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Effects of Villin on the Polymerization and Subunit Exchange of Actin

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We have investigated the Ca^{2+}-dependent interactions of villin, a protein of the intestinal microvillar core, with actin by monitoring resonance energy tranfer between fluorescently labeled actin subunits. In the presence of elevated free Ca^{2+} (~ 20 μM), villin affects both the nucleation and the elongation phases of actin polymerization. Consistent with previous reports, villin stimulates the nucleation process and will form stable nuclei under depolymerization conditions. Compared to the control, the net rate of polymerization is slightly inhibited at low concentrations of villin (villin/actin $\sim$ 1:400) but is stimulated at higher concentrations (villin/actin $>$ 1:100). Villin also significantly increases the critical concentration of actin polymerization. Addition of either villin or villin-actin complexes induces depolymerization of preassembled actin filaments. This villin-induced depolymerization is reversible upon removal of free Ca^{2+} or upon the addition of phalloidin. The exchange of actin subunits at steady state is inhibited at low concentrations of villin (villin/actin $\sim$ 1:200) but is stimulated at higher concentrations (villin/actin $\sim$ 1:50). None of the above effects is observed at $< 10^{-8}$ M free [Ca^{2+}].

Key words: actin, villin, fluorescence, energy transfer, polymerization, microfilament

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INTRODUCTION

In nonmuscle cells, actin exhibits amazing flexibility in its participation in both cytoplasmic structure and motility. Alone in test tubes, very few cellular activities can be reproduced with purified actin [for reviews on actin in nonmuscle cells see Taylor and Condeelis, 1979; Korn, 1982]. Presumably, it is the presence of numerous actin-binding proteins that confers to actin its remarkable diversity of functions [for reviews see Korn, 1982; Craig and Pollard, 1982; Weeds, 1982]. Among the most interesting of these proteins is a class of Ca^{2+}-sensitive actin-binding proteins identified in a wide variety of cell types, which regulate filament assembly, filament length, and filament-filament interactions.

One such Ca^{2+}-sensitive actin-binding protein is villin [also referred to as MV-95K or flaccin; Mooseker et al, 1980], a 95K-dalton protein of the actin filament bundle within microvilli of intestinal epithelial cells [Bretscher and Weber, 1979, 1980; Mooseker et al, 1980; Craig and Powell, 1980; Matsudaira and Burgess, 1982]. Although the in vivo functions of villin are not known, the in vitro interactions of this protein with actin have been intensively studied in a number of laboratories [for a review see Craig and Pollard, 1982]. These studies include analyses of the Ca^{2+}-dependent effects of villin on actin assembly, as well as its effects on preformed actin filaments. At free $[Ca^{2+}] < \sim 10^{-6}$ M, this protein has little effect on filament assembly [Mooseker et al, 1980; Glenney, Kaulfus, and Weber, 1981]. At $[Ca^{2+}] > \sim 10^{-6}$ M, villin increases the net rate of polymerization [as monitored by viscometry and UV spectroscopy; Mooseker et al, 1980; Craig and Powell, 1980; Glenney, Kaulfus, and Weber, 1981]. Recently, however, it has been observed that villin actually inhibits the rate of filament elongation by blocking monomer addition onto the fast growing ("barbed") end of actin filaments [Bonder and Mooseker, 1983].

The interaction of villin with preformed actin filaments (F-actin) is also Ca^{2+} dependent. At $< \sim 10^{-6}$ M Ca^{2+}, villin crosslinks F-actin into bundles [Bretscher and Weber, 1980; Mooseker et al, 1980; Matsudaira and Burgess, 1982]. In the presence of $> \sim 10^{-6}$ M Ca^{2+}, addition of villin to solutions of F-actin induces a rapid decrease in viscosity and a reduction in the sedimentability of actin filaments [Mooseker et al, 1980; Craig and Powell, 1980; Glenney, Kaulfus, and Weber, 1981]. The effects on viscosity and sedimendability are primarily owing to the reduction in average filament length, mediated by the disruption ("severing") of monomer-monomer interactions within the filament [Glenney, Kaulfus, and Weber, 1981; Bonder and Mooseker, 1983]. Changes in the extent of actin polymerization, however, have not been detected previously [Craig and Powell, 1980].

We have further investigated the Ca^{2+}-dependent effects of villin on actin using the technique of fluorescence energy transfer. In previous studies, we have used this technique to study the polymerization of actin and the exchange of actin subunits among filaments [Taylor et al, 1981; Wang and Taylor, 1981a]. Since this technique follows the association-dissociation of actin subunits directly, it gives a resolution much higher than that of viscometry or centrifugation. In addition, the technique involves very little mechanical perturbation to the solution during measurements, thereby minimizing possible artifacts caused by shear-induced filament breakage. In this study, we use energy transfer to investigate the effects of villin on the rate and the extent of actin polymerization. The results are compared to those of cytochalasin B. In addition, subunit exchange in the presence of villin is studied using a previously published protocol [Wang and Taylor, 1981a].

MATERIALS AND METHODS

Materials

The 5-iodoacetamidofluorescein and eosin-5-maleimide were obtained from Molecular Probes Inc. (Junction City, OR). ATP and phalloidin were purchased from Boehringer-Mannheim Corp. (Indianapolis, IN). Phalloidin was stored at 1 mM in 2% dimethylsulfoxide (DMSO). Cytochalasin B (Sigma Chemical Co., St. Louis, MO) was stored at 10 mM in DMSO.

Preparation of Proteins

Villin was prepared by the method of Mooseker et al [1980]. Actin was prepared from rabbit leg and back muscles according to the method of Spudich and Watt [1971] and was further purified by gel filtration through a Sephadex G-150 column. Fluorescent labeling of actin using 5-iodoacetamidofluorescein and eosin-5-maleimide was performed as described previously [Wang and Taylor, 1981a]. Actin was maintained for a maximum of 4 days by dialysis against buffer A[2 mM Tris-HCl, 0.5 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM dithiothreitol (DTT), 0.02% NaN_3, pH 7.8)]. Polymerization was induced by mixing 1 volume of G-actin in buffer A with 9 volumes of polymerization buffer (10 mM PIPES, 75 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, pH 7.2). The final free [Ca^{2+}] after mixing is $\sim 20\ \mu M$. Experiments at low free [Ca^{2+}]($< \sim 10^{-8}$ M) were carried out by adding 1 mM EGTA to the above mixture, resulting in a Ca^{2+}/EGTA ratio of 1:50. At the specified buffer condition and actin concentration, the bundling effect of villin was negligible. Mixing of solutions was carried out by a brief pulse (< 1 second) of gentle vortexing.

Fluorescence Measurements

Energy transfer was followed by measuring quenching of donor fluorescence at 520 nm while exciting the sample at 450 nm. The fluorescence signal was contributed primarily by the donor, but also included a small contribution from the acceptor ($\sim$20%). In addition, fluorescence of both donor-labeled F-actin alone and acceptor-labeled F-actin alone was affected to some extent by the presence of villin. Therefore, relative fluorescence intensity was used only as a qualitative indicator of the extent of actin polymerization-depolymerization. When accurate measurements of the extent of energy transfer were required, the contribution from the acceptor was measured separately and subtracted, and the effect of villin on donor-labeled actin alone was also corrected. Detailed description of the procedure has been published [Wang and Taylor, 1981a].

RESULTS

Effects of Villin on the Kinetics of Actin Polymerization

Actin polymerization is followed by fluorescence resonance energy transfer, using fluorescein as the donor and eosin as the acceptor. Both the donor and the acceptor are covalently linked to a sulfhydryl group on actin. Energy transfer is measured based on the quenching of donor fluorescence intensity [Wang and Taylor, 1981a]. When donor-labeled subunits are copolymerized with acceptor-labeled subunits, a *decrease* in donor fluorescence intensity is detected.

The effects of villin on actin polymerization are monitored by adding polymerization buffer, containing villin, to a mixture of donor- and acceptor-labeled actin subunits (Fig. 1). In the absence of villin, a lag phase of 2–3 minutes, previously

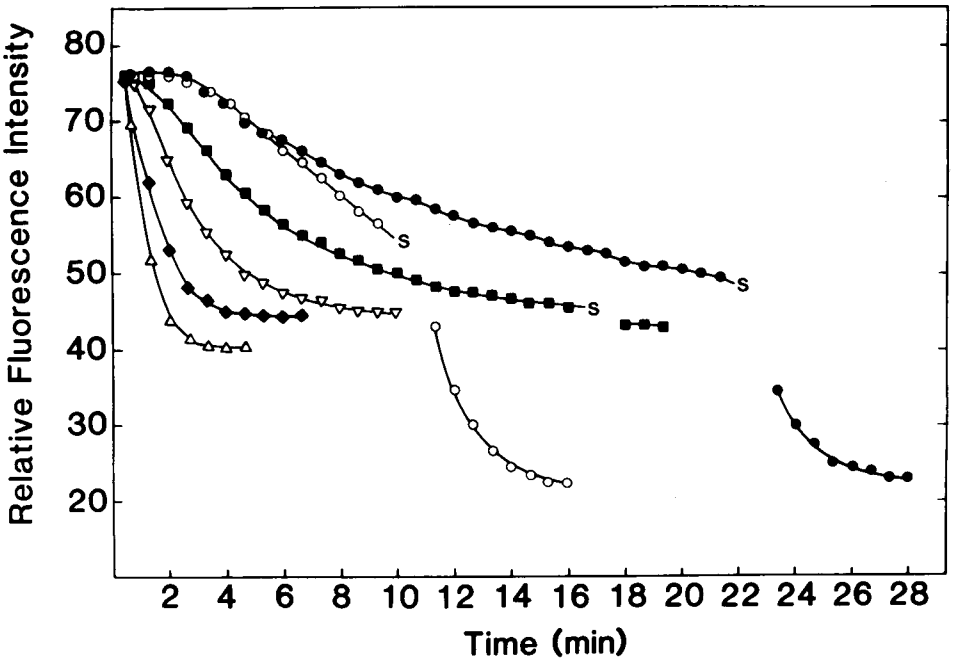

Fig. 1. Effects of villin on the kinetics of actin polymerization. At t=0, 9 volumes of polymerization buffer containing various concentrations of villin were added to 1 volume of a mixture of fluorescein- and eosin-labeled actin subunits. The solution was immediately transferred into a microcuvette in a spectrofluorometer. The final concentration of fluorescein-labeled actin was 0.5 μM, and of eosin-labeled actin was 5 μM. At specified time points ("S"), the solutions were sheared by two passes through Pasteur pipettes with narrow tips (diameter ~ 0.5 mm). Final concentration of villin: no villin (○), 12.5 nM (●), 50 nM (■), 100 nM (▽), 250 nM (◆), 500 nM (△).

interpreted as the time required to form oligomeric nucleation sites, is observed [Oosawa and Kasai, 1971; Pollard and Craig, 1982; Korn, 1982]. The subsequent polymerization of filaments is detected as a continuous decrease in donor fluorescence intensity. The rate of polymerization is very sensitive to mechanical shear. A dramatic stimulation of polymerization is induced by passing the solution through a pipette with a narrow opening. This observation can be explained readily by the generation of new filament ends during the shear and indicates that reliable kinetic information can only be obtained using techniques with minimal mechanical shear. A similar observation has been reported recently [Tait and Frieden, 1982]. The effect of shear is used in the present experiment to induce rapid polymerization in order to determine the steady state, or as a qualitative indicator of the length of the filaments, since short filaments are less sensitive to shear than long filaments.

In the presence of low concentrations of villin (villin/actin = 1:300–400), no apparent change in the lag phase is observed. However, the overall rate of polymerization is slightly inhibited as compared to the control. In addition, the donor fluorescence appears to approach a final extent significantly higher (ie, lower energy transfer) than that of the control. Upon shear, the donor fluorescence intensity approaches the control value.

At higher concentrations of villin, the lag phase is significantly reduced. The lag phase is no longer detectable at ratios of villin to actin greater than 1:50, and the net rate of polymerization is also dramatically increased. In addition, the final donor fluorescence at these higher villin concentrations reaches a plateau significantly higher than that of the control (ie, lower energy tranfer). Shearing these solutions has no effect on either the rate of polymerization or the final plateau of fluorescence intensity.

The effects of villin on the kinetics of actin polymerization are detectable only when excess free Ca^{2+} ions are present. If 1 mM EGTA is added to the polymerization buffer, no effect on polymerization can be detected even at a molar ratio of 1 villin to 20 actin molecules (not shown).

Similar experiments are performed using cytochalasin B as the actin-binding factor, which, like villin, has been suggested to bind to the "barbed" end of the actin filament [MacLean-Fletcher and Pollard, 1980; Flanagan and Lin, 1980; Pollard and Mooseker, 1981; Brown and Spudich, 1981]. Although cytochalasin B at concentrations up to 1 μM has no apparent effect on the lag phase, this drug does cause a decrease in the net rate of polymerization (Fig. 2). The results are qualitatively similar to those of villin at low concentrations (Fig. 1). At cytochalasin B concentrations higher than 1 μM, a decrease in the length of lag period and an apparent increase in the net rate of polymerization are observed (Fig. 2). This could be explained by the cytochalasin B-induced filament breakage, which would increase the number of ends available for assembly [Hartwig and Stossel, 1979; Wang and Taylor, 1981a]. However, the stimulatory effects of cytochalasin B on polymerization kinetics are

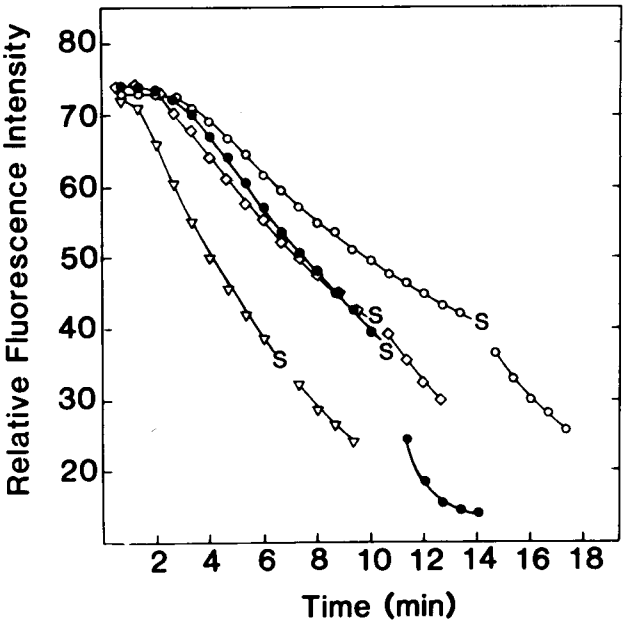

Fig. 2. Effects of cytochalasin B on the kinetics of actin polymerization. The procedure was similar to that in Figure 1, except cytochalasin B instead of villin was used. Final concentrations of cytochalasin B: no cytochalasin B (0.5% DMSO, ●); 1 μM cytochalasin B (○); 10 μM cytochalasin B (◇); 50 μM cytochalasin B (▽).

smaller compared to those of villin, and, unlike villin, the final extent of energy transfer is very similar to that of the control [see Table II of Wang and Taylor, 1981a].

Villin Induces the Formation of Actin Nucleation Sites Under Depolymerization Conditions

The stimulation of nucleation in the presence of villin could be caused solely by the "severing" of newly formed filaments. However, Glenney, Kaulfus, and Weber [1981] have provided evidence that villin will form oligomeric complexes with 2–3 actin molecules under depolymerization conditions [Glenney, Kaulfus, and Weber, 1981]. We therefore examined the possibility that such villin-actin complexes formed under depolymerization conditions could actually serve as nucleation sites for polymerization.

Villin is incubated with unlabeled actin under depolymerization conditions in the presence of 20–35 μM free [Ca^{2+}], followed by the addition of 1 mM EGTA, 75 mM KCl, 2.5 mM $MgCl_2$. The mixture is added immediately into a solution containing donor- and acceptor-labeled actin monomers to initiate polymerization. Stimulation of polymerization would be observed only if nucleation sites are assembled while under the depolymerization condition, since any subsequent effect of villin would be inhibited by decreasing the free [Ca^{2+}].

The experiments are carried out under several different conditions (Fig. 3). At an unlabeled actin concentration of 2.5 μM and villin concentration of 0.25 μM (with <1 minute incubation), only a slight stimulation of polymerization is observed. An

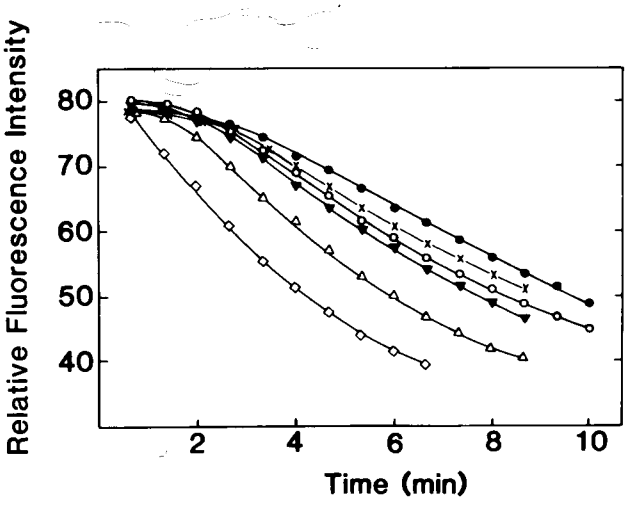

Fig. 3. Formation of villin-actin complexes in buffer A. Villin in buffer A was first mixed with unlabeled G-actin. Salt concentrations were subsequently raised to 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES, followed by immediate mixing of this solution (at t=0) with 1/9 volume of a solution containing 5 μM fluorescein-labeled actin and 50 μM eosin-labeled actin. The concentrations of villin and of unlabeled actin in the initial mixture at low ionic strength: no actin, 0.25 μM villin (●); 5 μM actin, no villin (×); 2.5 μM actin, 0.25 μM villin (▼); 2.5 μM actin, 0.5 μM villin (○); 5 μM actin, 0.25 μM villin (△, ◇). In one experiment, the mixture was incubated at room temperature for 20 minutes before adding salts (◇). Villin and unlabeled actin were diluted by a factor of 2.5 in subsequent steps.

identical result is obtained when the concentration of villin is increased to 0.5 μM. However, at a villin concentration of 0.25 μM and an actin concentration of 5 μM, a more significant stimulation of polymerization is observed. The stimulatory effect increases after an incubation for 20 minutes, suggesting that the formation of nucleation sites under depolymerization conditions is a relatively slow process.

Villin Increases the Critical Concentration of Actin Polymerization

It is observed in previous experiments that the final plateau of donor fluorescence intensity is significantly higher in the presence of villin than in its absence (Fig. 1). Therefore, we determine if the efficiency of energy transfer is actually reduced, and if so, why. We measure the efficiency of energy transfer at steady state as a function of villin concentration. As shown in Figure 4, the efficiency decreases sharply with increasing concentrations of villin, approaching a value of ~25% at villin/actin > 1:50.

The decrease in the efficiency of energy transfer could be caused by several possible mechanisms. Villin presumably binds to the "barbed" end of actin filaments. This could induce a dramatic increase in critical concentration. The binding could also induce a change in filament structure and an increase in the distance between neighboring subunits. In addition, villin has been shown to shorten the length of actin filaments, resulting in an increase in the number of donor-labeled subunits that are located at ends of the filament and are thus only partially surrounded by acceptor-labeled subunits.

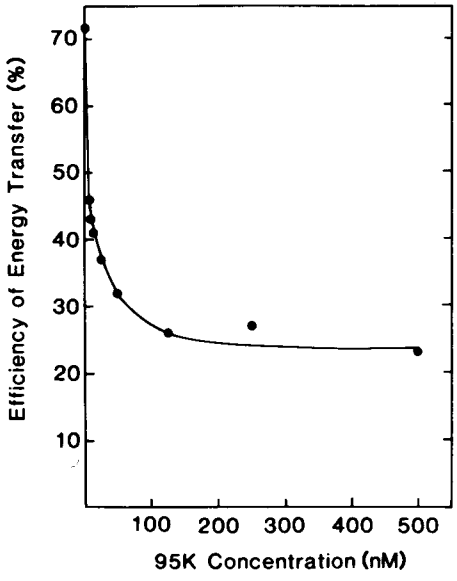

Fig. 4. Effect of villin on the efficiency of energy transfer at steady state. Assembled filaments were incubated with various concentrations of villin, followed by incubation at room temperature for 4 hours before measuring the efficiency of energy transfer. Intensity measurements were performed by taking multiple samples using microcapillaries. Detailed procedures for the microcapillary cuvettes and for the calculation of the efficiency of energy transfer have been described previously [Wang and Taylor, 1981a]. The final concentration of fluorescein-labeled actin was 0.5 μM, and of eosin-labeled actin was 5 μM.

TABLE I. Effect of Critical Concentration on the Reestablishment of Steady State After a Five-Fold Dilution of F-actin Solutions*

Critical concentration		Monomer-polymer distribution			Percent depolymerization
		Before dilution	After dilution	Steady state	
0.20 μM	[G-actin]	0.20 μM	0.04 μM	0.20 μM	8
	[F-actin]	9.80 μM	1.96 μM	1.80 μM	
1.00 μM	[G-actin]	1.00 μM	0.20 μM	1.00 μM	44
	[F-actin]	9.00 μM	1.80 μM	1.00 μM	

*The starting total actin concentration is assumed to be 10 μM.

To distinguish between these possibilities, we perform the following experiment (Table I). Assuming the critical concentration of actin alone to be 0.2 μM, then if we dilute a solution of 10 μM total actin concentration by a factor of 5, there should be an 8% depolymerization in order to reestablish the steady state (this net depolymerization would exhibit as an increase in donor fluorescence intensity). If villin induces an increase in critical concentration to 1 μM, then after dilution, a much more extensive depolymerization (44%) should be observed. However, if the change in the efficiency of energy transfer is caused by changes in filament structure or in filament length, then upon dilution one should observe a similar extent of depolymerization as in the control.

Only a slight increase (~ 10%) in donor fluorescence intensity is observed when the experiment is performed in the absence of villin (Fig. 5). This indicates that the final actin concentration (2 μM) is still much higher than the critical concentration. However, when villin is present in the solution, a much greater increase (>30%) in donor fluorescence is observed, which can only be attributed to an extensive depolymerization of the filaments. If the villin-actin mixture is diluted into polymerization buffer with 1 mM EGTA, so that the effect of villin on energy transfer is inhibited after dilution, a transient increase in the donor fluorescence intensity (ie, depolymerization) followed by a decrease in donor fluorescence (ie, net polymerization) is observed (Fig. 5). The final fluorescence intensity in the presence of EGTA is close to that observed in the absence of villin (data not shown).

In support of the notion that villin increases the critical concentration, we observe that addition of villin to preassembled filaments made by copolymerizing donor- and acceptor-labeled subunits causes a gradual increase (over 10 minutes) in donor fluorescence (Fig. 6). This result suggests that villin causes a shift to a new steady state. It is important to note that the time required to reach the new steady state (> 10 minutes) is much longer than that required for filament "severing" as measured by viscometry or electron microscopy [< 1 minute: Mooseker et al, 1980; Craig and Powell, 1980; Glenney, Kaulfus, and Weber, 1981]. In addition, the effect is readily reversible by the subsequent addition of EGTA or phalloidin. Phalloidin causes complete repolymerization within 20 seconds, whereas EGTA has a much slower effect.

A similar experiment is performed to study the depolymerization effect of villin precomplexed with unlabeled actin oligomers (Fig. 7). Filament depolymerization is also observed, although the rate is 4–5 times lower than that using uncomplexed villin. In a previous experiment, Glenney, Kaulfus, and Weber [1981] have shown

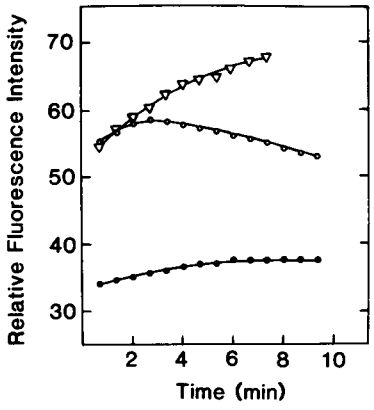

Fig. 5. Effect of dilution on the energy transfer of villin-actin complex. Fluorescein-labeled actin at 1 μM and eosin-labeled actin at 10 μM were copolymerized. Villin was subsequently added to attain a final concentration of 1 μM, followed by incubation at room temperature for 1 hour. At t=0, the mixture was diluted into 4 volumes of polymerization buffer with (○) or without (▽) 1 mM EGTA. In the control experiment, buffer instead of villin was added to the solution of copolymer; similar kinetics were observed without (●) or with (not shown) 1 mM EGTA.

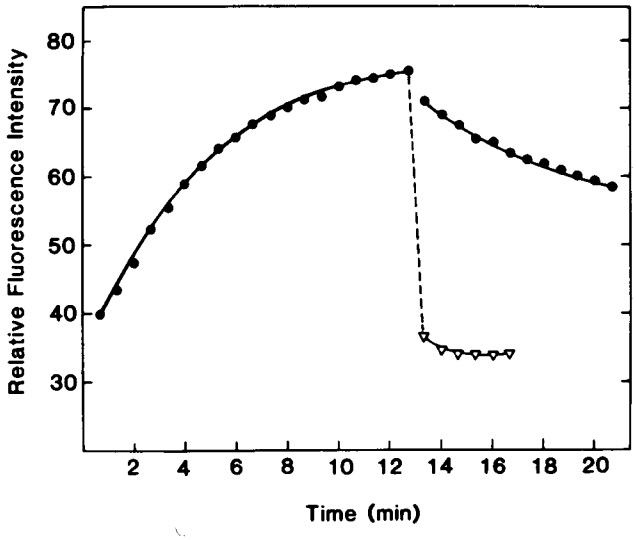

Fig. 6. Depolymerization induced by villin and its reversal by EGTA and phalloidin. Fluorescein-labeled actin at 0.5 μM and eosin-labeled actin at 5 μM were copolymerized. At t=0, 1/8 volume of villin was added to attain a concentration of 0.5 μM. The reversal was induced by adding EGTA to 1 mM (●), or by adding phalloidin to 6 μM (▽).

that such villin-actin complexes do not "sever" actin filaments as monitored by viscometry.

Effect on Actin Subunit Exchange

It is known that actin subunits undergo exchange at steady state under appropriate conditions, although the absolute rate has not been well established [Wang and

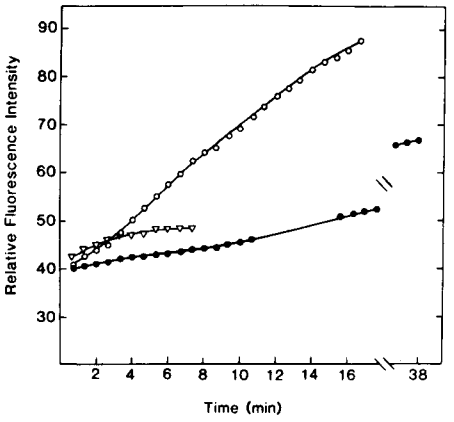

Fig. 7. Depolymerization induced by villin and by villin-actin complex. Fluorescein-labeled actin at 0.5 μM and eosin-labeled actin at 5 μM were copolymerized. At t=0, 1½ volumes of polymerization buffer containing villin (○) or villin precomplexed with 10-fold molar excess unlabeled F-actin (●) were added to attain a final villin concentration of 0.1 μM. Limited depolymerization was induced by adding polymerization buffer alone (▽).

Taylor, 1981a,b; Wegner and Neuhaus, 1981; Pardee et al, 1982]. The effect of villin on this process in the presence of 20 μM free Ca^{2+} is investigated using the "mixed-polymer" protocol, which involves mixing donor-labeled filaments with acceptor-labeled filaments and following the subsequent increase in energy transfer caused by the intermixing of labeled subunits [Wang and Taylor, 1981a]. At a total actin concentration of 5 μM and a molar ratio below 1 villin to 200 actin subunits, inhibition of exchange is observed (Fig. 8). In contrast, stimulation is observed at a molar ratio of 1 villin to 50 actin subunits.

DISCUSSION

Effects of Villin on Actin Polymerization Kinetics

The effects of villin on the kinetics of actin polymerization is qualitatively similar to those of cytochalasin B. At low concentrations, both factors cause a slight inhibition of the net rate of polymerization without affecting nucleation. At high concentrations, both factors stimulate nucleation and net polymerization. These effects can be explained by a combination of end blockage, filament "severing," and nuclei formation.

It has been proposed that both cytochalasin B and villin can cap the "barbed" end of the actin filament, as well as "sever" actin filaments [Craig and Pollard, 1982; Bonder and Mooseker, 1983]. End blockage would cause an inhibition of elongation. Since elongation is never completely inhibited by villin, it is consistent with the idea that only one end of the filament is blocked. "Severing," however, would stimulate the rate of polymerization due to the increase in the number of filament ends.

In addition to end blockage and filament "severing," direct stimulation of the nucleation process could also affect the kinetics of actin polymerization. It has been observed that villin can form complexes with multiple actin subunits under depolymerization conditions [Glenney, Kaulfus, and Weber, 1981]. Our experiments indicate

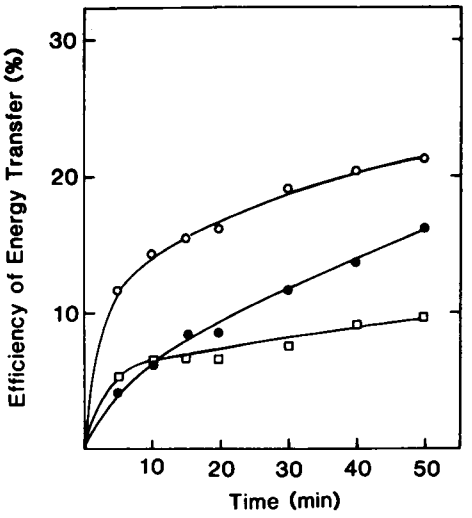

Fig. 8. Effect of villin on the exchange of actin subunits at steady state. Exchange was measured by following the increase in energy transfer when fluorescein-labeled actin filaments were mixed with eosin-labeled actin filament (at t=0). In order to calculate the efficiency of energy transfer properly, separate measurements were made by mixing fluorescein-labeled filaments with unlabeled filaments. The intensities of various solutions at specific time points were then used to calculate the efficiency [Wang and Taylor, 1981a]. Actin was polymerized at 5 μM in the presence of no villin (●), 25 nM villin (□), or 100 nM villin (○). The detailed procedure has been described previously [Wang and Taylor, 1981a].

that the complexes can either function directly as nucleation sites or can transform rapidly into nucleation sites when brought into polymerization conditions. Cytochalasin B, however, does not bind to actin under depolymerization conditions [Flanagan and Lin, 1980]. It is not known if cytochalasin B can induce the formation of nucleation sites under polymerization conditions.

Since end blockage, filament "severing," and nuclei formation can cause opposite effects on the rates of nucleation and elongation, the net effect on polymerization kinetics would be determined by the relative extent of end blockage versus "severing" and nucleation. The observed effects of both villin and cytochalasin B could be explained if, during the initial stage of polymerization, the concentration of ligand required for blocking the "barbed" end is lower than that required for the formation of nucleation sites or for "severing" the filaments. The stronger stimulatory effects of villin compared to cytochalasin B at high concentrations could be explained by a combination of more efficient "severing" and more effective nucleation induced by villin [Mooseker et al, 1980; Glenney, Kaulfus, and Weber, 1981; Craig and Powell, 1980; MacLean-Fletcher and Pollard, 1980].

Effects of Villin on the Critical Concentration

We have investigated the consequences of villin-actin interactions on the polymer-monomer equilibrium of actin. From our experiments, we conclude that villin causes a large increase in the monomer concentration. This conclusion is based on three pieces of data: a) depolymerization of villin-bound filaments upon dilution into a solution of high free [Ca^{2+}] (Fig. 5); b) net polymerization of villin-bound filaments

upon dilution into an EGTA-containing solution (Fig. 5); and c) relatively slow decrease in the extent of energy transfer upon addition of villin to F-actin (Fig. 6).

When F-actin solutions are diluted, there is a redistribution of the total amount of polymer and monomer in order to reestablish the critical concentration. The extent of depolymerization is determined by the critical concentration (higher critical concentration is coupled to more extensive depolymerization). In the absence of villin, this depolymerization is detected as a small increase in donor fluorescence intensity (Fig. 5). In the presence of villin, we observe a much more extensive increase in donor fluorescence as compared to the control (Fig. 5). This larger increase cannot be explained by the "severing" activity, which should decrease instead of increase upon dilution. In addition, the experiments are performed at the range of filament length where the efficiency of energy transfer is relatively insensitive to the concentration of villin (Fig. 4). We therefore conclude that villin induces an increase in critical concentration.

Dilution of villin-capped filaments into an EGTA-containing solution results in a net decrease in donor fluorescence over time. Immediately upon dilution there is a slight increase in donor fluorescence, which may be indicative of the time required for villin to dissociate from the filament end. The subsequent decrease in donor fluorescence (net polymerization) suggests that the concentration of monomer in the presence of villin and elevated free [Ca^{2+}], even after five-fold dilution, is still higher than the critical concentration in the EGTA-containing solution. In other words, in the presence of excess free Ca^{2+} ions, villin induces at least a five-fold increase in critical concentration.

Finally, addition of villin to the copolymer of donor- and acceptor-labeled subunits results in a relatively slow (>10 minutes) increase in donor fluorescence. Based on viscometry and electron microscopy, it is known that the severing activity of villin is very rapid—occurring within 1 minute. Therefore, the increase in fluorescence intensity observed over a period of 10 minutes cannot be attributed to filament severing and most likely reflects a depolymerization effect.

It has been shown that cytochalasin B does not cause a significant increase in critical concentrations [Wang and Taylor, 1981a; MacLean-Fletcher and Pollard, 1980]. Since both cytochalasin B and villin bind to the "barbed" end of actin filaments, identical critical concentrations, determined by the "pointed" end of the filament, should be observed. The difference between villin and cytochalasin B suggests that cytochalasin B even at high concentrations does not completely saturate the binding site at the end of actin filaments. Therefore, the critical concentration in the presence of cytochalasin B probably does not represent the actual critical concentration of the "pointed" end. It is interesting to note that, at low concentrations of villin, the critical concentration can be restored to a value close to the control by mechanical shear (Fig. 1). This can be explained by the generation of excess ends that cannot be saturated by the low concentration of villin.

Effects on Subunit Exchange

The results of Fig. 8 indicate that subunit exchange is inhibited at low concentrations of villin, but is stimulated at higher concentrations. In a previous study, Glenney, Kaulfus, and Weber [1981], using a different assay, reported a net inhibition

of subunit exchange at relatively low villin concentrations. However, the effect at higher villin concentrations was not reported.

The effects of villin on the rate of subunit exchange can be explained by a combination of depolymerization of filaments, capping of the "barbed" end, and severing of filaments. Both depolymerization and capping would cause a decrease in the number of ends that are active in subunit exchange. However, severing of filaments would cause an increase in the number of active ends. At low villin concentrations, the extent of severing is limited, resulting in a net decrease in the rate of exchange. At higher villin concentrations, severing becomes extensive. Therefore, even though most filaments are blocked at the "barbed" ends, there are many more free pointed ends than in controls, resulting in a net increase in the rate of exchange.

Possible Roles of Villin and of Proteins With Similar Activities

Although villin has so far been identified only in brush borders of epithelial cells, proteins with similar properties have been described in other nonmuscle cells. For example, gelsolin, originally purified from macrophages, has recently been identified in a large number of cell types [Yin and Stossel, 1979; Yin, Albrecht, and Fattoum, 1981]. It has a molecular weight similar to villin and "severs" actin filament in a calcium dependent manner [Yin, Zaner and Stossel, 1980; Yin, et al, 1981]. A similar protein has also been identified in blood platelets [Wang and Bryan, 1981]. Fragmin and a 40,000 dalton protein have been identified in slime molds Physarum polycephalum and Dictyostelium discoideum, respectively [Hasegawa et al, 1980; Brown, Yamamoto, and Spudich, 1982; Yamamoto et al, 1982]. They have molecular weights lower than villin but exhibit similar "severing" and depolymerizing activities. Therefore, biochemical properties exhibited by villin may be important for nonmuscle cells in general.

Two possible functions of villin, shortening of actin filaments and bundle formation, have been discussed in detail previously [Mooseker et al, 1980; Craig and Powell, 1980; Glenney, Kaulfus, and Weber, 1981; Matsudaira and Burgess, 1982]. However, other possibilities should also be considered. The possible role of actin polymerization-depolymerization in nonmuscle cell motility has long been recognized. The calcium-dependent nucleation activity of villin or villin-like proteins could regulate this process. Alternatively, they could affect the assembly state of preformed actin filaments by shifting the critical concentration. Villin or similar proteins could also serve as the attachment factor between actin filaments and other cellular components. This could be achieved by direct nucleation of actin polymerization off membranes, binding to the ends of preformed filaments, or by binding and severing preformed actin filaments along the length. Finally, if treadmilling occurs in the cell, villin-like proteins could regulate both the rate and the direction of the process. The exact role of villin-like proteins will be determined by characterizing the location and the concentration of these proteins, by measuring the extent of actin polymerization, and by determining the relationship of various actin binding factors.

In the present study, the energy transfer technique is used as a nonperturbing, high resolution assay to follow the association of actin subunits in the presence of villin. We have been able to confirm previous hypotheses as well as to obtain detailed new information about the interaction between villin and actin. The results establish

the value of this technique for studying not only the interaction between actin and villin, but also the interactions of actin with actin-binding factors in general.

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