Reconstitution and Regulation of Actin Gel-Sol Transformation with Purified Filamin and Villin

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Gel-sol transformation of actin filaments, a process essential for cell motility, can be reconstituted in vitro and regulated in a predictable fashion by the combined action of villin and filamin. Measurements made in a low shear falling ball viscometer show that mixtures of actin, villin, and filamin exist either as a gel (yield point ≥ 140 dynes/cm²) or as a low viscosity liquid depending on the relative ratio of villin:actin. Filamin induces gelation of F-actin by forming stable cross-links between actin filaments. Villin inhibits filamin-induced F-actin gelation, but the effect can be overcome by increasing the amount of filamin. Sedimentation assays show that villin does not inhibit gelation of actin by preventing filamin from binding to F-actin. Results from viscosity measurements and filament length determinations show that villin increases actin filament number by reducing the average filament length without altering the total amount of polymer. Because the gel point of a fixed amount of polymer is sharply dependent on the ratio of cross-links to number of polymers, the solation effect of villin might be explained by its effect on filament number. Based on the network theory of gel formation, calculations of the amount of additional cross-linker required to overcome the effect of a known increase in the number of actin filaments agree reasonably well with experimental findings. These results document the existence of cellular proteins which could regulate gel-sol transformation in vivo by their effect on actin polymer length and, therefore, on actin filament number.

The consistency of cytoplasm in intact cells transforms reversibly from that of a low viscosity liquid (sol) to that of a rigid gel. These consistency changes correlate with alterations in cell shape and motility, and convincing evidence shows that cytoplasmic sol-gel transformations form part of the structural basis for cell movements (recently reviewed by Taylor and Condeelis in Ref. 1). The discovery that naked cytoplasm from various cell types can undergo consistency changes in vitro depending on environmental factors such as pH, Ca²⁺, temperature, and ionic strength (2-5) has enabled identification of the molecular components responsible for production and control of gel-sol transformation. Fractionation and reconstitution studies showed that cytoplasmic gels are formed by F-actin and actin cross-linking proteins such as filamin (6, 7), macrophage actin-binding protein (8, 9), and the 58,000 plus 220,000 dalton proteins from sea urchin eggs (10).

Attention is now focused on identification of molecules which can regulate the development of cytoplasmic rigidity. The network theory of gel formation as put forth by Flory (11) predicts that the gel point of a fixed concentration of polymer will be sharply dependent on the ratio of the number of cross-links to the number of polymers. If cytoplasmic gels can be described by the network theory, as suggested by Hartwig and Stossel (12), then control of cytoplasmic gelation can be achieved by any mechanism which alters either the number of cross-links or the number of actin filaments. Already, two functionally distinct proteins which can regulate the gel point of F-actin have been described. Actinogelin (13) gels F-actin but only in the presence of M³⁺-Ca²⁺. Gelsolin, a protein from rabbit macrophage (14), does not gel F-actin, but confers Ca²⁺ sensitivity on gelation of F-actin by actin-binding protein or by filamin. At Ca²⁺ ≥ 10⁻⁷ M, gelsolin causes solation of actin/filamin or actin/actin-binding protein gels. Yin and Stossel (14) suggest, on the basis of indirect evidence, that gelsolin acts by breaking F-actin filaments thereby altering the critical cross-linker to filament ratio.

Having recently found that villin, a 95,000-dalton protein isolated from chicken intestinal epithelial cell brush borders, shortens the length of F-actin without affecting the total amount of polymer (15), we predicted that villin would regulate the gel point of F-actin/filamin mixtures. In this paper, we show that this prediction is experimentally verified and that the effect of villin on the gel point is directly related to its effect on the number average (and weight average) actin filament length. Therefore, one mechanism for regulation of gel formation can be based on the demonstrated existence of cellular proteins which regulate the length and thus the number of actin filaments formed by a fixed amount of actin.

EXPERIMENTAL PROCEDURES

Preparation of Proteins

Rabbit skeletal muscle actin was prepared from an acetone powder by the procedure of Spudich and Watt (16). Monomeric actin was further purified by gel filtration on Sephadex G-150 to remove trace components which modify actin polymerization (17). Villin (98% homogeneous) was prepared from chicken intestinal epithelial brush borders as described by Craig and Powell (15). Filamin was isolated from chicken gizzard smooth muscle by the method of Shizuta et al. (6) with the modifications described previously (18).

Biochemical Assays

Low Shear Viscometry—Low shear viscometry was performed with a falling ball viscometer as described by MacLean-Fletcher and Pollard (19). This viscometer is useful for measuring the apparent gel-sol transition of actin gels because the apparent viscosity of a solution increases sharply at the gel point (11). A gelled sample is defined as one through which the stainless steel ball does not fall when the capillary tube is held at an angle of 80°; such samples have a rigidity or yield strength ≥ 140 dynes/cm² (19).

High Shear Viscometry—These measurements were made in...
Ostwald capillary viscometers (Type 100 Cannon Instrument Co., State College, PA). Buffer flow times in different viscometers ranged from 53 to 62 s at 28°C. Specific viscosity is defined as flow time of sample solution divided by flow time of the corresponding buffer minus 1.0 (20).

Polyacrylamide Gels

Sodium dodecyl sulfate-polyacrylamide gels were run according to the procedure of Laemmli (21). The resolving gel was 7.5% acrylamide, 0.2% bisacrylamide; the stacking gel contained 3% acrylamide, 0.08% bisacrylamide. Gel slices were stained with Coomassie blue and scanned in a Gilford spectrophotometer at 590 nm.

Protein was measured by the dye-binding assay of Bradford (22) using bovine serum albumin as a standard.

Electron Microscopy of Number Average and Weight Average Length Measurements

Protein solutions were diluted to 0.05 mg/ml in polymerization buffer and applied (60 s) to carbon-shadowed grids which had been made hydrophilic by glow discharge or by pretreatment with 0.2 mg/ml of cytochrome c in 0.1% isomyl alcohol. Excess sample was removed; grids were floated three times in distilled water and then stained (30 s) with 0.75% uranyl formate, pH 4.3. Samples were photographed at 10,000 magnification in a Zeiss EM10A. Prints were made at a final magnification of 26,000. Collages were constructed to give an area large enough (23) for filament measurement (70 μm² for sample and control filaments to 220 μm² for control samples). An average of 950 filaments and no less than 400 filaments were measured for each sample. Number average length (Ln)/Ln, where n. = number of filaments of length, L, was calculated by dividing the total polymer mass in a given area (i.e. total filament length, measured with a linear tracer) by one-half the number of ends present in that area (24). Weight average length was determined by recording lengths of individual filaments and calculating [Ln/Ln]². Filaments shorter than 0.04 μm cannot be detected on the prints; hence they are excluded from analysis.

RESULTS AND DISCUSSION

As noted by others (9, 19), the transition of actin from a sol to a gel in the presence of an actin cross-linking protein such as filamin occurs sharply when the critical concentration of cross-linker is attained (Fig. 1A). At a molar ratio of 1 filament dimer:400 actin monomers, the actin/filamin mixture exists as a liquid with an apparent viscosity only slightly higher than F-actin alone. In contrast, at a molar ratio of 1 filament dimer:225 actin monomers, the mixture forms a gel with a static yield strength great enough to support the weight of a ball. In the presence of villin, the critical concentration of filamin required to gel the same amount of F-actin was found to increase (Fig. 1A). At a molar ratio of 1 villin monomer to 215 actin monomers, nearly a 5-fold increase in filamin concentration was required to gel the actin. Conversely, at fixed concentrations of filamin and actin (molar ratio filamin dimer:actin monomer 1:25 and 1:15) increasing amounts of villin are required to inhibit gelation (Fig. 1B). Thus, the transition from gel to sol state can be regulated by the relative amounts of F-actin, villin, and filamin.

How does villin inhibit gelation? Theoretically, villin could shift the gel point of actin/filamin mixtures by altering either the number of cross-links or the number of actin filaments. Measurement (by quantitative densitometry of Coomassie blue-stained gels) of the amount of filamin sedimented with F-actin in the presence and absence of villin showed that villin has no detectable effect on the binding of filamin to actin (Fig. 2) and, therefore, does not alter the number of cross-linkers. In contrast, a direct effect of villin on actin is detected by monitoring polymerization kinetics of villin/actin mixtures (15). Villin nucleates actin assembly (15) and reduces the steady state equilibrium viscosity of F-actin (Table 1). At the two villin:actin ratios used in the experiment shown in Fig. 1A, the final equilibrium high shear viscosity of F-actin was reduced by 14% (1 villin:360 actin monomers) and 40% (1 villin:215 actin monomers). We (15) have previously shown that the effect of villin on the equilibrium viscosity of F-actin is explained completely by its ability to shorten actin filaments since villin has no effect on the total amount of polymer as determined by the DNase 1 assay for actin monomer.

The effect of villin on F-actin length (and, therefore, filament number) was examined in detail by measuring the number average length (Lm) and the weight average length by DLS to a gel in the presence of an actin cross-linking protein such as filamin occurs sharply when the critical concentration of cross-linker is attained (Fig. 1A). At a molar ratio of 1 filament dimer:400 actin monomers, the actin/filamin mixture exists as a liquid with an apparent viscosity only slightly higher than F-actin alone. In contrast, at a molar ratio of 1 filament dimer:225 actin monomers, the mixture forms a gel with a static yield strength great enough to support the weight of a ball. In the presence of villin, the critical concentration of filamin required to gel the same amount of F-actin was found to increase (Fig. 1A). At a molar ratio of 1 villin monomer to 215 actin monomers, nearly a 5-fold increase in filamin concentration was required to gel the actin. Conversely, at fixed concentrations of filamin and actin (molar ratio filamin dimer:actin monomer 1:25 and 1:15) increasing amounts of villin are required to inhibit gelation (Fig. 1B). Thus, the transition from gel to sol state can be regulated by the relative amounts of F-actin, villin, and filamin.

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![Fig. 1. Low shear falling ball viscometry of actin, villin, and filamin mixtures. A, effect of increasing concentrations of filamin on the apparent viscosity of actin alone (C) or villin plus actin (molar ratio villin:actin = 1:360 (C) or 1:215 (B)). Actin, 9.0 μM; villin, 0.025 μM (C) and 0.04 μM (B). B, effect of increasing concentrations of villin on the apparent viscosity of actin/filamin mixtures. Actin, 7.0 μM; filamin, 0.15 μM (C). Actin, 8.5 μM; filamin, 0.30 μM (B). Calculations of molar concentrations of actin, villin, and filamin in all figures are based on their monomer molecular weights of 43,000, 95,000 and 250,000, respectively. The above experiments were done at 28°C. Buffer conditions were 10 mM Tris-HCl, 0.2 mM ATP, 0.2 mM CaCl₂, 0.5 mM dithiothreitol, pH 7.5. Actin polymerization was initiated by the addition of 2.5 M KCl stock solution to 50 mM. Duplicate samples were incubated for 1.5 h in 100 μl of Van-Lab micropipettes (WVR Scientific Inc., Univar Corp., San Francisco, CA), sealed with seal-seal (Clay Adams, Division of Becton, Dickinson and Co., Parsippany, NJ). Apparent viscosities were measured as described (19).](image-url)

![Fig. 2. Binding of filamin to actin in the presence and absence of villin. Mixtures of the proteins (50 μl total), actin (20 μg), villin (0.3 μg), and filamin (4 μg) in various combinations were polymerized and incubated under the same buffer and temperature conditions described in Fig. 1. After 1.5-h incubation, the samples were spun at 130,000 × g for 1.5 h in a Beckman Airfuge (room temperature). Supernatants were removed, pellets washed once, and wash combined with the supernatants. Pellets were solubilized directly into sodium dodecyl sulfate-polyacrylamide gel sample buffer. Supernatants were trichloroacetic acid-precipitated, then prepared for sodium dodecyl sulfate-polyacrylamide gel. Lanes a-f, supernatants; lanes g-l, corresponding pellets. a, actin alone; b, actin + villin; c, actin + filamin; d, actin + filamin + villin; e, filamin alone; f, villin alone; g, actin alone; h, actin + villin; i,actin + filamin; j, actin + filamin + villin; k, filamin alone; l, villin alone; m, starting mixture with all three proteins (no KC1 added). The positions of filamin, villin, and actin are marked with F, V, and A, respectively. The filamin band in lane e appears lower than in lanes i-m because the two separate slab gels do not precisely align. The villin band is barely visible in lanes b, j, and m because only 0.3 μg of villin was used in the assay.](image-url)
which different preparations of both actin and villin were used. The effect of a given amount of villin on F-actin length is not identical between experiments. However, if the percentage of reduction in F-actin viscosity is plotted as a function of the amount of villin (which normalizes for differences in polymerizability of the actin), the data from all three experiments fall on a common curve (Fig. 4A). Log-log plots of viscosity versus \((L/n)\) show that viscosity is linearly related to the square root of the number average filament length for \((L/n) \geq 0.65 \mu M\). For \((L/n) \leq 0.65 \mu M\), viscosity is a linear function of \(L^{1.4}\) (Fig. 4B). This linear relationship between viscosity and the number average filament length supports our assumption that calculation of changes in filament number based on electron microscopic measurement of changes in average filament length are relatively precise.

It should be noted that Staudinger's equation, \(\eta = KL^n\), is generally true only when the viscosity measurements have been extrapolated to zero protein concentration (intrinsinc viscosity) and to zero shear rate (20). The viscosity versus shear rate profile of polymeric actin is pseudoplastic and, therefore, non-Newtonian (25); as the shear rate approaches zero, viscosity approaches infinity. Thus, extrapolation of viscosity to zero shear rate is impossible. Nevertheless, our experimental results show that the relationship between F-actin viscosity measured in an Ostwald viscometer (which has a finite shear gradient across the radius of the capillary) and the measured number average filament length is described by \(\eta = KL^n\), where \(\eta = \eta_v\), rather than zero shear intrinsic viscosity. Whether the sharp break in the slope of the line (Fig. 4B) actually reflects a change in the flexibility of actin as predicted by theory (\(a = 0.5-1.0\) for random coils; \(a = 2.0\) for rigid rods) (20) will require independent analysis of flexibility.

It has been recently suggested by Hartwig and Stossel (12), that the network theory of gel formation proposed by Flory (11) can be used to calculate the predicted increase in cross-links required to gel a known increase in the number of polymers. Because of the effect of villin on actin filament length and, therefore, on actin filament number, we decided to determine whether the increased amount of filamin required to gel actin in the presence of villin could be predicted by the gel theory. Gelation depends on the mass of material involved, therefore, weight average rather than the number average filament length was used to determine the change in filament number. The weight average filament length for actin alone was 5.6 \(\mu M\) and for actin plus villin (215:1) it was 1.5 \(\mu M\). Histograms of the data used to obtain these average values are shown in Fig. 5. For actin alone, the ratio of weight average length/number average length is 2.0, indicative of an exponential filament length distribution. In the presence of villin, this ratio is 1.5, which suggests that villin may shift the actin filaments to a more homogeneous length distribution. However, the inability to detect filaments shorter than 0.04 \(\mu M\) might also account for the apparent change in the length distribution profile.

The number density of cross-links (PC) required for incipient network formation in a population of arbitrary length distribution (11) is equal to \(1/\bar{Xw}\), where \(\bar{Xw} = \) weight average degree of polymerization. This latter value is obtained by multiplying the weight average length (\(L/w\)) times the known value of 370 actin monomers/\(\mu M\) of F-actin (26). For F-actin alone the value of PC is \(4.8 \times 10^{-4}\). This number times the molar concentration of actin monomer in filaments (7.8 \(\mu M\)), corrected for the critical concentration, yields the theoretical amount of cross-linker (filamin) necessary for gelation, which is \(3.76 \times 10^{-9}\) \(M\). The experimentally determined amount of filamin dimer necessary to gel 7.8 \(\mu M\) actin is \(4 \times 10^{-9}\) \(M\). In the presence of villin (villin:actin, 1:215), PC is \(1.8 \times 10^{-9}\) and the theoretically calculated amount of cross-linker required is \(14 \times 10^{-9}\) \(M\). The actual amount of cross-linker necessary to

\[\text{TABLE I} \]
\text{Effect of villin on specific viscosity and number average length of F-actin}

Conditions of polymerization are described in the legend to Fig. 1. Number average length, \((L/n)\), and weight average length, \((L/w)\), measurements were made from electron micrographs as described under "Experimental Procedures." Protein preparations used in Experiment 2 were used on the same day to obtain the data in Figs. 1 and 2.

| Exp. | Villin \(\mu M\) | \((L/n)\) \(\mu M\) | \((L/w)\) \(\mu M\) | Reduction in \(\eta_v\) % |
|------|----------------|------------------|------------------|-----------------|
| 1    | 0              | 0.439            | 0.5              | 0               |
|      | 0.136          | 0.120            | 0.5              | 73              |
|      | 0.40           | 0.055            | 0.3              | 88              |
| 2    | 0              | 0.43             | 2.8              | 5.6             |
|      | 0.025          | 0.37             | 2.1              | 14              |
|      | 0.04           | 0.26             | 1.0              | 1.5             |
|      | 0.20           | 0.187            | 0.65             | 67              |

![Fig. 3. Electron micrographs of negatively-stained actin filaments in the presence and absence of villin. A, actin at 9.0 \(\mu M\) polymerized under the conditions described in Fig. 1. B, 9.0 \(\mu M\) actin + 0.04 \(\mu M\) villin. C, 9.0 \(\mu M\) actin + 0.09 \(\mu M\) villin. Bar, 1 \(\mu M\).](image)

![Fig. 4. Relationship of the specific viscosity (\(\eta_v\)) of F-actin to the number average filament length (\((L/n)\)) obtained in the presence of different amounts of villin. Data points from the same experiment are indicated by common symbols (\(\circ\), \(\bullet\), \(\square\), \(\bigcirc\)). Polymerization conditions for the three experiments are identical with those in Fig. 1. A, relationship between the concentration of villin and the steady state equilibrium viscosity of F-actin. B, logit plot of equilibrium viscosity versus number average filament length obtained in the presence of different amounts of villin. Slopes \((a)\) were obtained by least squares analysis. The results fit the equation \(\eta = KL^n\) with \((a) = 0.5\) when \((L/n) \geq 0.65 \mu M\); and \((a) = 1.4\) when \((L/n) \leq 0.65 \mu M\).](image)
overcome the effect of villin is $1.9 \times 10^{-7}$ M. Although the absolute difference between the theoretical concentration of filamin and the actual concentration of filamin is 10-13-fold, the relative increase (4.7-fold) in filamin concentration necessary to gel 7.8 $\mu$M actin in the presence of villin is close to the theoretical fold-increase (i.e. 3.7-fold). That the absolute concentration of filamin required for gelation is higher than theoretically predicted might be explained by the probable formation of cyclic structures that do not contribute to network formation (11), and by aggregation of filamin dimer (27).

In conclusion, these preliminary data indicate that the effect of villin on the gel point of actin/filamin mixtures can be partially accounted for by its effect on actin filament number. However, a more precise method for measuring the point of incipient gelation will be needed to rigorously test the conformity of this gel system to the network theory. Recently, two other proteins have been described which are functionally similar to villin. Gelsolin (14) confers Ca$^{2+}$ sensitivity on gelation of actin by filamin or by macrophage actin-binding protein. Indirect evidence suggests that gelsolin acts by restricting the length of actin filaments (14). Fragmin, a 50,000-dalton protein isolated from Physarum (28), exerts Ca$^{2+}$-sensitive control over the length of actin filaments. It is to be expected that fragment will have the same effect on in vitro actin gelation systems as do villin and gelsolin. Thus, a class of regulatory proteins has been defined which may well be important in the control of cytoplasmic rigidity and, therefore, of cell shape and motility.

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Addendum—While this manuscript was being revised, a report by Yin and Stossel ((1980) J. Biol. Chem. 255, 9490-9498) appeared which provides direct evidence that gelsolin acts to shorten F-actin filaments and that this action can partially account for its ability to shift the gel point of F-actin and actin-binding protein mixtures.

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