

Purification and Properties of Transgelin: A Transformation and Shape Change Sensitive Actin-gelling Protein

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Abstract. We have purified the transformation and shape change sensitive isoform of an actin associated polypeptide doublet previously described by us (Shapland, C., P. Lowings, and D. Lawson. 1988. *J. Cell Biol.* 107:153–161) and have shown that it is evolutionarily conserved as far back as yeast. The purified protein: (a) binds directly to actin filaments at a ratio of 1:6 actin monomers, with a binding constant (K_a) of $\sim 7.5 \times 10^5 \text{ M}^{-1}$; and (b) causes actin filament gelation within 2 min. Although these activities are controlled by ionic strength (and may be mediated by

positively charged amino acid residues) the molecule remains as a monomer irrespective of ionic conditions. EM reveals that the addition of this protein to actin filaments converts them from a loose, random distribution into a tangled, cross-linked meshwork within 1 min, and discrete tightly aggregated foci after 10 min. By use of an “add-back” cell permeabilization system we can rebind this molecule specifically to actin filaments in cells from which it has previously been removed. Since the protein is transformation sensitive and gels actin, we have named it transgelin.

ACTIN is crucial for a variety of cellular events such as motility, division and cell surface receptor movement (40, 43, 55, 56). Actin organization can be controlled by a large number (~ 70 are so far known) (3, 37) of actin-associated proteins which act by bundling, cross-linking, severing, gelating, sequestering monomers, or preventing actin polymerization (3, 9, 37, 40, 43, 44, 49). These molecules, acting either individually or in concert, regulate (a) the physical status of actin (that is the ratio of globular to filamentous actin) (43, 56), (b) actin geometry (5, 19, 20, 27, 44), and (c) provide both the fine control and the driving force required for the cellular events mentioned above.

Disruption of the actin network is known to accompany events such as neoplasia (21, and see reference 41) and, in this instance, can involve alterations to both the actin microfilament network itself (21) and the expression of selected actin-associated proteins (45). However, only seven of the 70 or so proteins associated with actin are thus far known to be affected by transformation; namely, the higher molecular weight tropomyosins (11), nonmuscle caldesmon (14), smooth muscle myosin light chain 2 (16), gelsolin and actin-binding protein (17), protein C4 (41), and gelsolin (50). Since some actin-associated proteins probably act synergistically to control and organize actin, e.g., tropomyosin, gelsolin, and caldesmon (50), it seems very likely that the major changes to the actin microfilament network that occur following transformation may reflect the coordinated down-regulation of several of these important molecules rather than alterations to the ratio of globular: filamentous actin itself.

We have previously identified a transformation-sensitive

polypeptide doublet (protein C4^{h,1}) present in all cells and tissues apart from skeletal muscle, red blood cells and neurons, and have shown that the higher relative molecular weight polypeptide (C4^h) is down regulated when mesenchymal cells are (a) transformed by DNA or RNA viruses or (b) switched to nonadherent culture conditions (41). We have now purified this higher molecular weight isoform (C4^h) from sheep aorta and used a variety of assays to study the interactions of this transformation sensitive isoform which our amino acid sequence analysis shows is highly homologous to SM22 α (33). Since we have demonstrated that this polypeptide is transformation sensitive and that it rapidly gels actin, we have named the molecule transgelin.

Materials and Methods

Protein Purification

Fat and connective tissue were stripped from fresh sheep aorta which was then stored at -196°C . All further purification steps were performed at 4°C , in the presence of $2 \mu\text{g/ml}$ leupeptin, chymostatin, and pepstatin. Frozen aorta was weighed, fractured into small pieces, homogenized in a blender (Waring Commercial, New Hartford, CT) in 5 vol of buffer A + 0.5% CHAPS (60 mM KCl, 4 mM MgCl_2 , 10 mM Imidazole, pH 7, 1 mM NaN_3 , 0.5 mM EGTA, 5 mM DTT, 0.1 mM EDTA, 0.2 mM PMSF), using 3×10 s runs of the blender set at high speed, left on ice for 1 h, and the detergent-insoluble material removed by centrifugation at 23,000 g for 5 minutes. The supernatant was filtered over glass wool, dialysed for 16 h against 2 liters of Buffer B (50 mM Na Acetate, 4 mM MgCl_2 , 0.5 M KCl, pH 4.8), clarified by centrifugation at 48,000 g for 2 h, dialyzed against 25 mM ethanolamine HCl, pH 9.6, and fractionated on a 1.6×70 cm chromatofocusing column, using a pH 9–7 gradient. The peak containing transgelin was identified by SDS-PAGE/immunoblotting with monoclonal anti-transgelin antibody (41), dialyzed overnight against 20 mM potassium

phosphate buffer, pH 6.8, and as a final purification and concentration step, applied to a small (1 × 1 cm) hydroxylapatite column, previously equilibrated in 20 mM phosphate buffer, and eluted with 100 mM phosphate buffer. SDS-PAGE/immunoblotting of aorta and rat embryo fibroblasts (REFs)¹ was carried out as previously described (18, 41).

Actin was extracted from an acetone powder preparation of rabbit skeletal muscle (a kind gift from Dr. J. Sleep, Medical Research Council Biophysics Unit, Kings College, London) as previously described (31, 42), and purified by column chromatography as described (31).

Viscosity Assay

G actin, prepared as described previously (31, 42), was clarified by centrifugation at 148,000 *g* for 1 h 40 min, adjusted to a concentration of 9.3 μM, and polymerized by the addition of buffer P (2 mM MgCl₂, 1 mM ATP, 1 mM EGTA, 12 mM K phosphate buffer, pH 6.8), in the presence or absence of varying amounts of transgelin (up to 4.4 μM). Ionic strength was adjusted by the addition of KCl (up to 100 mM) or sodium triphosphate (up to 4 mM). Low shear viscosity experiments were carried out using falling ball viscometry as described by MacLean-Fletcher and Pollard (24, and see reference 36). All measurements were made at 25°C and at an angle of 50°. The capillary tubes were loaded with actin solution 30 min before measurement.

Light Scattering Assay

9.3 μM G actin ± 2.4 μM transgelin was polymerized in buffer P ± 100 mM KCl. The increase in 90° light scattering intensity was measured in a spectrofluorimeter (MPF 3L; Perkin-Elmer Corp., Norwalk, CT) with excitation and emission wavelengths set at 360 nm and slit widths at 2 nm (13).

EM

0.5 μM G actin was incubated for 1 or 10 min ± 2.4 μM transgelin and then fixed by the addition of 5% glutaraldehyde in buffer P to a final concentration of 0.5%. Aliquots (18 μl) of filaments were allowed to settle onto carbon formvar grids, pre-treated with cytochrome c, negative stained as described (29), and viewed in an electron microscope (100 CXII; JEOL USA, Peabody, MA) operating at 80 kV.

Sedimentation Assay

The interaction of transgelin with F actin was examined using the pelleting assay described by Yamashiro-Matsumura and Matsumura (58). G actin, prepared as above, was induced to polymerize by the addition of buffer P, incubated with varying concentrations of transgelin in a total volume of 100 μl for 1 h at 25°C, and then centrifuged in an airfuge (Beckman Instruments, Fullerton, CA) at 148,000 *g* for 1 h at 4°C. Pellets and supernatants were separated carefully, suspended in 150 μl of Laemmli SDS sample buffer, electrophoresed on 12% SDS-PAGE, stained with Coomassie blue, destained for 1 h, and scanned with a densitometer (GS 300; Hoefer Scientific Instruments, San Francisco, CA) linked to a MacIntosh SE30 using a GS 370 electrophoresis data reduction system to determine the amounts of protein in each band. The densitometer was calibrated by scanning SDS-PAGE gels containing known amounts of transgelin. From these data the concentration of bound versus free protein, and therefore the binding constant *K*_a were estimated (58).

Sucrose Density Gradients

Sucrose density gradient centrifugation was carried out by the method of Martin and Ames (25). Briefly, 0.5 ml of transgelin at a concentration of 0.5 mg/ml in buffer P ± 100 mM KCl, was loaded on to a 10 ml, 5–20% (wt/vol) linear sucrose gradient in the same buffer and centrifuged at 150,000 *g* for 43 h at 4°C. Protein standards run in parallel gradients were soy bean trypsin inhibitor 21,000 *M*_r, ovalbumin 43,000 *M*_r, BSA 68,000 *M*_r, and transferrin 80,000 *M*_r. 1-ml fractions were collected from the bottom of each tube as described (25) and the protein concentration monitored by absorbance at 280 nm. Fractions corresponding to peak readings at 280 nm were then analyzed by SDS-PAGE.

Amino Acid Sequencing

Purified transgelin was applied directly to an automated amino acid sequencer (477A with on line 120A PTH analyzer; Applied Biosystems, Foster City, CA) using modified chemistry cycles (47).

Immunofluorescence

Detergent Extraction of Transgelin. Secondary cultures of rat embryo fibroblasts were plated on glass coverslips at a concentration of 2.5 × 10³ cells/ml in DME, 10% FCS for 4 d, rinsed briefly in cold buffer P ± 60 mM KCl, and then extracted at 4°C for various times (15 s to 15 min) in buffer P ± 60 mM KCl + 0.5% CHAPS, rinsed in buffer P ± 60 mM KCl, plunged into -20°C methanol, and then blocked for 10 min in 3% BSA, and 100 mM lysine in PBS. Control coverslips were rinsed briefly in cold buffer P + 60 mM KCl, plunged into methanol, rehydrated, and blocked as above. Antibody labeling and immunofluorescence were as previously described (41).

Transgelin rebinding to permeabilized cells. For rebinding experiments, cells were detergent extracted for 15 min, rinsed and blocked as above, and then incubated at 4°C for 30 min in either 1.5 or 3 μM transgelin in buffer P ± 60 mM KCl, in a total volume of 60 μl. Coverslips were then rinsed/plunge fixed in 7 ml of 1% glutaraldehyde for 30 s at 4°C, rapidly transferred to 2% formaldehyde in PBS for 5 min at room temperature, rinsed briefly in PBS, reduced in sodium borohydride (1 mg/ml in PBS at 37°C for 10 min), rinsed in PBS, and processed for immunofluorescence as above, and antibody labeled as previously described (41). To obtain a fluorescence exposure base line for these experiments, the exposure time required for control cells was measured and used for photographing all other preparations.

Immunoblotting/Polyclonal Anti-Transgelin Antibody. Samples of *Saccharomyces pombe*, kindly supplied by Prof. J. Hyams, (Biology Department, University College London), and 5 μg of purified transgelin were immunoblotted as described (18, 41). Polyclonal anti-transgelin was raised by cutting strips containing the protein from aorta electrophoresed on 3-mm thick SDS-PAGE gels. These were then electroeluted into SDS running buffer in dialysis tubing, acetone precipitated, and injected into rabbits over a 3-mo period. A DEAE prepared IgG fraction was passed over an affinity column of purified transgelin (2 mg) coupled to Affigel 10, eluted with 50 mM DEAE, pH 11.5, immediately neutralized with 1 M Tris buffer, pH 7.5, dialyzed into PBS, concentrated by Millipore filtration (Millipore Corp., Bedford, MA), and used at a concentration of 2 μg/ml. Goat anti-rabbit IgG was purchased from Cappel Dynatech (Malvern, PA), affinity purified as above and 100 μg conjugated to ¹²⁵I as described (18).

Results

Purification of Transgelin

We have previously shown that aorta is a rich source of transgelin, with the lower molecular weight polypeptide present in negligible amounts (41). To isolate transgelin from aorta we have exploited our finding that the molecule is highly detergent soluble (41), (Fig. 1, *a* and *b*). A high salt, low pH step which removes actin by precipitation (Fig. 1 *d*), leaving transgelin in the supernatant (Fig. 1 *e*) was followed by chromatofocusing with a final purification and concentration step on hydroxylapatite (Fig. 1 *f*) to yield a preparation containing a single band with no apparent degradation as shown by SDS-PAGE and immunoblotting (Fig. 1, *f* and *g*). This band migrates at the same position in immunoblots as the higher molecular mass band present in fibroblasts, which also contain the lower molecular mass isoform (Fig. 1 *h*). In addition, reverse phase chromatography indicated the presence of only a single protein peak (not shown). By using this protocol we obtained, from 10 g of aorta, ~1 mg of transgelin, in its native state as defined by its capacity to (*a*) bind to purified actin in vitro, (*b*) gel actin, and (*c*) bind to actin in a permeabilized cell system.

1. Abbreviation used in this paper: REF, rat embryo fibroblast.

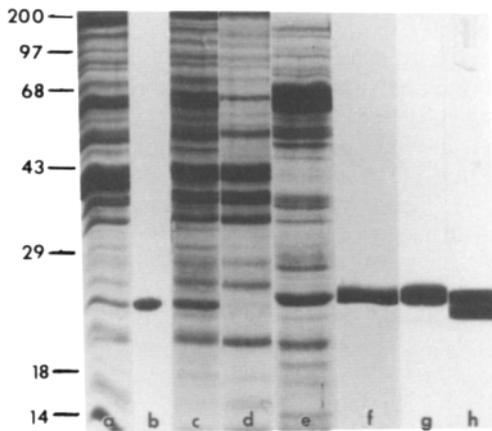


Figure 1. Purification of transgelin. (a) Total aorta. (b) Immunoblot of total aorta incubated with monoclonal anti-transgelin antibody showing that transgelin is the principal isoform present in this tissue. (c) Detergent-soluble supernatant after incubation in 0.5% CHAPS for 30 min and centrifugation at 23,000 *g* for 5 min. Note the presence of transgelin in this lane. (d) Pellet after 16-h incubation in 0.5 M KCl at pH 4.8, and then centrifugation at 48,000 *g* for 48 h. While little transgelin is precipitated by this treatment most contaminating actin is removed. (e) Supernatant after incubation in 0.5 M KCl at pH 4.8 and centrifugation as above, showing a significant enrichment of transgelin. (f) Purified transgelin after column chromatography using chromatofocusing and hydroxylapatite. (g) Immunoblot of purified transgelin labeled with monoclonal anti-transgelin antibody showing that only the high molecular mass isoform is present. Compare with lane *h* where both isoforms are present. No transgelin degradation is detectable. (h) Immunoblot of total REF labeled with monoclonal anti-transgelin antibody, showing the presence of both isoforms.

Viscometric Analysis and Control of Transgelin-induced Actin Gelation

Falling ball viscometry showed that the addition of increasing amounts of transgelin (up to 4.2 μM) to 9.3 μM actin resulted in gelation of the actin filaments at a concentration of 2.4 μM transgelin, with the ball bearing remaining stationary immediately beneath the meniscus (Fig. 2 *a*). In contrast, purified actin filaments at a concentration of 9.3 μM showed that in this system the ball bearing fell at an average rate of ~ 30 s/cm after an initial lag phase of at least 1 min (Fig. 2 *b*), and gelation was never observed (Fig. 2 *b*). In contrast, the addition of transgelin to actin caused their gelation within 2 min (Fig. 2 *b*), but here too, there was an initial lag phase of at least 1 min during which time the rise of apparent viscosity of actin \pm transgelin was essentially the same (Fig. 2 *b*). Pre-incubation of transgelin with G actin for 30 min before the addition of polymerization buffer did not affect these findings, neither did the presence or absence of calcium, nor variations in pH between 5 and 10 (not shown).

However, increased ionic strength completely and reproducibly inhibited the gelating activity of transgelin. A series of viscometric experiments showed that the addition of 10 mM KCl to buffer P blocked the gelating activity of transgelin and the addition of 30 mM KCl to Buffer P reduced the apparent viscosity of transgelin and actin to that of actin alone (Fig. 2 *c*). The addition of 1 mM Na tripolyphosphate to buffer P similarly almost totally inhibited the actin gelation induced by transgelin (Fig. 2 *c*).

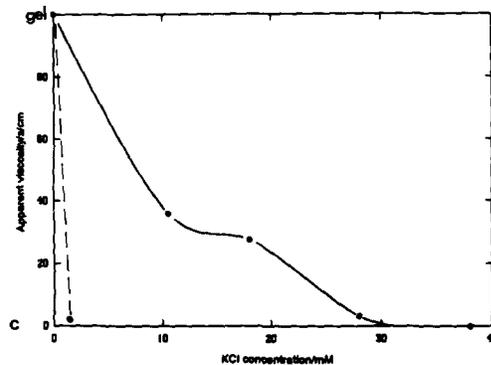
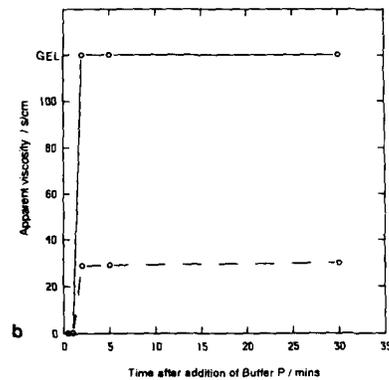
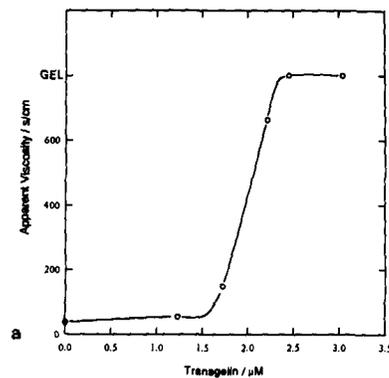


Figure 2. (a) Effect of transgelin on actin viscosity. G actin (9.3 μM) was incubated with varying concentrations of transgelin in buffer P. Viscosity, which is proportional to the velocity of the ball bearing through a capillary tube containing a solution of actin and transgelin, was measured after 30-min incubation. Gelation (defined here as the ball bearing remaining stationary immediately beneath the meniscus) occurred at a concentration of 2.4 μM transgelin. Note the sharp transition from liquid to gel which occurs as a function of transgelin concentration. (b) Time course of actin gelation \pm transgelin. Viscosity was measured as in *a* at various times after the addition of 2.4 μM transgelin to G actin (9.3 μM) in buffer P. After an initial lag phase of 1 min when there was no appreciable increase in viscosity, the viscosity of actin alone (----) increased to 30 s/cm by 2 min, and remained unchanged after 30 min. In the presence of transgelin (—) a similar lag phase of 1 min was seen, but by 2-min gelation of actin filaments had been induced. (c) Ionic strength control of transgelin induced actin gelation. G actin (9.3 μM) was incubated with transgelin (2.4 μM) in buffer P + various concentrations of KCl (—) up to 40 mM. Transgelin-induced actin gelation is inhibited by 50% when 7 mM KCl was added to buffer P and is lost at higher ionic concentrations. The addition of 1 mM $\text{Na}_5\text{P}_3\text{O}_{10}$ (----) to buffer P similarly almost totally inhibits transgelin induced actin gelation. These values are normalized with respect to actin controls.

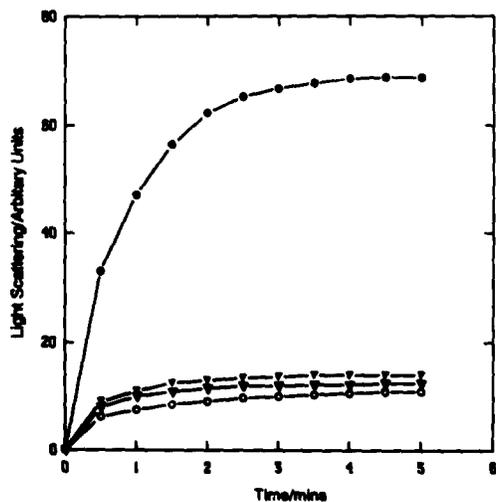


Figure 3. Effect on transgelin on actin filament light scattering. The increase in light scattering when actin ($9.3 \mu\text{M}$) was induced to polymerize by incubation in buffer P + (\circ) or (∇) 100-mM KCl was measured, and compared with actin ($9.3 \mu\text{M}$) + transgelin ($2.4 \mu\text{M}$) in buffer P + (∇) or (\bullet) 100 mM KCl. Note the rapid increase in light scattering when actin and transgelin are incubated in buffer P (\bullet) and the loss of this effect in buffer P + 100 mM KCl (∇).

Light Scattering Assay

When $2.4 \mu\text{M}$ transgelin was added to $9.3 \mu\text{M}$ actin in buffer P a rapid increase in light scattering to 60-fold above control levels occurred within 3–4 min (Fig. 3). This effect was totally abrogated when the reaction was carried out in buffer P + 100 mM KCl (Fig. 3).

EM

Actin filaments, formed by polymerization of G actin for 10 min in buffer P and then examined by negative stain EM, formed a random loose meshwork over the entire grid with no clear areas visible (Fig. 4 a). In contrast, actin polymerized in buffer P for only 1 min, but in the presence of $2.4 \mu\text{M}$ transgelin, was aggregated into large, cross-linked tangles between which large clear areas of grid were now visible (Fig. 4 b). When actin and transgelin were incubated for 10 min, the large tangles seen in Fig. 4 b were now tightly aggregated and cross-linked into discrete foci (Fig. 4 c), in which individual actin filaments could be seen only at the edges (Fig. 4 d).

Sedimentation Assay

Incubation of transgelin at concentrations between 0.6 and $12 \mu\text{M}$ with $9.3 \mu\text{M}$ actin in buffer P followed by ultracentrifugation and analysis of pellets and supernatants by SDS-PAGE revealed that apparent saturation of transgelin binding occurred at a molar ratio of $1.5 \mu\text{M}$ transgelin to $9.3 \mu\text{M}$ actin (Fig. 5), showing that 1 transgelin molecule binds: 6 actin monomers. The apparent binding constant (K_a) of $\sim 7.5 \times 10^5 \text{ M}^{-1}$ was estimated as the inverse of (free transgelin) at which the binding reaches half saturation (Fig. 5) (58).

Sucrose Density Gradients

Linear sucrose gradients (25) showed that transgelin remains as a monomer (banding at an identical position to soy bean trypsin inhibitor $21,500 M_r$) in ionic conditions which either promote (buffer P) (Fig. 6) or prevent (buffer P + 100 mM KCl) (Fig. 6) the actin binding/gelating activity of the molecule. In further control gradients, run in parallel, we

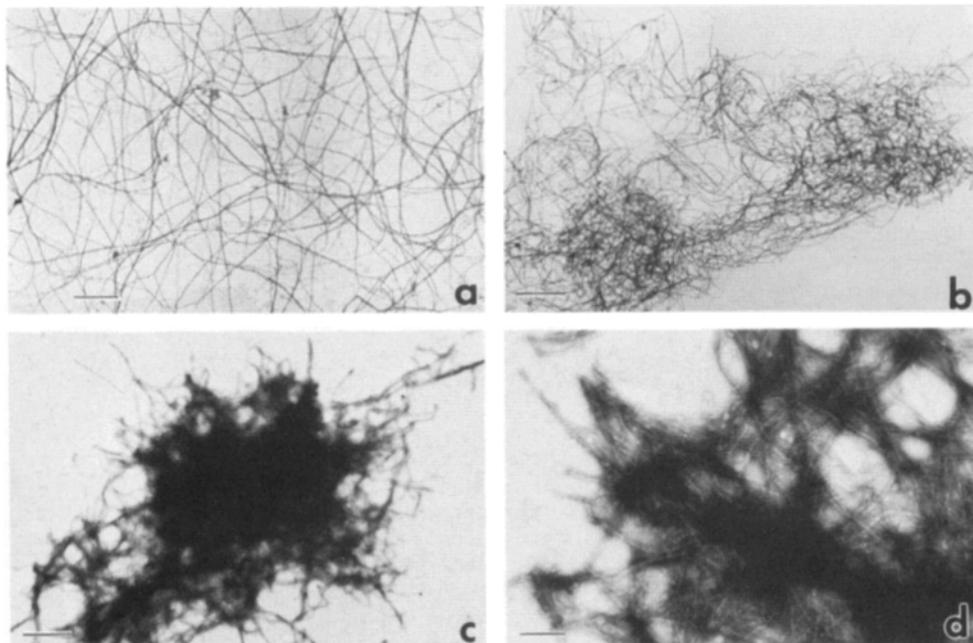


Figure 4. Negative stain EM of actin filaments \pm transgelin. (a) Actin ($0.5 \mu\text{M}$) was polymerized for 10 min in buffer P. Note the essentially random, loose meshwork of filaments covering the grid. (b) Actin ($0.5 \mu\text{M}$) was polymerized for 1 min in buffer P + $2.4 \mu\text{M}$ transgelin. Actin filaments are now tangled and loosely aggregated. Large areas devoid of actin filaments are visible. (c) Actin ($0.5 \mu\text{M}$) was polymerized for 10 min in buffer P + $2.4 \mu\text{M}$ transgelin. The actin filament tangles seen in c are now tightly aggregated into smaller, dense, geodome like foci, in which individual actin filaments are visible at the edges. (d) High power image showing the close meshwork of individual actin filaments which form the foci in Fig. 6 c. Bars: (a and d) $25 \mu\text{m}$; (b) $2.8 \mu\text{m}$; and (c) $0.8 \mu\text{m}$.

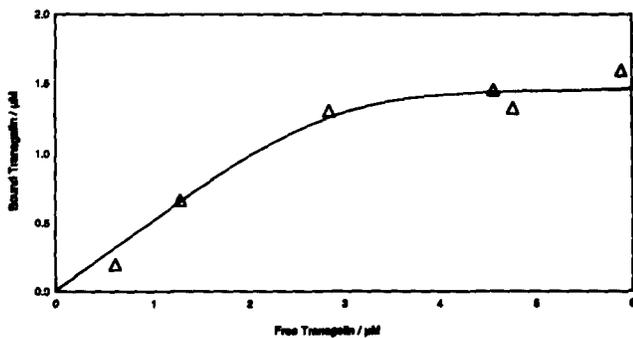


Figure 5. Binding constant of transgelin/actin. G actin ($9.3 \mu\text{M}$) was incubated for 30 min at 25°C with increasing amounts of transgelin in buffer P. After centrifugation at $148,000 g$ for 1 h in a Beckman Airfuge, supernatants and pellets were resuspended in equal volumes of SDS sample buffer and analyzed on 12% SDS-PAGE gels. Protein concentrations were determined by densitometry. The apparent binding constant of transgelin to actin, K_a , was estimated at $7.5 \times 10^5 \text{ M}^{-1}$. Saturation of binding occurred at a molar ratio of 1 transgelin molecule: 6 actin monomers.

loaded ovalbumin, BSA, and transferrin, with molecular masses of 43, 68, and 80 kD, respectively. These distinctly and reproducibly banded at lower points (that is higher sucrose densities) in the gradients. An arrow indicates the position of ovalbumin in Fig. 6.

Amino Acid Sequence

Automated sequence analysis of purified transgelin gave a single amino acid sequence, KGPSYGMSREVV, with a starting signal of 6 pmoles. A search for homologous sequences contained in the Swiss Prot (release 23) and the PIR database (release 34) showed this sequence to be unique. However, a high degree of homology was detected to the amino terminus of the 22-kD avian SM22 α protein, which has the sequence ANKGPAYGMSRDV (33), and calponin with a sequence RGPAYGLSAEV (48).

Immunofluorescence

Detergent Removal of Transgelin. Control cells incubated with anti-transgelin antibody show intense uniform staining on actin stress fiber bundles (Fig. 7 a). Cultures of rat embryo fibroblasts, permeabilized for varying times with 0.5% CHAPS in buffer P + 60 mM KCl revealed that after 1 min in CHAPS most transgelin staining was significantly reduced (Fig. 7 b), and after 10–15-min extraction little detectable transgelin remained in the cell (Fig. 7 c). Identical experiments but using buffer P without additional KCl showed that there was a significant enhancement of transgelin retention in permeabilized cells even after 10-min extraction in CHAPS (Fig. 7 d). This level of transgelin retention remained unchanged when cells were extracted in a volume of $60 \mu\text{l}$ rather than 2 ml (not shown). Actin filament stress fiber bundle integrity appeared to be unaffected by these manipulations (not shown).

Transgelin Rebinding to Permeabilized Cells. When purified transgelin at a concentration of $3 \mu\text{M}$ in buffer P + 60 mM KCl was added to detergent extracted cells from which endogenous transgelin had been removed (Fig. 7 c), very little rebinding was ever seen (Fig. 7 e) compared with control cells (Fig. 7 a). In complete contrast a dramatic and

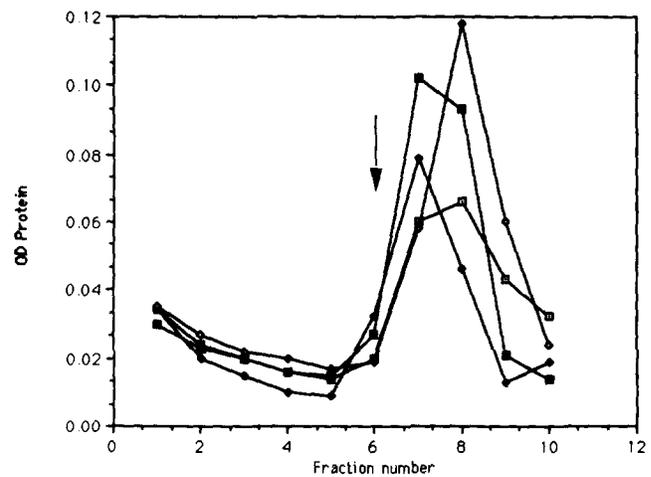


Figure 6. Sucrose density gradient centrifugation of transgelin. Purified transgelin in 12 mM buffer P (■) was layered on to a 5–20% linear sucrose gradient in the same buffer and centrifuged at $150,000 g$ for 43 h at 4°C . Transgelin in buffer P + 100 mM KCl (◇), soy bean trypsin inhibitor in buffer P (◆), and soy bean trypsin inhibitor in buffer P + 100 mM KCl (□) were run in parallel gradients. Transgelin bands at the same level as soy bean trypsin inhibitor, which has an equivalent of 21 kD, in either buffer P or buffer P + 100 mM KCl, indicating that transgelin remains as a monomer regardless of ionic conditions. Arrow marks the position of ovalbumin.

reproducible increase in transgelin rebinding, to levels indistinguishable from control cells, was found when $3 \mu\text{M}$ transgelin was added in buffer P without additional KCl (compare Fig. 7 f with a).

Evolutionary Conservation of Transgelin

Whole fission yeast *S. pombe* (Fig. 8 a), immunoblotted, and probed with affinity-purified polyclonal rabbit anti-transgelin antibody showed that transgelin is present in *S. pombe* (Fig. 8 b), where it has the same molecular mass of 21,000 in SDS-PAGE as transgelin purified from aorta (Fig. 8 c) and immunoblotted (Fig. 8 d).

Discussion

We have previously investigated a molecule of 21 kD which we termed protein C4 and have shown that: (a) it is present as a detergent soluble doublet (C4^{h,l}) associated with actin filaments in all cells and tissues investigated apart from neurons, red blood cells, and skeletal muscle; (b) the higher relative molecular weight polypeptide (C4^h) is absent in both transformed mesenchymal cells where actin stress fiber bundles are reduced in number or absent, and in nonadherent cells such as lymphocytes; and (c) expression of this higher relative molecular weight isoform can also be blocked by switching normal mesenchymal cells from adherent to suspension culture with cyclical reexpression occurring 24 h after these cells are returned to normal adherent culture (41). These observations dictated that we purify this high molecular weight isoform (C4^h) which we now term transgelin and study its function. To achieve these aims, we detergent permeabilized aorta, and in combination with chromatofocusing, were able to rapidly obtain a high yield of purified transgelin in its native state. We excluded possible contamination

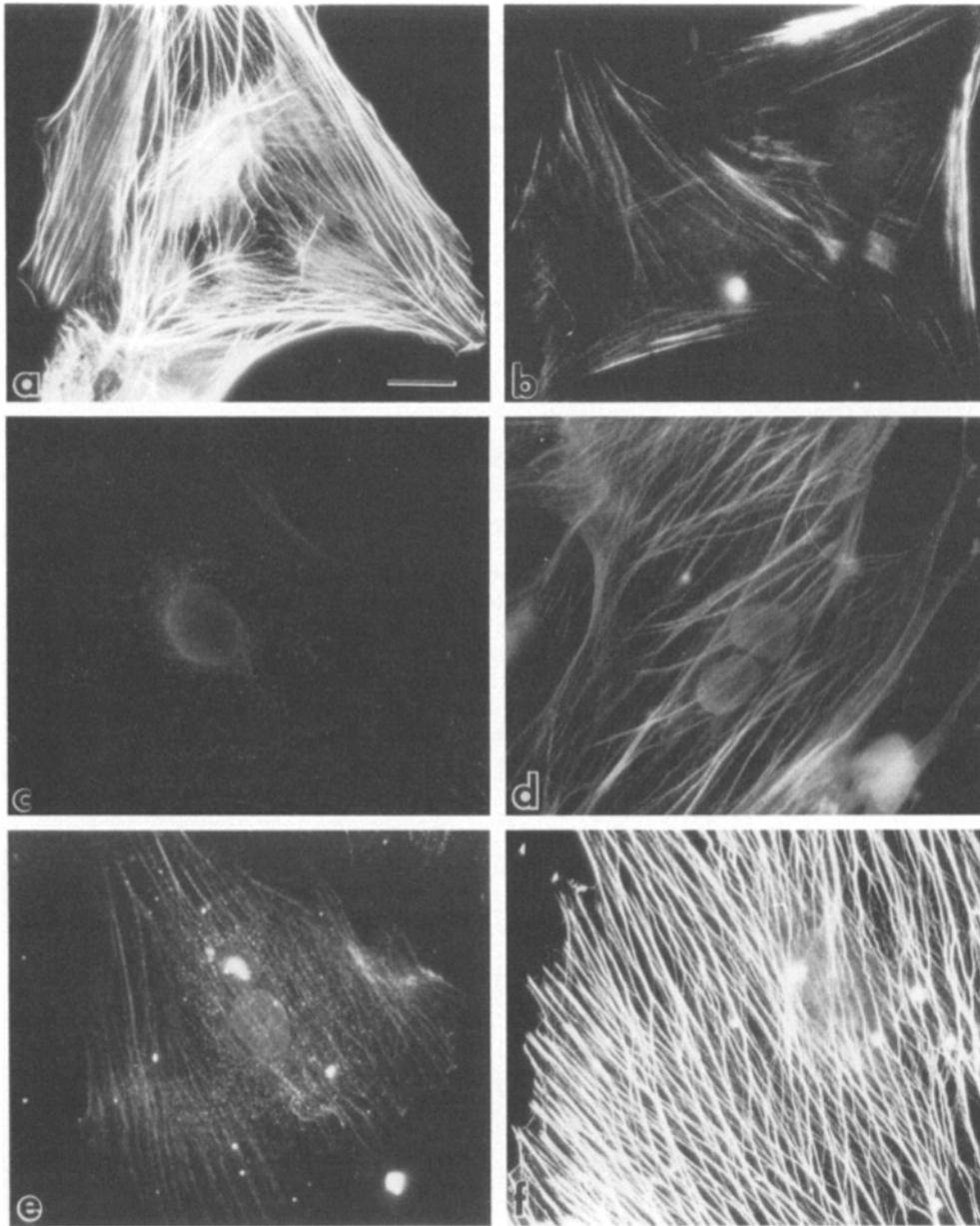


Figure 7. Ionic dependence of transgelin removal and rebinding in detergent-permeabilized REFs. (a) Methanol permeabilized REF showing transgelin's distribution on actin stress fiber bundles. (See also reference 32). All other micrographs were photographed using the exposure measured for these cells. (b) REF permeabilized for 1 min in 0.5% CHAPS in buffer P + 60 mM KCl. Most transgelin has been removed by this time. (c) REF permeabilized as above but for 10 min. Little detectable transgelin staining is now visible. (d) REF permeabilized for 10 min in 0.5% CHAPS in buffer P without KCl. A significant retention of transgelin binding is seen in these cells. (e) REF detergent extracted as in c and then incubated with 3 μ M transgelin in buffer P + 60 mM KCl. Very little transgelin rebinding is ever seen in these cells. (f) REF permeabilized as in c and then incubated with 3 μ M transgelin in buffer P alone. The amount of transgelin binding is indistinguishable from the control cell in a. Note also that in these cells rebound transgelin is found only on actin stress fiber bundles. Bar, 20 μ m.

in our preparation of transgelin with the lower, more acidic, relative molecular weight isoform which we have previously defined (41) by (a) using chromatofocusing—the two isoforms have different pIs (41)—and (b) purifying transgelin

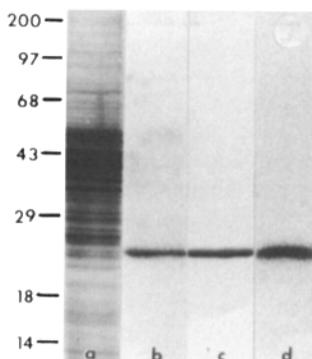


Figure 8. Evolutionary conservation of transgelin. (a) Coomassie blue-stained track of total *S. pombe*. (b) Immunoblot of lane a labeled with polyclonal anti-transgelin antibody showing one band only running in parallel with purified transgelin (in lanes c and d). (c) Coomassie blue-stained lane of purified transgelin. (d) Immunoblot of lane c labeled as in lane b.

from aorta, a tissue where it is the major isoform present (41, and this study).

Our results using viscometric analysis shows that transgelin interacts directly with actin filaments and causes their rapid gelation within 2 min. This finding was unaltered by preincubation of transgelin with G actin. Neither variations in pH between 5 and 10, nor the presence or absence of Ca^{2+} had any effect on these results; a finding reinforced (in the latter case) by the use of $^{45}\text{Ca}^{2+}$ (not shown). The rapid increase in viscosity induced by transgelin in these experiments was not due to either nucleating activity or enhanced rate of monomer addition to pre-existing actin filaments, as measured by the incorporation of pyrene-labeled actin monomers (not shown).

We found that the activity of transgelin was controlled by ionic strength. The ability of transgelin to gel actin was totally abolished when KCl was added to buffer P. Furthermore, this inhibition is not due to chloride ions, since eleva-

tion of ionic strength by K phosphate had the same effect. Similar results have been found for the 21-kD NH₂-terminal fragment of myosin (28). Our immunofluorescence rebinding experiments (discussed below) exclude the possibility that these results are due to bound yet functionally inactive transgelin in these ionic conditions, in favor of the alternative, namely an absence of binding. While we are aware that the ionic conditions which favor transgelin-induced actin gelation are not those found in the cell, (and at present have no explanation for this apparent discrepancy) there is evidence that some actin-associated proteins bind to actin in vivo (32) and yet are unable to gel/bundle actin in vitro in ionic conditions which more closely resemble those inside cells (2, 6). Either the co-operative nature of actin associated protein interactions (50) or the possibility that transgelin is part of a regulatory system that is highly sensitive to ionic conditions may well explain this caveat.

Since our preliminary analysis of cDNA clones coding for transgelin revealed that the transgelin molecule contains a cluster of positively charged amino acid residues, we further investigated the nature of transgelin's interaction with actin by blocking these sites with a polyanion, sodium tripolyphosphate. Falling ball viscometry clearly showed that the addition of 1 mM sodium tripolyphosphate not only totally abrogated actin gelation induced by transgelin but reduced the viscosity of actin + transgelin to control levels. These data strongly suggest that either the actin binding site or the functional "gelation site" (see below) of transgelin is associated with these amino acid residues. This interaction is therefore likely to be essentially electrostatic in nature with the effect of the polyphosphate based on the competition of these polyanions with actin for this cluster of positive amino acids. Noncoulombic forces are probably less involved here (28, 49). Similar results have been found for the 21-kD NH₂-terminal fragment of myosin (28).

A major possibility arising from these studies was that the gelling activity of transgelin was controlled via the formation of dimers or oligomers at low ionic strength. We have excluded this by the use of linear sucrose gradients which show that transgelin remains as a monomer irrespective of these ionic conditions. To define unequivocally whether or not there are two functional sites involved in transgelin activity—one actin binding and another gelating demands the use of peptides against specific regions of the molecule and we are currently investigating these possibilities.

Our viscometric and light scattering assays strongly suggested either a bundling (57) or cross-linking role (13) for transgelin in the formation of the actin gels seen in these studies. To extend and clarify these observations we used electron microscopy which clearly showed that transgelin rapidly induced the formation of an actin filament meshwork (a characteristic and well-described feature of actin gels formed by cross-linking proteins) (8) well within the time required for the rapid increases in viscosity seen in our viscometric and light scattering assays. There was no evidence of actin bundle formation by transgelin. Although most actin cross-linking proteins are large molecules (4, 10, 27, 53), there is at least one other protein of similar molecular mass to transgelin (26) which is known to function as an actin cross-linker. Furthermore, it is well documented that the sharp transition point from a viscous liquid to a gel is a major feature of many actin cross-linkers as they induce a gelling

actin polymer network (36) and this is also a major characteristic of the actin gelation induced by transgelin.

Apparent saturation of transgelin binding at a concentration of 1 transgelin molecule: 6 actin monomers is in line with our previous observations (41). The concentration of transgelin (an average of 2.4 μ M) required to gel 9.3 μ M actin is slightly more than the apparent saturation concentration (1.5 μ M) which we found in our experiments. While this may well be due to actin induced self association of transgelin, the level of saturation is less than that of an actin bundling protein found in *Limulus* (46) and similar to dematin (12). The interaction of transgelin with skeletal muscle actin is not surprising, even although it is clearly absent from this tissue in vivo (41), given the high degree of conservation between actin isoforms in different tissues and widely divergent species (15, 34, 35, 44, 56).

Since both our sedimentation assay data, which shows that transgelin binding is saturable, and our viscometric assay, which defines the gelling activity of the molecule, strongly suggested that our purification protocol did not denature the protein we decided to develop an in vivo rebinding assay which, in addition to visually investigating the rebinding ability of transgelin, would also give us positional information and allow us to investigate whether or not the loss of gelation in elevated KCl discussed above was due to either functional inactivation or loss of transgelin binding. To achieve these aims we used cultures of rat embryo fibroblasts which we have previously shown contain large bundles of actin stress fibers (19, 41). Coverslips of such cells, from which most, if not all, endogenous transgelin had been removed by detergent extraction, were then incubated in different ionic conditions with purified transgelin at various concentrations, and examined by immunofluorescence. These experiments reinforced our biochemical and functional data and show that transgelin rebound specifically to F actin filaments in a distribution and at a level indistinguishable from control cells.

While our preliminary sequence analysis suggests that calponin is partially homologous to transgelin (*a*) calponin has an extra seven amino terminal residues and four different residues within the region of overlap (48), (*b*) calponin and transgelin have very different molecular weights and pIs (41, 52), (*c*) calponin binds calcium (52) while transgelin does not, and (*d*) these proteins have different functions (52, and this manuscript). However, preliminary sequence analysis shows that transgelin has a high degree of homology with the amino terminus of the 22 kD avian SM22 α protein (30, 33), which is found mainly in smooth muscle and has a molecular weight and pI similar to transgelin (22, 23). Similar homology was also found with WS3-10, a protein present in normal and senescent fibroblasts which may well represent the human homologue of SM22 α (45), and p27, a protein thought to be fibroblast specific (1, 38). In no instance was any function ascribed to these proteins (1, 22, 23, 30, 33, 38, 45).

The inherent conservation of cytoskeletal proteins (4, 27, 39) (and mentioned above) led us to investigate the evolutionary conservation of transgelin. Our previous studies using a mAb had shown that the molecule was present at least as far back in evolution as *molluscs* and *crustacea* (41). However, since a mAb only recognizes a limited number of epitopes (7, 54) we raised and affinity purified a polyclonal antibody to transgelin. This extended our previous observations and

showed that (a) transgelin is present in yeast and (b) the molecule has the same apparent molecular weight in SDS-PAGE. These preliminary experiments, which we are at present pursuing at the level of the gene, suggest that transgelin has been conserved since the evolution of multicellular eukaryotes from single celled *Protista* over the last 2,000 million years.

The *in vitro* gelating/cross-linking role played by transgelin, and its *in vivo* distribution on actin filaments strongly suggests that this molecule plays an important *in vivo* role in cytoskeletal organization in normal tissue-associated cells. This may well be reflected by the down regulation of transgelin expression in oncogenically transformed cells (41) where cytoskeletal activation, reorganization and abnormal migrations (51) are an important feature of metastasis.

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