Gelsolin is a downstream effector of rac for fibroblast motility

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Rac, a member of the rho family of GTPases, when activated transmits signals leading to actin-based membrane ruffling in fibroblasts. Compared with wild-type fibroblasts, gelsolin null (Gsn–) dermal fibroblasts have a markedly reduced ruffling response to serum or EGF stimulation, which signal through rac. Bradykinin-induced filopodial formation, attributable to activation of cdc42, is similar in both cell types. Wild-type fibroblasts exhibit typical lamellipodial extension during translational locomotion, whereas Gsn– cells move 50% slower using structures resembling filopodia. Multiple Gsn– tissues as well as Gsn– fibroblasts over-express rac, but not cdc42 or rho, 5-fold. Re-expression of gelsolin in Gsn– fibroblasts by stable transfection or adenovirus reverts the ruffling response, translational motility and rac expression to normal. Rac migrates to the cell membrane following EGF stimulation in both cell types. Gelsolin is an essential effector of rac-mediated actin dynamics, acting downstream of rac recruitment to the membrane.

Keywords: actin/gelsolin/rac/motility/ruffling

Introduction

The recent convergence of two research programs promises to advance our understanding of the cell surface movements involved in cell motility and shape changes. One of these fields concerns cellular actin assembly, fundamental to nearly all cell surface movements, and its regulation by actin-binding proteins. The other encompasses the spectrum of GTPases involved in cell signal transduction. GTPases, especially the rac, rho and cdc42 members of the ras superfamily and their numerous binding partners, appear to contribute importantly to the way in which particular surface stimuli elicit specific types of actin restructuring phenomena that lead to specific cell protrusions (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; Tapon and Hall, 1997). Since actin is the structural element that establishes and maintains the architecture of surface protrusions, the factors directly regulating actin assembly and the three-dimensional configurations of assembled actin most likely operate downstream of the GTPases. Although numerous proteins have been identified which interact with rho GTPases (for reviews see Lamarche and Hall, 1994; Cerione and Zheng, 1996; Macara et al., 1996; Tapon and Hall, 1997), the challenge is to better establish the connections between GTPases and actin assembly.

The GTPase which appears most likely to control cell surface protrusive activity associated with translational locomotion is rac (Ridley et al., 1992). Overexpression of constitutively active rac promotes the formation of ruffling lamellae, which classically appear at the leading edge of crawling fibroblasts. In contrast, constitutively active cdc42 elicits extension of hairlike filopodia (Kozma et al., 1995; Nobes and Hall, 1995), but these structures can progress to lamellae and appear early during translocation motility in many cell types. Constitutively active rho causes formation of adhesion plaques and bundles of actin filaments (stress fibers) (Ridley and Hall, 1992), but since the abundance and size of these structures correlate negatively with cell locomotion (Herman et al., 1981), rho seems more likely to be involved in adhesion than locomotive responses to external stimulation.

Actin-regulating proteins that cap the fast growing ends of filaments are potentially downstream of rac in the pathway to actin assembly and locomotion (Schafer and Cooper, 1995; Barkalow et al., 1996). One founding member of the family of actin capping proteins is the ubiquitously expressed protein gelsolin (Yin and Stossel, 1979; Kwiatkowski et al., 1988). Gelsolin, activated by calcium ions and/or protons, severs the noncovalent bonds between actin subunits in a filament, thereby promoting rapid actin filament shortening (Yin and Stossel, 1979; Yin et al., 1981). After severing, gelsolin binds avidly to the fast-growing (barbed) ends of actin filaments, preventing filament elongation. Activated gelsolin can also nucleate actin filament assembly from monomers, creating filaments capped at the barbed end and growing at the pointed end. D3 and D4 phosphoinositides (PPI) remove gelsolin from actin filament barbed ends, permitting filament elongation (Jannney and Stossell, 1987; Hartwig et al., 1995). These in vitro observations provide a rationale for the importance of gelsolin in actin dynamics in vivo.

To explore gelsolin’s in vivo role, we recently generated mice null for gelsolin by gene knockout techniques (Witke et al., 1995). Analysis of the mice demonstrated the importance of gelsolin in the dynamic actin responses of blood platelets and leukocytes, although the mice had normal development and viability. In addition, although tissue architecture appeared normal in the mice, their cultured fibroblasts had large actin stress fiber bundles and reduced motility in Boyden chamber and tissue culture wounding models.

In this paper we describe results that point to a close relationship between rac and gelsolin in the control of actin assembly and locomotion. All cells and tissues that
normally express gelsolin have a compensatory over-expression of rac in Gsn- animals. Despite rac overexpression, Gsn- fibroblasts have markedly depressed ruffling activity and motility, although EGF stimulation translocates rac from the perikaryon to the cell periphery in normal fashion. Re-expression of gelsolin in these cells normalizes motility and rac expression, confirming that the lack of gelsolin leads directly to the overexpression of rac. These findings establish that gelsolin is a downstream effector of rac for motility, and that absence of gelsolin influences rac expression.

Results

Motility in Gsn- dermal fibroblasts
We have previously demonstrated that tissue culture wound healing and Boyden chamber transmigration were significantly impaired in Gsn- adult dermal fibroblasts in comparison with wild-type (WT) controls of similar origin (Witke et al., 1995). Since membrane ruffling is a process closely linked to cell crawling activity and can be measured quantitatively using assays of macropinocytosis, we investigated macropinocytosis in these cells using the tracking compounds FITC-dextran and HRP-peroxidase (Table I). Unstimulated uptake of FITC-dextran from the media was markedly reduced in Gsn- fibroblasts compared with WT cells and the difference between the two cell types was accentuated by application of EGF or PDGF. Similar differences in stimulated uptake were seen when HRP-peroxidase was used as the tracking compound (Table I). Similar qualitative observations were made for uptake of Lucifer Yellow.

To investigate the motility of these cells in greater detail, videomicroscopy was used. Figure 1 provides a representative panel of frames of Gsn- and WT cells, which were initially serum-starved and then stimulated with EGF and followed for 15 min. Several regions of ruffling activity can be seen in the WT cell over the 15 min interval (large arrows). In contrast, the Gsn- cell is larger and flatter and displays no ruffling activity in any region. Motility differences could also be appreciated in co-culture experiments (Figure 2). When Gsn- and WT fibroblasts were cultured on the same coverslip, serum-starved and then stimulated with EGF, ruffles containing gelsolin in the WT cells could be appreciated, whereas the Gsn- cells had no ruffles.

Since the Gsn- cells had previously been shown to move at about half the rate of WT cells in a tissue culture wounding model, we monitored these cells by videomicroscopy after wounding to assess their translocalional motility. Linear wounds were made in a subconfluent dish of cultured cells and the cells were monitored in the constant presence of serum over 12 h (Figure 3). WT cells translocated through a process involving regions of peripheral ruffling, followed by a broad lamellar protrusion and attachment to the substratum, followed by migration of the nucleus and retraction of the tail of the cell. The Gsn- cells moved much less in general than the WT cells and those which did move proceeded through a different progression. Cell protrusions occurred without ruffling activity and were much narrower in shape, though larger than classic filopodia or microspikes. Following extension of these narrow protrusions there was a broadening of the protrusion with attachment to substratum and forward movement of the cell body with delayed retraction of the tail. Measurement of nuclear translocation in these video images confirmed a reduced rate of movement in the Gsn- cells compared with WT. WT cells moved at 0.902 ± 0.164 µm/s; Gsn- cells moved at 0.514 ± 0.171 µm/s (P<0.01, 10 cells each tracked).

Rac expression in Gsn- dermal fibroblasts and animals: recovery with gelsolin transfection
Since the rac GTPase is implicated as a critical signalling intermediate in cells undergoing ruffling, we investigated expression of rac in Gsn- cells. Monoclonal antibodies against rac were prepared through the use of the recombinant his-tagged protein as immunogen in mice. Several hybridomas secreting antibodies reactive with the immunogen were identified, and monoclonal 7G3 was used for all further studies presented here. Specificity of this monoclonal antibody was demonstrated by immunoblot and ELISA binding to bacterially expressed rac, and lack of binding to similarly expressed rhoA or cdc42a, as well as its identification of a single 21 kDa band in immunoblot analysis of human and murine tissues.

Immunoblot analysis demonstrated that rac was overexpressed by ~5-fold in cultured Gsn- dermal fibroblasts and aortic smooth muscle cells, in comparison with WT cells (Figure 4A, left). To confirm that this observation did not occur as an artifact related to culturing of the cells, analyses of murine tissue extracts were performed (Figure 4A, right). Ten Gsn- and WT tissues demonstrated a consistent difference in the expression of rac, again with an ~5-fold increase in the Gsn- tissues. Rac expression was variable from tissue to tissue in WT animals, as expected. A mild increase in rac expression in Gsn- liver was also seen, even though gelsolin expression was below the detection limit in this particular immunoblot. We examined the livers of these animals by immunohistochemistry, using antibodies against gelsolin and rac. We observed expression of both proteins predominantly in the bile duct and arterial vascular cells, with increased rac expression in these cells in the Gsn- liver (data not shown).

<table>
<thead>
<tr>
<th>Cell</th>
<th>FITC-dextran control</th>
<th>+EGF</th>
<th>+PDGF</th>
<th>Horseradish peroxidase control</th>
<th>+EGF</th>
<th>+insulin</th>
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<tr>
<td>WT</td>
<td>7.0 ± 2.0</td>
<td>11.0 ± 2.4</td>
<td>10.9 ± 1.0</td>
<td>0.29 ± 0.15</td>
<td>1.31 ± 0.60</td>
<td>1.01 ± 0.57</td>
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<tr>
<td>Gsn-</td>
<td>2.2 ± 0.5</td>
<td>2.8 ± 1.1</td>
<td>3.0 ± 1.0</td>
<td>0.22 ± 0.12</td>
<td>0.52 ± 0.20</td>
<td>0.27 ± 0.15</td>
</tr>
<tr>
<td>G1</td>
<td>7.6 ± 1.9</td>
<td>12.7 ± 2.0</td>
<td>11.5 ± 2.2</td>
<td>0.15</td>
<td>1.31 ± 0.60</td>
<td>1.01 ± 0.57</td>
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Table I. FITC-Dextran and horseradish peroxidase uptake by Gsn-, WT and revertant (G1) dermal fibroblasts. Absorbance per ml of extract is shown.
To establish a direct connection between gelsolin expression and increased rac expression in the dermal fibroblasts, a gelsolin expression vector was transfected into the Gsn– cells and stable transfectant lines obtained. As shown in Figure 2D, gelsolin expression was restored to levels similar to those seen in WT fibroblasts. Notably, rac expression reverted to normal levels in the gelsolin-transfected cell lines. Analysis of these revertant Gsn+ lines showed that they were now smaller in volume and spread size, comparable with WT fibroblast cells, and that they displayed increased motility (Figure 4D) in a tissue culture wounding assay. The wound closure rate of the revertant gelsolin-expressing cells surpassed that of the WT cells in some cases, and roughly correlated with the level of gelsolin expression, similar to what we have previously seen in NIH-3T3 cells (Cunningham et al., 1991). The lack of a perfect correlation between gelsolin expression levels and motility in these sublines is due, we suspect, to spontaneous drift in the motility phenotype of these clonal cell populations, reflecting their extended passage.

To confirm a causal connection between lack of gelsolin and increased rac and motility, we also re-introduced gelsolin expression into Gsn– fibroblasts through the use of a recombinant gelsolin-expressing adenovirus. As shown in Figure 1 (right), gelsolin-expressing, adenovirus-infected Gsn– cells displayed ruffling activity in response to EGF after serum starvation that was similar to that seen in WT fibroblasts. In contrast, control adenovirus-infected cells showed no increase in ruffling activity under similar stimulation (data not shown). Concomitant with the recovery of gelsolin expression, rac expression also declined to normal in the gelsolin-expressing, adenovirus-infected cells (Figure 4C).

**Bradykinin signalling in Gsn– cells: cdc42 and rhoA expression**

To examine signalling through cdc42, we stimulated serum-starved cells with bradykinin, and examined filopodial formation by videomicroscopy (Figure 5). In both Gsn– and WT cells filopodia appeared, but at lower levels
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than reported in Swiss 3T3 cells (Kozma et al., 1995; Nobes and Hall, 1995) probably reflecting differences in strain and tissue of origin. The numbers of filopodia did not differ significantly between the WT and Gsn− cells (0.29 ± 0.13 per cell versus 0.21 ± 0.07 per cell respectively). In the Gsn− cells filopodia did not progress to ruffling activity, consistent with the lack of response to EGF and PDGF seen above. In the WT cells progression of filopodial to ruffling activity could be seen in most cells, although the ruffling activity was not as striking as was seen following stimulation with PDGF or EGF. Expression levels of cdc42 and rhoA in Gsn− and WT fibroblasts were indistinguishable (Figure 4B).

**Signalling through rac in the Gsn− dermal fibroblasts**

To explore the mechanism of arrest of cell motility in dermal fibroblasts we examined the in situ distribution of rac and the kinetics of actin nucleation in WT and Gsn− cells. Antibody staining with the anti-rac antibody demonstrated that in resting, serum-deprived cells, rac was seen in both WT and Gsn− cells in a perinuclear location (Figure 6, top level). In response to EGF stimulation (Figure 6, second and third levels), in both cells there was migration of rac to the cell periphery, with focal concentration in WT cells in regions that were undergoing ruffle formation. Rac was also clearly overexpressed in the Gsn− cells in comparison with WT cells. Therefore, recruitment of rac to the cell membrane occurs in both cell types appropriately but downstream events are impeded in the Gsn− cells. Treatment of both cell types with wortmannin (Arcaro and Wymann, 1993) prior to EGF stimulation blocked the movement of rac to the cell membrane in both cell types (Figure 6, bottom level). This observation suggests that PI 3-kinase is involved in rac translocation, and that again both the Gsn− and WT cells could perform this event, implicating gelsolin’s effect as downstream to these early events in rac-mediated signalling for motility.

To examine actin dynamics in these two cell populations in response to EGF stimulation, quiescent cells in suspension were treated with EGF and actin nucleation sites were measured in a pyrene-actin fluorimetric assay (Hartwig et al., 1995). As shown in Figure 7, the actin monomer assembly rate in unstimulated Gsn− cells was reduced compared with WT cells and showed no change following EGF stimulation. The actin monomer assembly rate in unstimulated WT cells was higher, and rose 3-fold 5 min after treatment with EGF. Assembly in the WT cells was completely inhibited by the addition of cytochalasin B to the assay, demonstrating that actin monomers were being assembled at the barbed end of actin nuclei only. Both Gsn− and WT cells responded to treatment with bradykinin during a 10 min interval with a ~3-fold increase in actin monomer assembly rate.

**Discussion**

We have provided evidence that gelsolin is a necessary downstream protein molecule (‘effector’) required for motility signalling through rac in adult murine dermal fibroblasts. First, there is a decrease in motile activity in the Gsn− cells compared with WT as measured by several motility assays. Secondly, this defect is repaired by expression of gelsolin at normal levels in the Gsn− cells. Thirdly, rac expression is increased in the Gsn− cells, and despite this overexpression, motility is still reduced; overexpression is reverted by expression of gelsolin in the same cells. Fourthly, rac overexpression is also seen in multiple tissues derived from the adult Gsn− mouse. Finally, Gsn− cells fail to respond to EGF by exposure of actin barbed
end nuclei. These actin polymerization sites therefore drive actin assembly in ruffles. The results indicate that lack of gelsolin results in a pronounced defect in rac-mediated motility in these cells. Rac overexpression occurs, apparently in compensation for the reduction in motility, but is incompletely effective in relieving the motility defect. High level rac expression may account for the ability of the Gsn⁻ cells to translocate by extension of blunt filopodia (Figure 3). ‘Run-off’ stimulation of rho, also downstream of rac, could partially explain the abundant actin stress fibers present in the Gsn⁻ cells, although in quiescent Gsn⁻ cells we saw no evidence of constitutive rac activation as indicated by rac translocation to the membrane.

Actin polymerization is a necessary component of most forms of cell movement involving the leading edge of the cell, including protrusive activity and ruffling (Condeelis, 1994). The process of cell crawling or translocation involves other cellular activities in addition to actin polymerization, including adhesion events, force generation, release of adhesion complexes and retraction in the posterior portion of the cell (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Actin polymerization in vivo appears to be a complex process, involving associated proteins with at least three potential activities: (i) creation of an actin nucleation complex or release of a barbed end of a pre-existing filament, both to serve as sites for rapid addition of actin monomers; (ii) provision

Fig. 3. Videomicroscopy analysis of Gsn⁻ and WT fibroblast response to tissue culture wounding. Frames collected at 4 h intervals after creation of a wound in a subconfluent population are shown. WT fibroblasts (left) demonstrate both more translocation and a broad leading edge (arrowheads). Gsn⁻ fibroblasts (right) demonstrate less translocation and narrow regions of protrusion (arrowheads) that gradually broaden.
of actin monomers charged with ATP for incorporation in filaments; and (iii) F-actin binding ability to organize actin polymers into three dimensional arrays as required for the particular type of cell process being generated.

The source of actin filament nucleation sites or barbed ends for actin assembly into filaments has been widely debated (Lauffenburger and Horwitz, 1996; Welch et al., 1997) and is difficult to examine in vivo. In platelets there is good evidence for a critical role for gelsolin. Gelsolin severs actin filaments during an initial Ca$^{2+}$ flux in platelets and then is released by a rac-mediated rise in PPIs of the D4 type. This creates the actin nucleating sites that appear during the shape change reaction (Hartwig et al., 1995). The increase in D4-containing PPIs also blocks the binding of other PPI sensitive barbed end binding proteins (capping protein, capG) during this process (Schafer and Cooper, 1995; Barkalow et al., 1996).

Our observations suggest that gelsolin also mediates the formation of sites for rapid actin polymerization during cell protrusive and ruffling activity in murine dermal fibroblasts. The formation of actin microspikes and actin polymerization into filaments; and (iii) F-actin binding ability to organize actin polymers into three dimensional arrays as required for the particular type of cell process being generated.

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Our observations suggest that gelsolin also mediates the formation of sites for rapid actin polymerization during cell protrusive and ruffling activity in murine dermal fibroblasts. The formation of actin microspikes and actin assembly observed in Gsn$^{-}$ cells following stimulation with bradykinin indicates that cdc42-mediated signalling follows a different pathway, not involving gelsolin. It also demonstrates that other critical components for actin assembly are present and functional in the Gsn$^{-}$ cells, including actin monomers and the family of proteins that modulate assembly of actin monomers into filaments such as profilins, thymosins, cofilin and others (Sun et al., 1995; Carlier et al., 1997). These results are also consistent with recent observations in which gelsolin did not appear necessary for Listeria motility in Xenopus oocytes (Rosenblatt et al., 1997).

Previous studies in neutrophils have demonstrated the translocation of rac from the cytosol to membrane fractions following stimulation (Bokoch et al., 1994), but direct in situ evidence for rac movement has not been obtained. In quiescent fibroblasts, we observed rac in a central, perinuclear location, correlating with the inactive state, and its translocation to the cell periphery to apparent regions of ruffling activity upon stimulation. Wortmannin completely blocked this translocation, indicating that it is dependent on PIP3 generation (Aracaro and Wymann, 1993). The translocation could be a diffusion driven process, following release of racGTP from rhoGDI, were a favorable site for racGTP binding created at sites to become ruffles, but could also be mediated by an adapter protein, such as grb2 for ras (Bourne et al., 1991). The specific association of rac to regions of ruffles, suggests that a specific transport and/or binding mechanism is involved.

Based upon these observations we propose the following model for induction of ruffling and motility in normal WT fibroblasts. EGF interaction with receptor leads to activation of PI 3-kinase (Hawkins et al., 1995; Rameh et al., 1995) which, by some as yet undefined mechanism, leads to activation and recruitment of rac to membrane sites where ruffling and motility are to occur. Activated rac at the membrane recruits a multi-protein complex including PI(4)P-5 kinase (Tolias et al., 1995), and stimulates PI(4,5)P$_2$ synthesis. Calcium ion or proton transients and regulated changes in PI(4,5)P$_2$ levels lead to cycles of activation of gelsolin, correlating with severing and partial dissolution of existing actin filament networks, and inactivation providing actin filament nucleation sites for actin assembly. Our demonstration that both rac and gelsolin are recruited to the sites of ruffling activity (Figures 2 and 6) suggests the possibility of physical interaction between gelsolin and rac with membrane lipids in these regions.

The upregulation of rac in the Gsn$^{-}$ animals and cells indicates that some mechanism exists for regulation of expression of this gene and protein. Presumably regulation of rac expression occurs due to some feedback mechanism that senses the motile capacity or actin organization of the cell. Such regulation is unusual among the major members of the ras and rho families of GTPases and exploration of its mechanism will be of considerable interest. In addition, the putative role of rac in transformation (Qiu et al., 1995) suggests the possibility that the Gsn$^{-}$ animals will be at increased risk of developing malignancies. Thus far, without carcinogenic insult, no increase in tumor formation has been observed in these animals.

**Materials and methods**

**Reagents**

EGF (used at 100 ng/ml) and PDGF (used at 10 ng/ml) were purchased from R&D systems (Minneapolis, MN). Cytochalasin B, bradykinin (used at 100 ng/ml), FITC-labeled dextran, glutathione agarose beads, wortmannin (used at 10 nM), phallacidin, phenylmethylsulfonylfluoride (PMSF), horse radish peroxidase, Lucifer Yellow, and TRITC-labeled phallolidin were from Sigma (St. Louis, MO). His Bond Resin was obtained from Santa Cruz Biotechnology.

**Gsn$^{-}$ mice and cell culture**

The Gsn$^{-}$ trait is maintained in mixed Sv129-BALB/c and Sv129-C57/bl backgrounds. Matched littermates that are Gsn$^{-}$ and WT, derived from Gsn$^{-}$/– matings, are used for all experiments including cell line derivation.
Tissue extracts from the mice were prepared by homogenization in 1% Triton X-100, 50 mM Tris–HCl, pH 7.5, 1 mM PMSF, 0.2 µg/ml aprotinin, 4 mM EDTA. Dermal fibroblasts are cultured as explants from the abdominal and dorsal cutaneous tissue of 1–2 month old mice and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL) supplemented with 10% fetal calf serum. Cultured cells were used for all experiments by culture passage 2, apart from the clonally gelsolin-transfected sublines. Tissue culture motility measurements were made by wounding a subconfluent culture dish with an 18 gauge needle as described previously (Cunningham et al., 1991).

**Videomicroscopy**
Cells were plated onto 25 mm acid washed glass coverslips and serum starved for 16–24 h in CO₂ independent medium without serum. Coverslips were placed on the microscope stage and maintained at 37°C using a TC102 temperature controller and incubation chamber (Medical System Corp., Greenvale, NY). Differential interference contrast (DIC) microscopy was performed on a Zeiss Axiovert 405M inverted microscope with a 40×0.9 numerical aperture plan-neofluor lens. For time-lapse recordings, images were collected using a Hamamatsu C2400 video camera (Photonic Microscopy Inc., Bridgewater, NJ) and recorded on a Panasonic TQ-3038F video recorder.

**Fluorescence microscopy**
Cells grown on coverslips were fixed with 4% paraformaldehyde/PBS for 5 min at room temperature. Coverslips were rinsed in PBS and cells were treated with sodium borohydride (0.5 mg/ml) in PBS for 10 min and blocked with 5% normal goat serum/PBS for 60 min at room temperature. The coverslips were incubated with primary antibodies for 1 h at room temperature. After being washed with PBS five times, the coverslips were incubated with FITC-labeled goat anti-rabbit or anti-mouse antibody for 1 h at room temperature with or without added 100 ng/ml TRITC-phalloidin. The coverslips were rinsed five times with PBS and mounted onto glass slides using Aqua polymount (Polyscience) and viewed on an Olympus BH3 microscope.

**Production and purification of recombinant proteins**
RhoA, rac and cdc42 (G25 isotype) were expressed as glutathione S-transferase and/or six histidine fusion proteins in bacteria. A rhoA cDNA clone was a gift from S.Narumiya, Kyoto University; and a rac cDNA clone was a gift from A.Hall, University College London. A human cdc42 (G25k isoform) cDNA was obtained by PCR amplification of human brain cDNA, and confirmed by sequencing.

**Generation of antibodies**
Polyclonal anti-mouse gelsolin antibodies were prepared by immunizing rabbits with recombinant mouse gelsolin, and were affinity purified prior to use. Monoclonal antibodies (anti-rac and anti-rho) were produced according to standard procedures. In brief, 6-week-old BALB/c mice were immunized with bacterially expressed protein three times with adjuvant at 3 week intervals. Spleen cells were fused with mouse myeloma cell (SP2) and hybridoma clones secreting anti-rac or anti-rho antibodies were selected by ELISA and confirmed by immunoblotting of samples of all three recombinant proteins as well as cellular extracts.

**Transfection methods**
To express gelsolin in dermal fibroblasts, two methods were used. First, we subcloned the murine gelsolin cDNA into the expression vector LKH, electroporated 10⁶ cells and selected stable transfectant cell lines using hygromycin B. Six clonal lines were derived, of which four stably
expressed murine gelsolin. Secondly, the murine gelsolin cDNA was cloned into the pacCMV vector which contains the CMV early promoter to drive transcription and a downstream fragment of the SV40 genome that includes the small t antigen intron and the polyadenylation signals. This gelsolin-containing plasmid and pJM17 were co-transfected into 293 cells. Recombinant adenovirus was isolated, titered and amplified as described (Becker et al., 1994).

**Immunoblotting**
Proteins were SDS–PAGE fractionated and transferred onto immobilon P membrane (Millipore Corporation, Bedford MA). Membranes were incubated in 50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (TBS-T) (pH 8.0) containing 10% (w/v) non-fat dry milk at 4°C overnight followed by a 1 h incubation with an affinity-purified polyclonal (gelsolin, 1 µg/ml; cdc42 1 µg/ml) or monoclonal (rac, 50 ng/ml; rho, 100 ng/ml) antibody diluted in TBS-T, 5% dry milk (TTM). Following three rinses, horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody was diluted in TTM and added to the membranes for 60 min at room temperature. The membranes were then washed five times for 5 min and bound antibodies were detected using enhanced chemiluminescence (ECL).

**Pinocytosis assays**
Cells were plated at three different densities (2×10⁴, 4×10⁴ and 8×10⁴ cells/25 mm dish). The cells were serum-starved overnight in DMEM and incubated in PBS at 37°C for 30 min. FITC-dextran, horse radish
peroxidase, or Lucifer yellow was added (final concentrations 2, 1 and 1 mg/ml, respectively) with or without EGF (10 ng/ml) and incubated at 37°C for 60 min. As a control the same set of cells were incubated at 4°C. After washing with chilled PBS three times, the cells were lysed in 50 mM Tris pH 8.5, 0.1% Triton X-100 for 30 min at room temperature. Fluorescence measurements were made with a Perkin-Elmer LS50B fluorescence spectrophotometer to assess uptake of FITC-dextran (excitation 495 nm, emission 514 nm). Horse radish peroxidase activity was measured by incubation with o-dianisidine at 0.1 mg/ml and quantitation of absorbance at 460 nm. Lucifer yellow uptake was assessed by fluorescence microscopy.

**Measurement of actin filament ends**

The number of exposed actin filament barbed ends was determined by measuring the rate and extent of pyrene-labeled actin polymerization in detergent lysates (Hartwig et al., 1995). Cells were cultured in conventional tissue culture dishes, serum-starved overnight, detached by treatment with 5 mM EDTA, and maintained as a suspension at 10^6 cells/ml at 37°C in DMEM. After stimulation with 10 ng/ml EGF or 100 ng/ml bradykinin, they were permeabilized with 0.1 volume of PHEM buffer (60 mM PipES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl₂, pH 7.4) containing 1% Triton X-100, 1 µM phallacidin, and protease inhibitors (Schiwa et al., 1981). To 100 µl of this detergent lysate, 185 µl of 100 mM KCl, 0.2 mM MgCl₂, 0.1 mM EGTA, 0.5 mM ATP, 10 mM Tris–HCl, 0.5 mM dithiothreitol pH 7.0 were added. Assembly of added pyrene-actin (final concentration 1 µM) into filaments was monitored with a Perkin-Elmer spectrophotometer at excitation and emission wave lengths of 366 and 386 nm, respectively. The data are expressed as actin assembly rate per cell (monomers into filaments/s) as previously described (Hartwig et al., 1995).

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**References**


