernatant. Protein content in pellet samples of lanes 2, 4, 5 and 6-13 was 10  $\mu$ g, while the protein content of supernatants was not estimated because loaded volumes had to be identical to pellet samples. (B) Western blotting of samples shown in A with ID10.

**Figure 8. Motility assay of microtubules along kinesin-coated slides.**

(A) Untreated microtubules gliding over kinesin-coated slides. Microtubules move with speeds ranging from 0.4 to 0.6  $\mu$ m/sec. Arrows and arrowheads indicate some specific microtubules. Numbers in the top left corner are time in seconds. Bar: 5  $\mu$ m. (B) TGase-treated microtubules moving along kinesin-coated slides. Microtubules move with speeds around 0.2-0.3  $\mu$ m/sec. Arrows and arrowheads indicate moving microtubules. Black arrowhead indicates putatively one microtubule bundle. Numbers in the top left as in (A) corner are time in seconds. Bar: 3  $\mu$ m.

**Figure 9. Reactivity of microtubules with pollen TGase in the presence of putrescine (Pu).**

(A) Effects of 1 mM putrescine. Lane 1, microtubules (10  $\mu$ g). Lane 2, pollen TGase (1  $\mu$ g). Lane 3, supernatant after incubation of microtubules with pollen TGase. Lane 4, corresponding pellet; arrowheads indicate high molecular mass polypeptides. Lane 5, supernatant after incubation of microtubules with pollen TGase and 1 mM putrescine. Lane 6, corresponding pellet; the high molecular mass aggregates are more visible. Lane 7, supernatant after incubation of microtubules with putrescine only. Lane 8, corresponding pellet. Lane 9, supernatant after incubation of microtubules only. Lane 10, corresponding pellet. Protein content in pellet samples of lanes 3-10 was 30  $\mu$ g, while the protein content of supernatants was not estimated because loaded volumes had to be identical to pellets. The gel is Coomassie-stained. (B) DIC view of microtubules after TGase treatment (pellet in lane 4 of gel A). (C) Microtubules after treatment with pollen TGase and 1 mM putrescine (pellet in lane 6 of gel A). Bar for B-C: 4  $\mu$ m. (D) Effects of 0.1 mM putrescine. Lanes contain samples equivalent to the gel in A; protein content was also comparable. Arrows indicate the presence of high molecular mass aggregates. The gel is Coomassie-stained. (E) Microtubules after TGase treatment (pellet of lane 6 in gel D). (F) Microtubules after treatment with pollen TGase and 0.1 mM putrescine (pellet of lane 4 in gel D). Bar for E-F: 4  $\mu$ m.

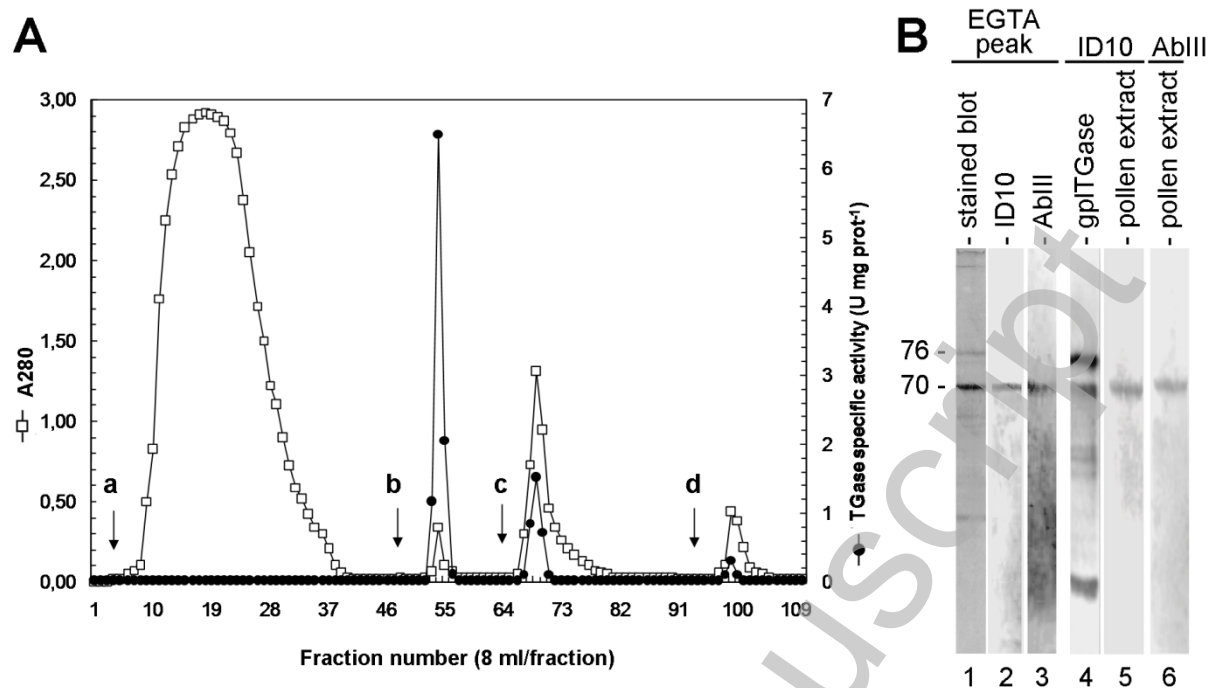


FIGURE 1

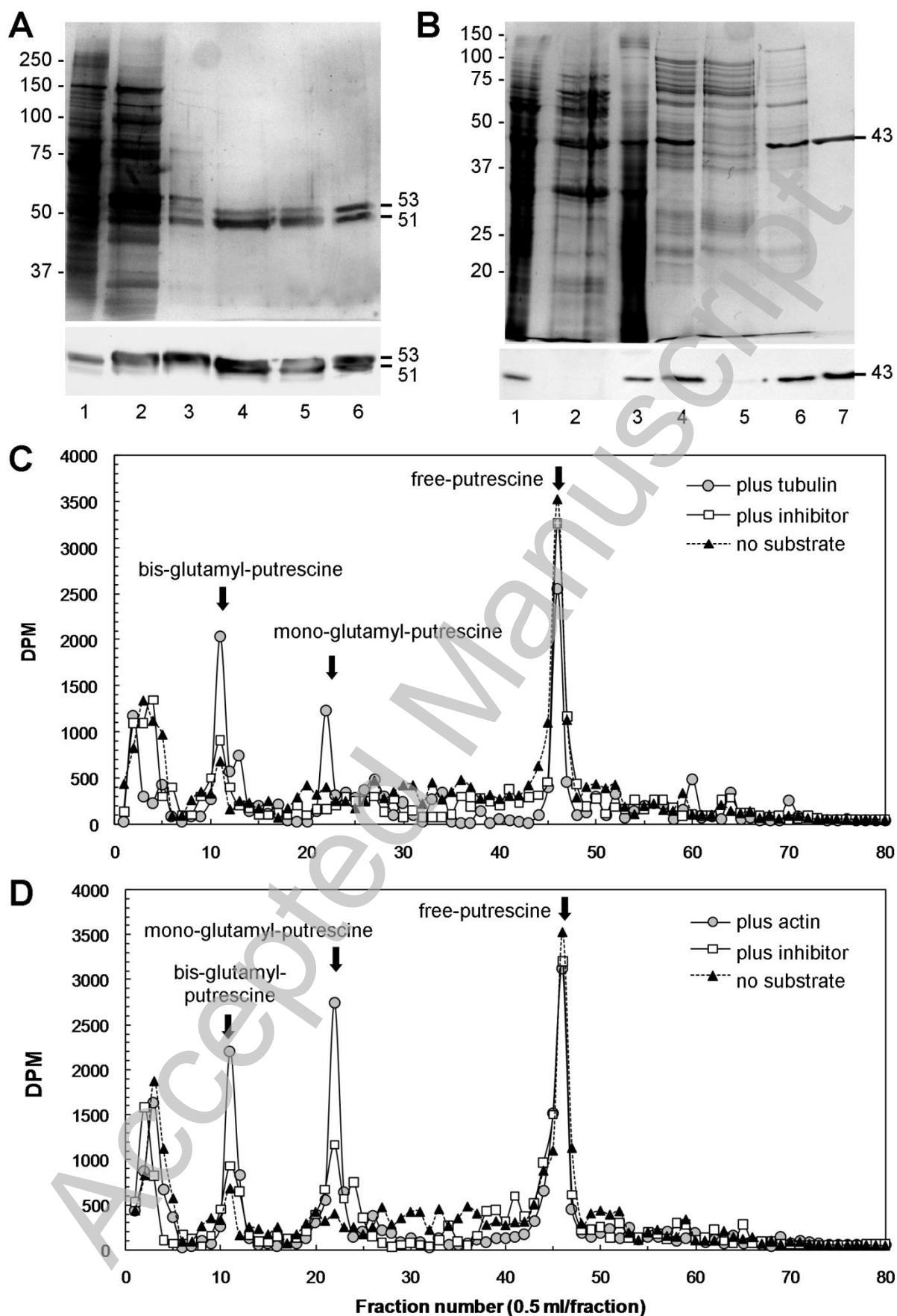
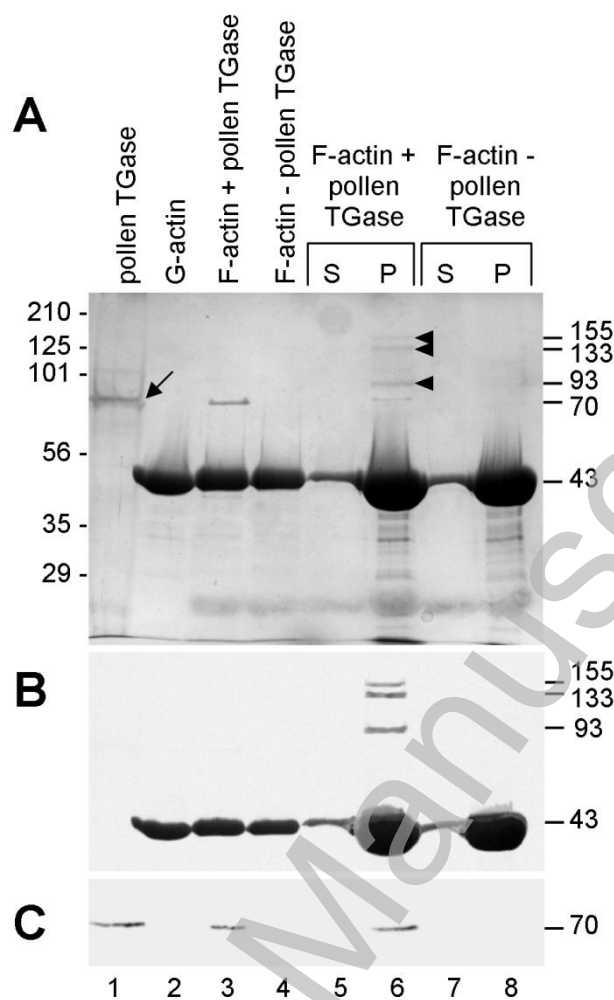
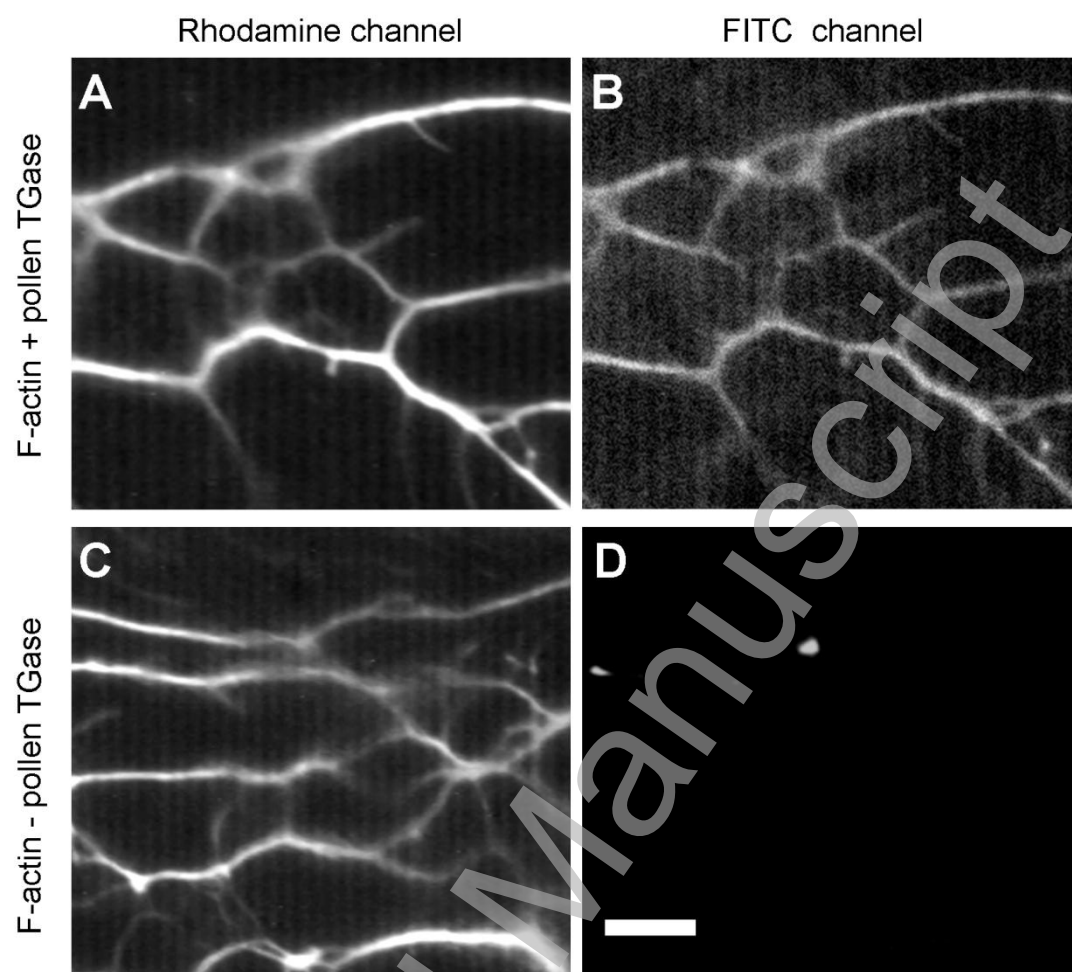


FIGURE 2

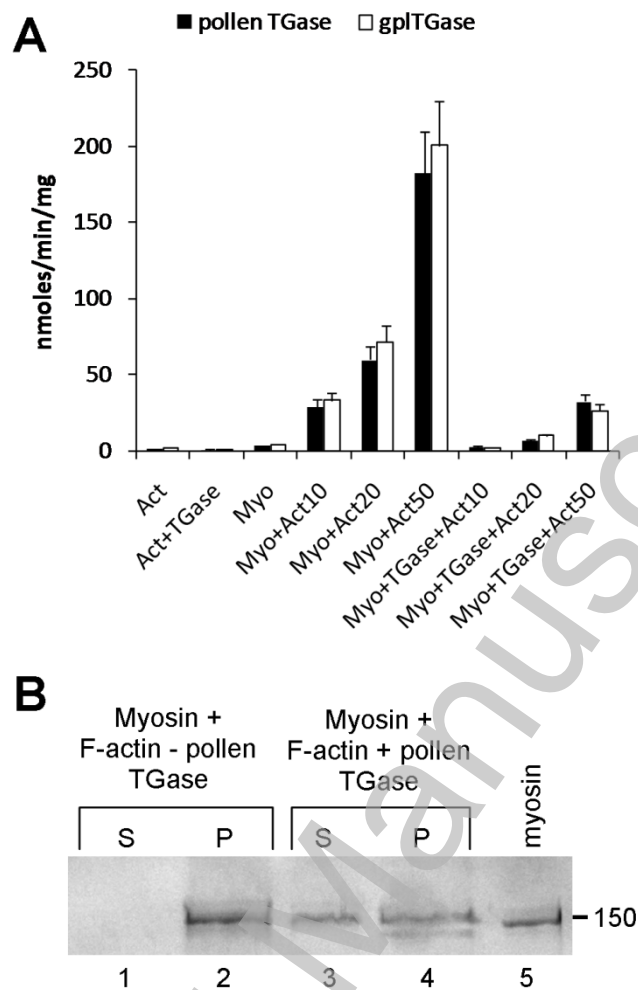




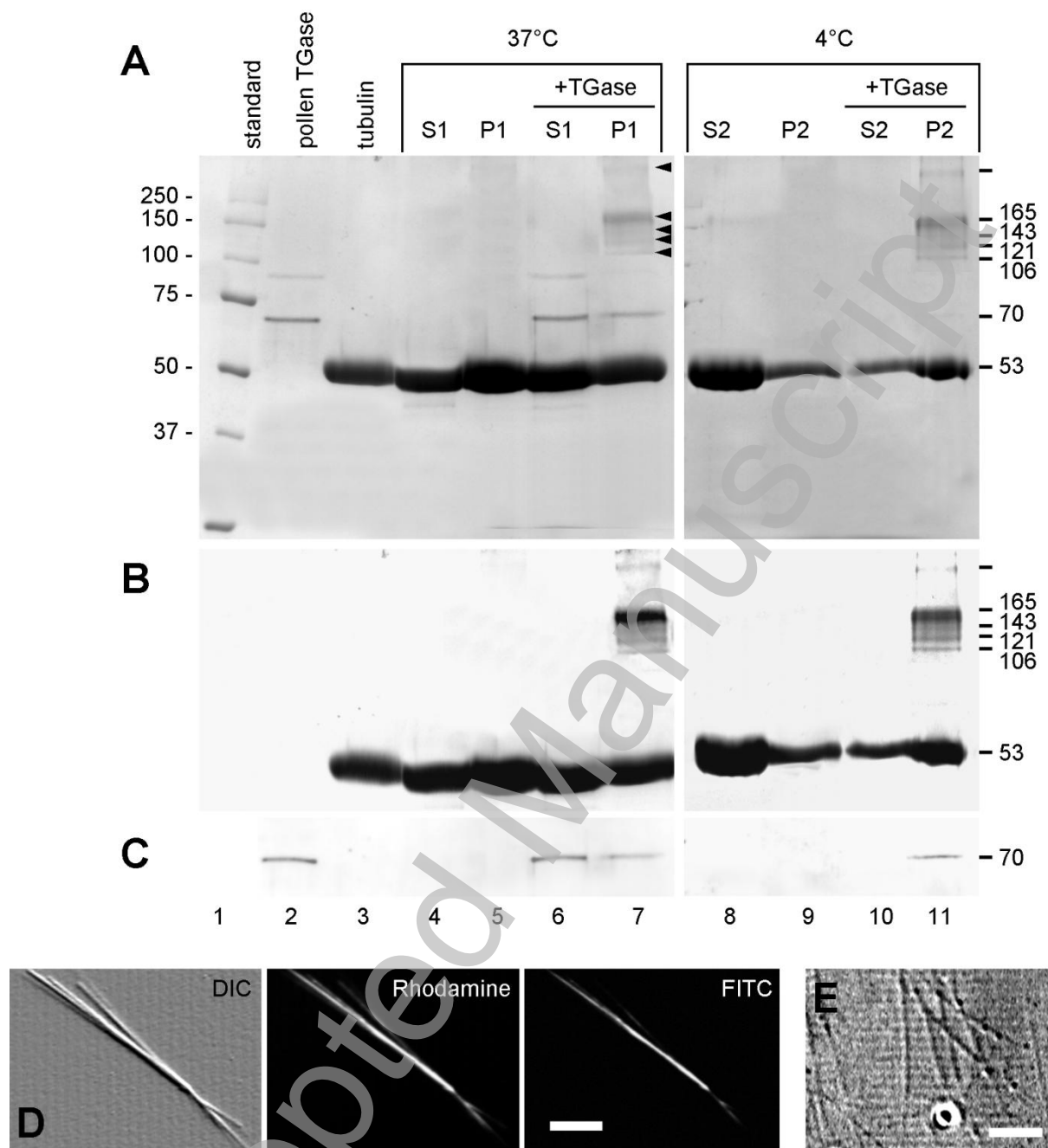
**FIGURE 3**



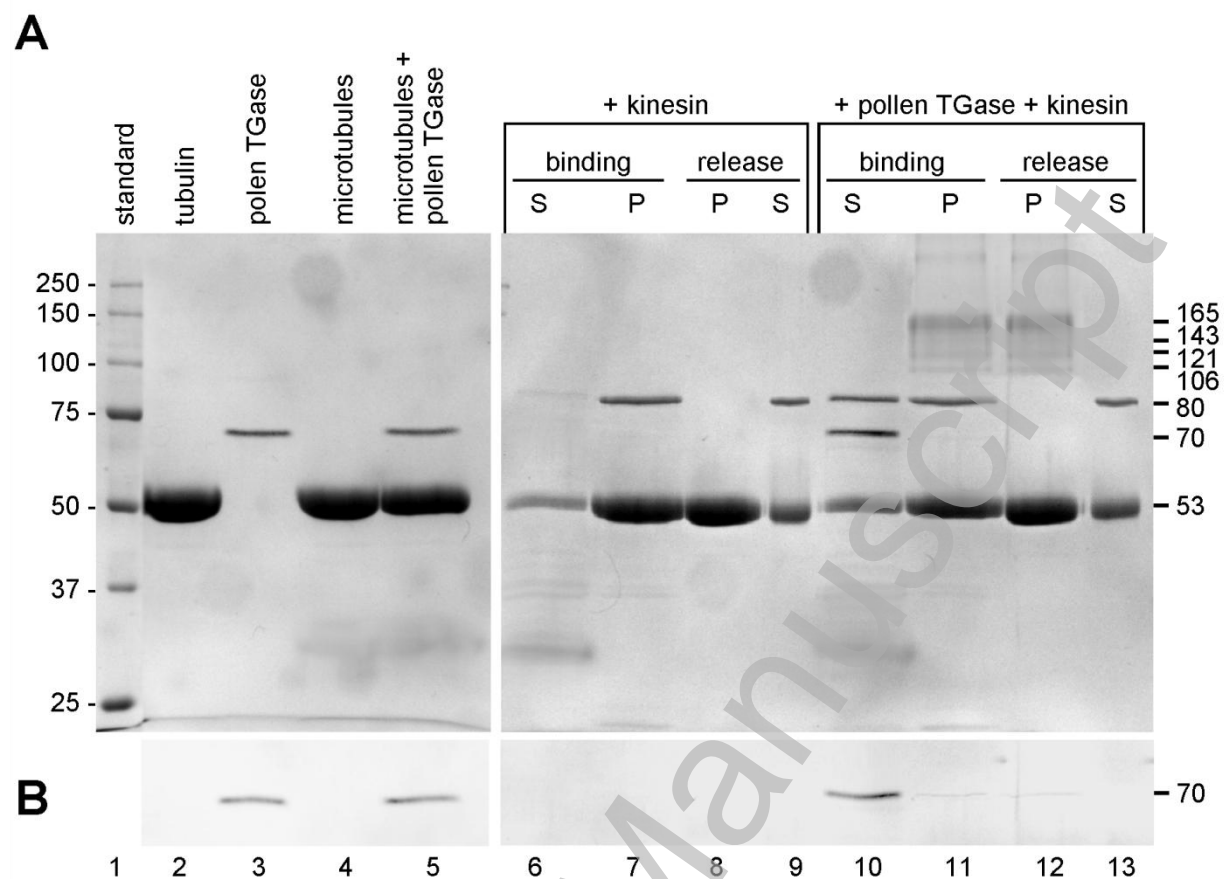
**FIGURE 4**



**FIGURE 5**

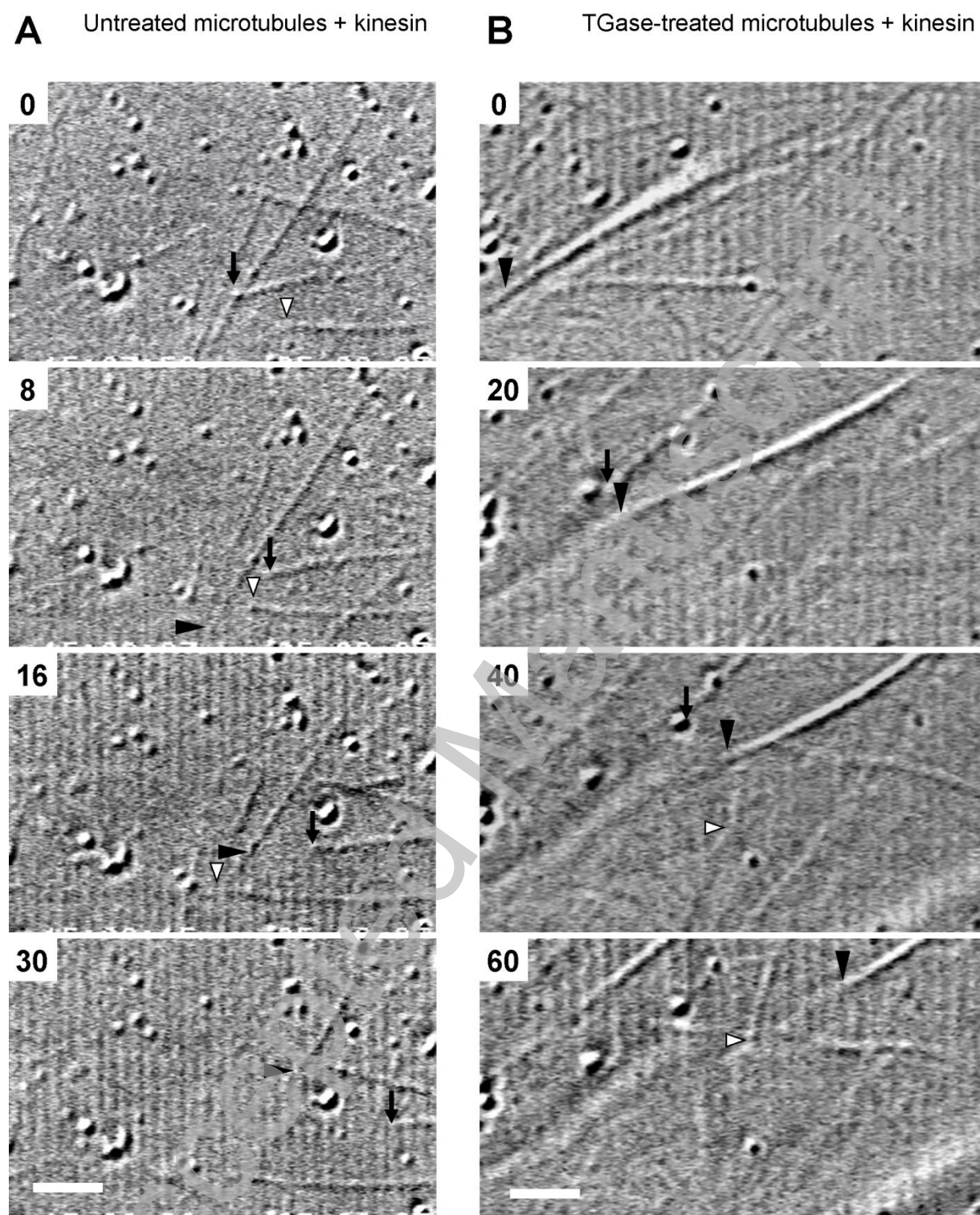


**FIGURE 6**

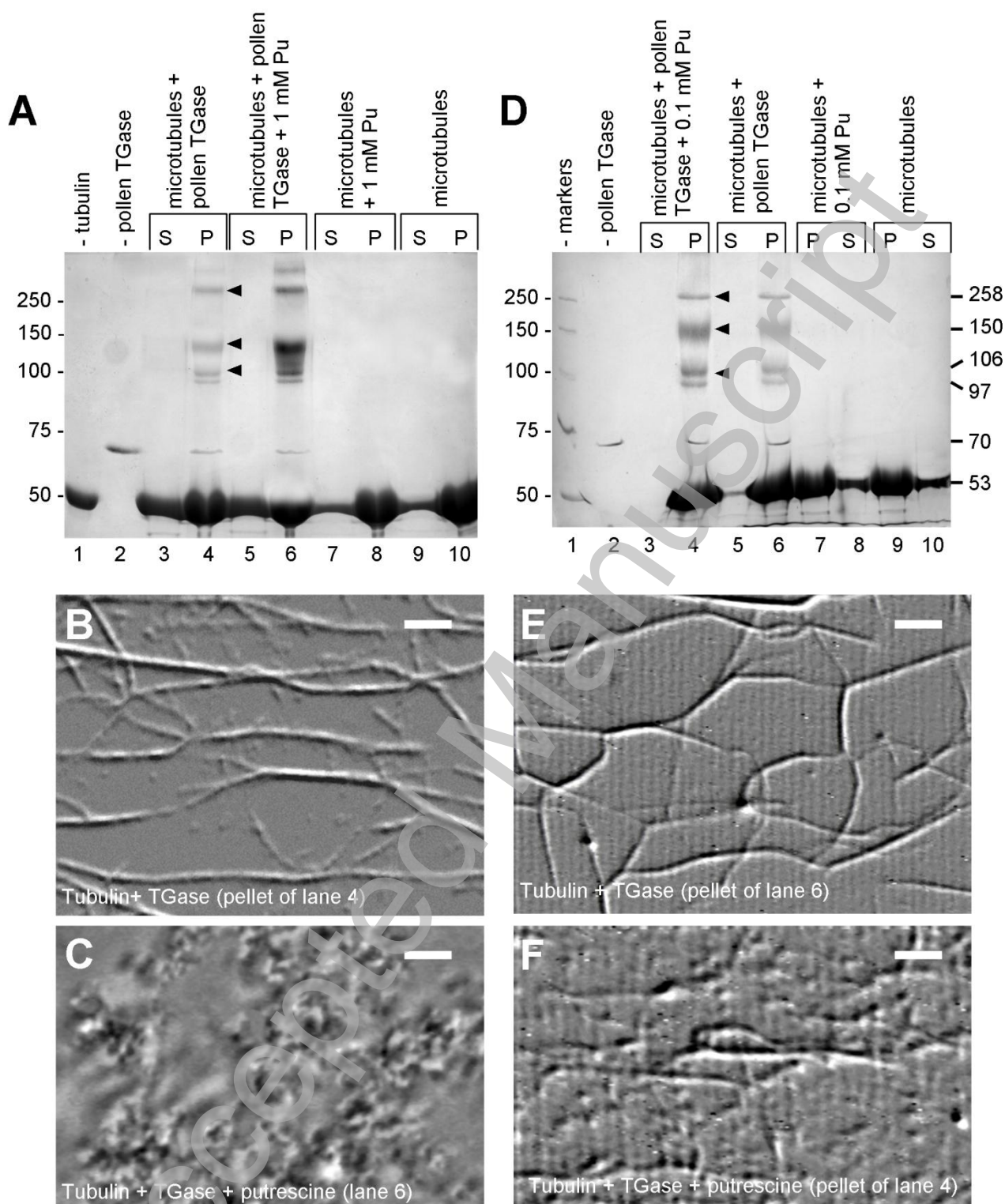


**FIGURE 7**





**FIGURE 8**



**FIGURE 9**