Effect of Villin on the Kinetics of Actin Polymerization†

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ABSTRACT: The effect of villin on the critical concentration of actin and on the kinetics of its polymerization has been measured. In the presence of villin and 10 μM calcium, the critical concentration of actin increased from 0.2 to 0.9 μM. This effect of villin on the critical concentration was shown to be the result of its well-documented ability to block the "barbed" end of actin filaments, i.e., the "high-affinity end" of a polymer with a different monomer binding constant at each end. Thus, below 0.8 μM actin polymerization was prevented when the ratio of villin to actin was about 1 in 1000. Furthermore, the effect of villin was saturable; i.e., the critical concentration remained constant with increasing villin concentration once the maximal change had been obtained. In addition, fragmentation of actin filaments previously capped with villin, producing uncapped filaments, caused a rapid, transient fall of the monomer concentration. With the disappearance of the uncapped filaments the actin monomer concentration returned to that measured before fragmentation. The binding constant of villin to the barbed end of the actin filament was calculated to be greater than 10^{11} M^{-1}. The rate constants of elongation and of depolymerization at each end of an actin filament were measured. The depolymerization rate constant from the barbed end was about 10 times greater under conditions leading to complete depolymerization than under steady-state conditions. We discuss a possible explanation for the finding and its implication for possible regulatory mechanisms.

The state of assembly of actin filaments in nonmuscle cells may be regulated by the action of various types of actin binding proteins (Korn, 1982; Craig & Pollard, 1982; Weeds, 1982). One of the most intensively studied proteins in this group is villin (Bretscher & Weber, 1980), also referred to as MV-95K (Mooseker et al., 1980), the 95 kdalton protein of the brush border microvilli core. The in vitro effects of villin on actin have been recently reviewed (Craig & Pollard, 1982). In the presence of micromolar calcium (Glenney et al., 1981) villin speeds actin polymerization (Mooseker et al., 1980; Craig & Powell, 1980; Glenney et al., 1981) presumably by promoting nucleation (Glenney & Weber, 1981). On the other hand, villin lowers the rate of filament elongation by blocking monomer addition to the fast growing, "barbed"[1] filament end (Glenney et al., 1981; Pollard & Craig, 1982; Bonder & Mooseke, 1983). Addition of villin to polymerized actin, in the presence of calcium, causes rapid filament shortening together with an increase in filament number (Bretscher & Weber, 1980; Mooseker et al., 1980; Craig & Powell, 1980; Matsudaira & Burgess, 1982), presumably the result of villin-induced filament breakage (Glenney et al., 1981; Weber & Glenney, 1982; Mooseker et al., 1982; Bonder & Mooseker, 1983). In low calcium ($<10^{-7}$ M) villin has little detectable effect on actin polymerization (Mooseker et al., 1980; Glenney et al., 1981; Glenney & Weber, 1981; Bonder & Mooseker, 1983) but cross-links actin filaments into tight bundles (Bretscher & Weber, 1980; Mooseker et al., 1980; Matsudaira & Burgess, 1982).

We have used actin labeled with fluorescent probes that report its state of polymerization to study the effect of villin on the critical concentration of actin. Some of these results have been described in a preliminary report (Weber et al., 1982). These experiments complement recent studies on the action of villin using electron microscopy (Bonder et al., 1983). Titration of the change in critical concentration with increasing concentrations of villin suggests that the only action of villin in the presence of calcium is blocking the high-affinity end of a treadmilling polymer (Wegner, 1976, 1982; Kirschner, 1980; Hill & Kirschner, 1982), i.e., a polymer with a different monomer binding constant at each filament end. Villin binds to the barbed end with very high affinity; a minimal value for the binding constant of 10^{11} M^{-1} was calculated.

Use of the same fluorescent actin to measure the kinetics of polymerization and depolymerization in the presence and absence of villin gave the unexpected result that the rate constant of depolymerization at the barbed end was not the same under conditions of steady state and of net depolymerization at concentrations of total actin well below the critical concentration. The possible implications of this observation are discussed.

Materials and Methods

Protein Preparations. Actin was prepared from acetone powder according to Spudich & Watt (1971) modified as described by Murray et al. (1981). Actin, if labeled after modification with the fluorescent probe, was chromatographed through Bio-Gel P-150, equilibrated with 5 mM triethanolamine hydrochloride at pH 7.5, 0.5 mM ATP, 0.2 mM $CaCl_2$, 0.1 mM Ca-EDTA,[2] and 3 mM NaN_3 (Wegner, 1976). Early fractions which contained traces of contaminating actin binding proteins were discarded [see also Maclean-Fletcher & Pollard (1980)]. If actin had not been modified with a fluorescent probe, 0.5 mM dithiothreitol was included in the elution buffer. Actin was stored at 4 °C in the elution buffer and was used within a period of 2–4 weeks. Although we have found actin to be more stable at 14 °C than at 4 °C, bacterial growth was lower at 4 °C.

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[1] Named after the appearance of myosin decorated actin filaments.

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Actin modified with *N*-ethylmaleimide (NEM) or with NEM and 7-chloro-4-nitro-2,1,3-benzoxadiazole (NEM–NBD–actin) was prepared as previously described (Detmers et al., 1981). Actin modified with pyrene attached to Cys-374 (pyrene–actin) was prepared according to Kouyama & Mihashi (1981) as follows. After removal of dithiothreitol by overnight dialysis, actin in 10 mM Tris-HCl, pH 8.0, 0.5 mM ATP, and 0.2 mM $CaCl_2$ (depolymerizing solution) was mixed with 1.0 mM pyrenyliodoacetamide in 33% dioxane–67% acetone to a final concentration of 50 μM actin and 50 μM pyrenyliodoacetamide followed by the addition of $MgCl_2$ and KCl to final concentrations of 2 and 100 mM, respectively. After a 2-h incubation at room temperature, actin was centrifuged for 2 h at 100000*g*. The pellet was then depolymerized by dialysis against depolymerizing solution. Actin was checked for unreacted Cys-374 by incubation with [^{3}H]-*N*-ethylmaleimide for 5 h (Porter & Weber, 1979). Incorporation of [^{3}H]NEM into actin was always less than 10 % (mol/mol).

Villin was prepared according to Mooseker et al. (1980). The purified protein was stored in liquid nitrogen after dialysis against 75 mM KCl–10 mM imidazole at pH 7.2. Preparations were not refrozen after thawing.

Protein concentrations of villin and actin were determined either by Lowry's method (1951) or, in the case of actin, from E_{290} = 24.9 mM^{-1} cm^{-1} (Wegner, 1976).

Fluorescence and Kinetic Measurements. Fluorescence was measured in a Perkin-Elmer double-beam fluorometer, Model MPF-2L. Excitation/emission wavelengths were 470/530 nm for NEM–NBD–actin and 370–410 nm for pyrene–actin. The protein samples were exposed to the exciting light only while recording since continuous exposure caused photobleaching. On incorporation of NEM–NBD–actin into filaments, there was a 2.2–3-fold increase in fluorescence intensity without any change in absorption (Detmers et al., 1981). Pyrene–actin exhibited increases in both absorption and emission; the increase in fluorescence intensity varied between 15-fold and 30-fold for different preparations. The reason for the variation is not clear. Inner filter effects became significant (>2%) at concentrations of polymerized pyrene–actin above 1.0 μM and for NEM–NBD–G-actin and F-NEM–NBD–F-actin above 2.0 μM (1-cm path length). Light scattering artifacts were minimized by placing cutoff filters (below 390 and 490 nm, for pyrene–actin and NEM–NBD–actin, respectively) in the path of the emitted light.

Unless stated otherwise, kinetic experiments were carried out at 14 °C, in polymerizing solution containing 0.5 mM ATP, 10 mM Tris-HCl, at a pH as indicated, 1.0 or 2.0 mM $MgCl_2$, 0.1 M KCl, and $CaCl_2$ as indicated.

Evaluation of the Effect of Labeling on Actin Kinetics. NEM–NBD–actin had a shorter lag period and polymerization was completed in a shorter time than was the case for pyrene–actin. Nevertheless, the time course of fluorescence increase was identical when the two proteins were mixed in equal proportions, indicating that the two proteins copolymerize randomly (Figure 1) [see Tait & Frieden (1982a–c)]. In view of the demonstration that there was a marked change in the kinetics of both labeled actin preparations when they were mixed, it is significant that a 2–3-fold dilution of pyrene–actin with native actin did not markedly alter the kinetics (cf. Cooper et al., 1983a,b) notwithstanding the relatively small changes observed by Frieden (1982).

Critical concentrations were determined for NEM–NBD–actin and for mixtures of pyrene–actin plus native actin and pyrene–actin plus NEM–actin from the increase in fluorescence as a measure of F-actin formation as illustrated in Figure

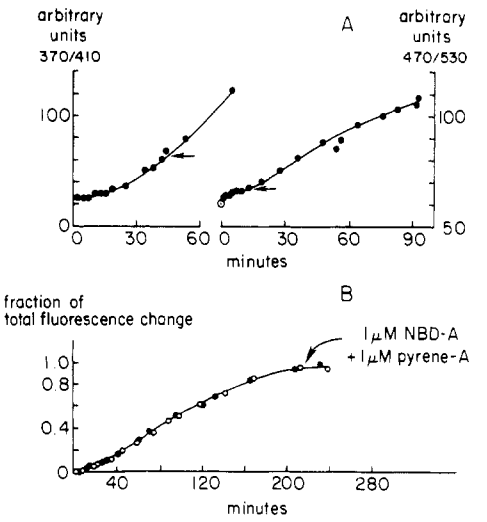

FIGURE 1: Polymerization and copolymerization of NEM–NBD–actin and pyrene–actin. (A) Comparison of the polymerization of 100% pyrene–actin (left) and 100% NEM–NBD–actin (right); 2.0 μM total actin in each case. Arrows indicate 5% polymerization (maximal fluorescence 730 and 125 for pyrene–actin and NEM–NBD–actin, respectively). Only the initial phase of polymerization is shown since the fluorescence intensity measurements of pyrene–F-actin are increasingly distorted by inner filter effects above 1.0 μM (i.e., the value of 730 units for the end point is too low in comparison to the initial fluorescence intensity). The initial rise of fluorescence of NEM–NBD–actin is caused by an effect of magnesium on the free monomer and occurred also below the critical concentration (Frieden et al., 1980; Frieden, 1982). (B) Copolymerization of a mixture of 1.0 μM each actin. During copolymerization fluorescence was measured alternately with the wavelength pairs 370/410 nm and 470/530 nm for pyrene–actin and NEM-NBD-actin, respectively. Conditions for (A) and (B) as described under Materials and Methods; pH 7.4, 10 μM calcium.

2. An increase in the intercept of the two slopes was taken to indicate an increase in the critical concentration only when the slopes remained the same. Full equilibrium in polymerizing solution required 1–2 days. In the absence of villin the end point of polymerization was checked by sonicating for 5 s. Sonication caused a rapid increase in polymerization (and therefore fluorescence) when the end point had not yet been reached, because the fragmentation of filaments greatly increased the concentration of nuclei (Asakura et al., 1963). In the presence of villin sonication had a different effect (see Figure 4) and therefore was not used to test for the end point. Polymerization reached steady state more rapidly in the presence of villin (<12 h) presumably as a result of its nucleating action (Glenney & Weber, 1981).

In the presence of villin under conditions where a true increase in the critical concentration had been established as described above, the critical concentration was sometimes measured by a simpler method. The concentration of monomeric actin was calculated as the difference between the total actin concentration and that of polymerized actin determined from a single fluorescence measurement (in triplicate). For increased accuracy actin concentrations were used that were relatively close to the anticipated value for the critical concentration, e.g., 1.2–1.5 μM.

Elongation kinetics were measured in terms of the increase in fluorescence in the presence of a relatively large concentration of preformed nuclei. Villin–actin nuclei were formed by preincubating actin with villin in depolymerizing solution (Glenney & Weber, 1981) for a few hours before initiating elongation by the addition of KCl and $MgCl_2$ (cf. Wang et al., 1983). At the concentrations used, villin caused so much nucleation that the effect of spontaneous nucleation became

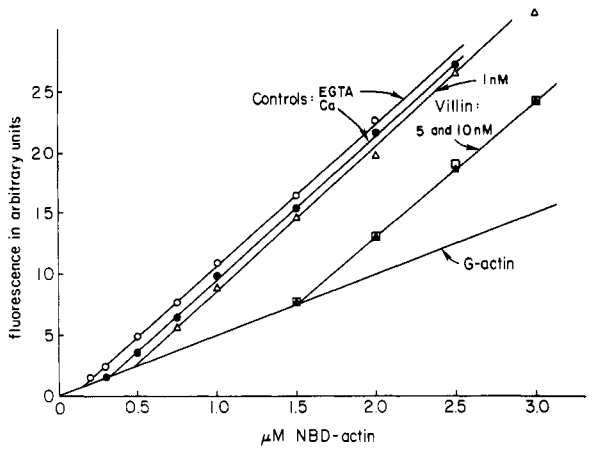

FIGURE 2: Effect of villin on the critical concentration of NEM–NBD–actin. Fluorescence was measured at increasing concentrations of actin. The slope of the fluorescence of NEM–NBD–actin changes at the critical concentration; i.e., it is different for G- and F-actin. The critical concentration is indicated by the intersect of the two slopes. Triethanolamine, pH 8.0; (●, ▲, △, □) 200 μM calcium or (○) 1.0 mM EGTA. Villin was mixed with actin before polymerization was initiated by the addition of KCl and $MgCl_2$. Measurements were made in the concentration range 0.07–3.0 μM actin (points at subcritical concentrations not shown for clarity). The critical concentration in the absence of villin was exceptionally high for this actin preparation.

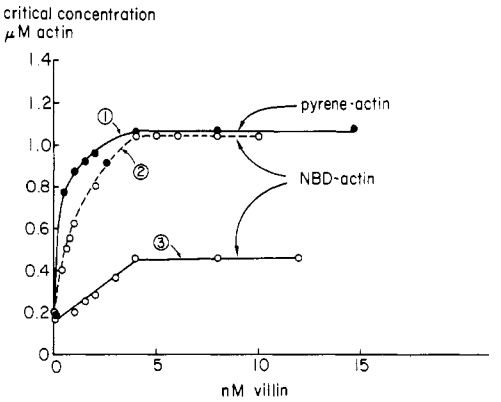

FIGURE 3: Titration of the critical concentrations of NEM–NBD–actin and pyrene–actin with increasing concentrations of villin. Ordinate: critical concentration. Abscissa: total villin concentration. (Curve 1) Pyrene + NEM actin and (curve 3) NEM–NBD + NEM–actin: pH 7.4, 10 μM calcium. (Curve 2) NEM–NBD–actin: pH 8.0, 200 μM calcium. Different villin preparations for curves 2 and 3; same villin for curves 1 and 3; different preparations of NEM–NBD–actin in curves 2 and 3; same NEM–actin for curves 1 and 3. Critical concentrations were measured for curve 2 as in Figure 2 and for curves 1 and 3 with constant actin (1.3 μM) as described under Materials and Methods.

insignificant. Thus, in the experiment of Figure 7, polymerization in the presence of 17 nM villin was 50% complete after 10 min at which time only about 10% of the NEM–NBD–actin had polymerized in the absence of villin. The end point, which is needed to analyze the time course of elongation, was obtained by continuing the incubation for 12 h after which no further change in fluorescence was observed.

Depolymerization kinetics were measured after diluting actin to concentrations (0.05 μM) calculated to be so far below the critical concentration that polymerization did not significantly affect the rate of depolymerization during the initial third of the reaction. Since the rate of depolymerization depends on the number concentration and length distribution of filaments, the following standard procedure for preparing filaments was established. Actin was polymerized at 1.0–2.0 μM because at higher concentrations paracrystal-like aggregates may form. To obtain an initial equilibrium length distribution (cf. Results), pyrene–actin was incubated overnight while NEM–NBD–actin was sonicated for 5 s 2 h before the measurements. The latter procedure was followed because, for reasons not yet understood, the rate of NEM–NBD–actin depolymerization decreased markedly to very low rates at long times after initial polymerization or sonication. In order not to alter the length distribution before the final dilution step, a gentle stirring procedure was adopted consisting of 25–30 gentle vertical strokes with a plastic spoon. Control experiments (see Figure 8, bottom) showed that applying this stirring procedure during depolymerization did not change its time course, suggesting that significant filament breakage did not occur.

Length distribution of the actin filaments after the sonication procedure used with NEM–NBD–actin was determined by electron microscopy. Two hours after sonication 2 μM NEM–NBD–actin was negatively stained on copper grids coated with collodion and stabilized by a thin carbon film. Immediately before use the grids were glow discharged to improve wetting. A drop of actin solution was negatively stained 30 s after placing it on the grid with unbuffered 1% aqueous uranyl acetate. Grids were examined on a Phillips 201 or 301 electron microscope at an accelerating voltage of 80 kV. Electron micrograph montages of about 400 μm square areas were then constructed and filament lengths directly measured from the micrographs.

Results

Critical Concentration. In the presence of calcium villin increased the critical concentration of NEM–NBD–actin to 1.5 μM (Figure 2), and the effect was maximal at a concentration of 5 nM villin (Figure 3). Raising the villin concentration to higher values did not increase the critical concentration any further. While the maximal values for pyrene–actin quite reproducibly were between 0.75 and 1.1 μM, those for NEM–NBD–actin varied over a 3-fold range from 0.5 to 1.5 μM (Figure 3). It should be noted that, at a concentration of 0.5 nM, 1 villin molecule prevented the assembly of about 1000 molecules of pyrene–actin (Figure 3).

Although calcium alone raised the critical concentration (Figure 2), its effect was readily distinguished from that of villin. This effect of calcium occurred primarily at concentrations above 10 μM whereas the effect of villin on the critical concentration was saturated at 10 μM calcium (experiments not shown). Villin had no effect on the critical concentration when the calcium concentration was very low, i.e., in the presence of 1.0 mM EGTA.[2] Furthermore, the effect of villin on the critical concentration was completely reversed by the removal of calcium.

The binding constant of villin for the barbed end of actin filaments must be higher than 2×10^8 M^{-1} since the filament ends of both NEM–NBD–actin and pyrene–actin were completely capped at 5 nM villin (Figure 3). A further refinement of the estimate for the binding constant giving a minimum value of 10^{11} M^{-1} will be described under Discussion.

Effect of Filament Fragmentation on the Critical Concentration in the Presence of Villin. If villin raises the critical concentration by capping the high-affinity end of a treadmilling polymer, then fragmentation of the filaments should cause an immediate fall in the monomer concentration, maximally to the critical concentration in the absence of villin, followed by a slow return to the critical concentration typical for the presence of villin (Figure 4). When NEM–NBD–actin was sonicated for 5 s after polymerization had reached steady state in the presence of 5 nM villin, the fluorescence rapidly increased, indicating an increase in the state of polymerization

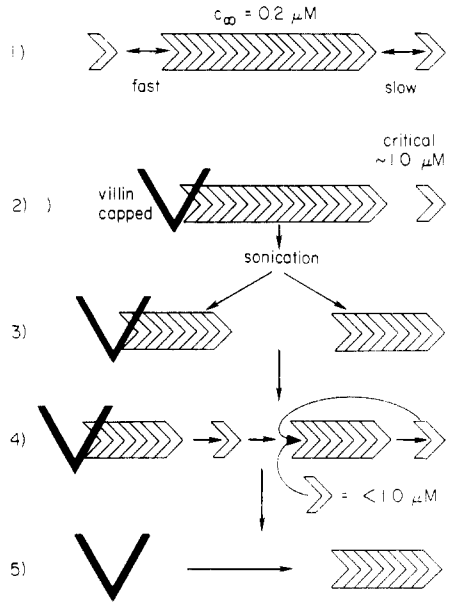

FIGURE 4: Sketch of the anticipated events after sonication of treadmilling actin filaments which are capped at the high-affinity end by villin (V). (1) Treadmilling actin filament in the absence of villin; (2) villin-capped filament at steady state with 1.0 μM monomers; (3) after sonication coexistence of villin-capped and villin-free actin filaments; (4) the newly formed free barbed ends exposed to a monomer level above their critical concentration rapidly take up monomers, thus lowering the monomer concentration and causing the capped filaments to depolymerize; (5) when they are completely depolymerized, the released villin caps free barbed ends.

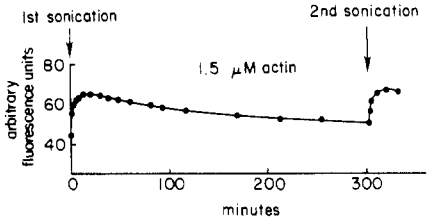

FIGURE 5: Effect of sonication on the steady-state monomer concentration in the presence of 5 nM villin; triethanolamine, pH 7.5, and 200 μM calcium. The increase in fluorescence indicates a decrease in the monomer concentration from 1.0 to 0.5 μM. The critical concentration in the absence of villin was 0.2 μM.

and a decrease in the monomer concentration (Figure 5). Presumably the monomers were taken up by the newly formed free barbed filament ends (Figure 4). [While in this experiment the monomer concentration did not fall to the critical concentration in the absence of villin, such a decrease has been observed after more vigorous sonication (data not shown).] Thereafter, the monomer concentration slowly increased again, returning to the original concentration of 1.0 μM after 5 h.

The slow net depolymerization during recovery from sonication was not due to denaturation of actin. This was demonstrated by a second sonication (Figure 5) which resulted in a repeat of the first response. Furthermore, sonication of actin in the absence of villin did not lower the steady-state monomer concentration. Control experiments (in collaboration with Dr. C. Franzini-Armstrong) ruled out that sonication caused transient denaturation of villin. Electron microscopic examination of filament length demonstrated that sonicated villin was capable of preventing restoration of the fragmented filaments to their original length; a process which takes minutes in the absence of villin.

Rate Constants. One of our aims was to obtain a minimum value for the binding constant of villin to barbed filament ends from the data of Figure 3. This requires a value for the ratios

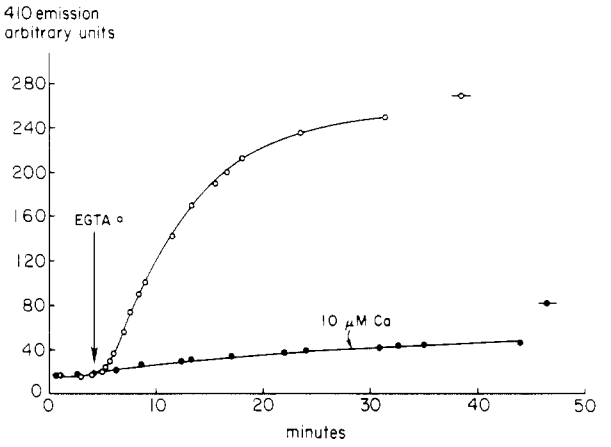

FIGURE 6: Time course of elongation of 2.5 μM pyrene-actin NEM-actin (28% pyrene) onto preformed villin-actin nuclei with and without villin. (●) Polymerization in the presence of 9.9 nM villin, 10 μM calcium, and 10 mM Tris-HCl at pH 7.4; (○) polymerization after inactivation of villin by the addition of EGTA at the arrow. Initiation of polymerization by the addition of $MgCl_2$ and KCl.

of the rate constants for elongation and for depolymerization of the barbed and the pointed ends. They were determined by fluorescence measurements with villin to block the barbed filament end. To determine the ratio of the rate constants in the absence and presence of villin for either elongation or depolymerization, the concentration of nuclei or filaments must be the same under both conditions. The exact values, however, need not be known. In the following, first, the ratios of these rate constants in the absence and presence of villin are reported together with the absolute values for the rate constants of elongation. A surprising result is that the effect of villin on the rates is not consistent with its effect on the critical concentration. Second, the absolute values for the rate constants of depolymerization were calculated in order to discover which of the rate of constants is responsible for the discrepancy. This leads to the conclusion that the rate constant of depolymerization at the barbed filament end during steady state is different from that during the initial phase of net depolymerization.

Polymerization Kinetics. The ratio of the rate constants of elongation in the absence and presence of villin was determined by using as nuclei villin-actin dimers and trimers (Glenney & Weber, 1981) and villin-actin minifilaments (Figure 6). In practice villin was always present, and "absence" or villin means that villin had been inactivated by the addition of EGTA. That is justified by control experiments showing that rates of polymerization in the presence of EGTA were not altered by the addition of villin (Figure 7) and that the rates of polymerization were similar in the presence of EGTA and of 10 μM calcium when villin had not been added. Villin-actin nuclei were generated by the same procedure for both curves in Figure 6 (Materials and Methods). Elongation rates in the absence of villin were measured after the nuclei had elongated to short filaments (average length of 14 monomers, assuming one nucleus for two villin molecules; see below). There was a short lag period of 30–120 s after addition of EGTA before the rate of elongation increased about 10-fold (Figure 8). This may have been the time necessary for villin inactivation. After that, the time course of elongation was described by a single exponential function, suggesting that the nuclei concentration remained constant.

The ratio of the rate constants of elongation k_{+b}/k_{+p} (b and p = barbed and pointed ends, respectively) calculated from the maximal slopes after the lag period was 13 for the ex-

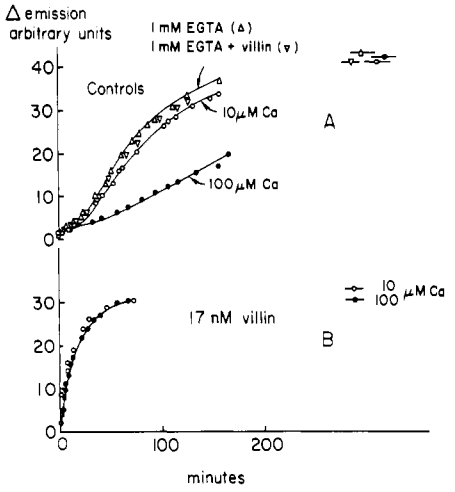

FIGURE 7: Time course of polymerization of 2.4 μM 50% labeled NEM–NBD–actin, with and without villin. (A) Polymerization at different calcium concentrations in the absence of villin (●, ○, △) and when villin was inactivated in the presence of 1.0 mM EGTA (▽). (B) Polymerization in the presence of villin and two different calcium concentrations. Polymerization was initiated by the addition of $MgCl_2$ and KCl, pH 7.2.

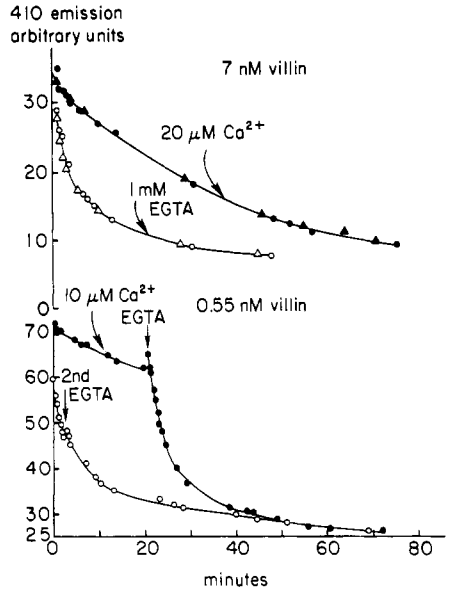

FIGURE 8: Effect of villin on the rate of depolymerization. (Top) Pyrene–actin was diluted from 2.0 to 0.05 μM (●) into 7 nM villin and (○) into medium without villin. All solutions contained 0.5 mM ATP, 10 mM Tris-HCl, pH 7.6, 10 μM calcium, 0.1 M KCl, and 2.0 mM $MgCl_2$. Triangles and circles indicate duplicates, showing the reproducibility of the measurements. Tenfold inhibition of the initial rate of depolymerization by villin. (Bottom) 4.0 μM pyrene–actin plus 47 nM villin in 10.0 μM calcium was diluted to 0.05 μM actin and 0.8 nM villin into medium containing (●) 10 μM calcium or (○) 2.0 mM EGTA. A second addition of EGTA to the control assay (arrow) did not affect the time course of depolymerization (see Materials and Methods). Villin inhibited the initial rate of depolymerization 20-fold. After 20 min villin was inactivated by the addition of EGTA (arrow) to a final concentration of 2.0 mM (closed circles continued).

periment of Figure 6. The average was close to 10 for both types of fluorescent actin (7–15 for pyrene–actin).

Absolute values for the rate constants of elongation in the presence and absence of villin were also calculated. It was estimated that one nucleus was formed for two villin molecules on the basis of electron microscopic measurements (in collaboration with Dr. C. Franzini-Armstrong) which showed that actin filaments with an average length of 40 monomers were produced during polymerization with an actin–villin ratio of

Table I: Rate Constants for Fluorescent Actin[a]

	measurements by			
constants	fluorescence + villin	EM,[b] p	fluorescence − villin	EM,[b] b + p
k_+ (μM^{-1} s^{-1})	0.06–0.32	1.5	0.6–3.2	13.8
observed k_- (s^{-1})	0.08–0.3	0.7	0.9–4.4	2.7
calculated[c] k_- (s^{-1})	0.04–0.35		0.12–0.64[e]	
observed c_∞ (μM)	0.7–1.1	0.6	0.18	
calculated[d] c_∞ (μM)	1.0–1.3	0.5	1.3–1.4	0.2

[a] The values for fluorescent actin refer to both NEM–NBD–actin and pyrene–actin. b and p indicate barbed and pointed ends, respectively. [b] EM, electron microscopy by Bonder et al., (1983). [c] $k_- = c_\infty k_+$. [d] $c_\infty = k_-/k_+$; k_+ and k_- represent either the rate constants of the pointed end of the filament (in the presence of villin) or the sums of the rate constants for both ends (in the absence of villin). [e] A calculated value of 0.04–0.34 for k_{-b} was obtained from $(k_{-b} + k_{-p})_{calcd} - (k_{-p})_{obsd}$, referring to the values in the absence and presence of villin, respectively.

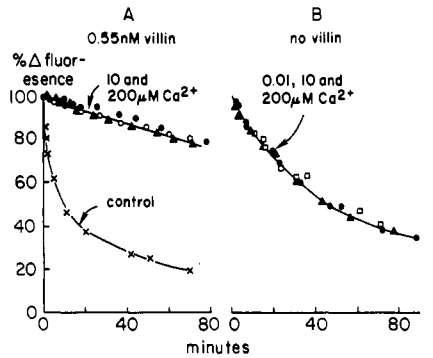

FIGURE 9: Effect of calcium on the rate of depolymerization in the absence and presence of low concentrations of villin. (A) 4.0 μM pyrene–actin (100%) plus 47 nM villin, preincubated with 10 μM calcium, was diluted to 0.05 μM actin and 0.55 nM villin into 1.0 mM EGTA (control, i.e., villin inactivated) or 10 or 200 μM calcium as indicated. (B) 1.0 μM pyrene–actin (100%) was diluted to 0.05 μM into 2 mM EGTA (0.01 μM calcium), 10 and 200 μM calcium as indicated, and 10 mM imidazole, pH 7.4. The control rate in (A) was faster than in (B) because the average filament length was shorter due to the preincubation with villin at a ratio 1/100.

20 as compared to very large filaments in the absence of villin. The rate constants of elongation in the presence and absence of villin were not significantly different for NEM–NBD–actin and pyrene–actin. [Since the rates of elongation of pyrene–actin were not significantly affected by dilution with unlabeled actin (cf. Cooper et al., 1983a,b), the wide spread in values (Table I) is most likely due to differences in the actin preparations themselves rather than the result of modification.]

In order to compare these values with those from other laboratories obtained at different temperatures, we determined the temperature coefficient between 14 and 25 °C to be 3.8 in a single experiment (average of three measurements).

Depolymerization Kinetics. To determine the ratio of the depolymerization rate constants in the absence and presence of villin, depolymerization must always start with the same filament concentration. Three different procedures were used to obtain this initial condition. First, depolymerization was initiated by diluting actin filaments from the same stock solution to well below the critical concentration into a calcium containing solution either with or without villin [Figures 8 (top) and 9B]. For the second procedure, used for the calculation of rate constants, polymerized actin was incubated with villin overnight [Figures 8 (bottom) and 9A]. Villin was thus bound to the filaments prior to depolymerization, and the average filament length was modified by appropriate changes in the

villin/actin ratio. To measure the rate of depolymerization in the presence of villin, the actin was diluted into a calcium-containing medium. To measure the control rate in the "absence" of villin, villin was inactivated by dilution into an EGTA-containing medium. That was justified by control experiments showing, first, that in the presence of EGTA villin did not affect the rate of depolymerization (data not shown) and, second, that in its absence the rate of depolymerization of both pyrene–actin and NEM–NBD–actin was insensitive to the presence of calcium (Figure 9). The third procedure consisted of inactivating villin at various times during the course of depolymerization by the addition of a small amount of concentrated EGTA (Figure 8, bottom).

All three procedures gave comparable results which were similar for NEM–NBD–actin and pyrene–actin. The only difference between the methods was in the length of the lag periods. When actin was diluted into villin, the lag period presumably was the time required for villin binding to the filament ends since the lag period decreased with increasing villin concentrations (data not shown) and was absent after preincubation of the actin with villin. The lag period which was observed sometimes after the addition of EGTA presumably reflected the time required for the inactivation of villin.

Villin reduced the initial rate of depolymerization of different actin preparations 6–20-fold, on the average about 10-fold (Figures 8 and 9), i.e., by the same factor as it reduced the rate of elongation. This reduction of both rates by the same factor was quite reproducible, in spite of the wide spread in the absolute values of the rate constants (Table I) and was observed for both pyrene–actin and NEM–NBD–actin. A reduction by villin of both rates by the same factor is incompatible with the villin-induced 5-fold increase in the critical concentration.[3]

The absolute values for the rate constants of depolymerization were obtained by the second procedure described above and were calculated on the basis of one filament/two villin molecules as described for the rate constants of elongation. In the presence of villin the critical concentration calculated from the ratio of the rate constants of depolymerization and elongation was comparable to the observed critical concentration in the presence of villin(Table I, compare column 1). In the absence of villin, the calculated critical concentration was about 6–7 times greater than the observed critical concentration in the absence of villin (Table I, column 3), suggesting that one of the rate constants at the barbed filament end was not the same under conditions of net change and during steady state. The time course of elongation in the presence of villin was described by a single exponential function until steady state was reached, indicating that the rate constant of elongation was the same under the two conditions. Thus, it appears that the rate constant of depolymerization at the barbed filament end may have been higher under conditions of net depolymerization than during steady state.

The time course of depolymerization is sensitive to the length distribution of the filaments. If the length distribution is exponential, the time course of depolymerization is described by a single exponential function (to be published), and any deviations from that indicate nonuniformity of the actin population or a change of rate constants with time.

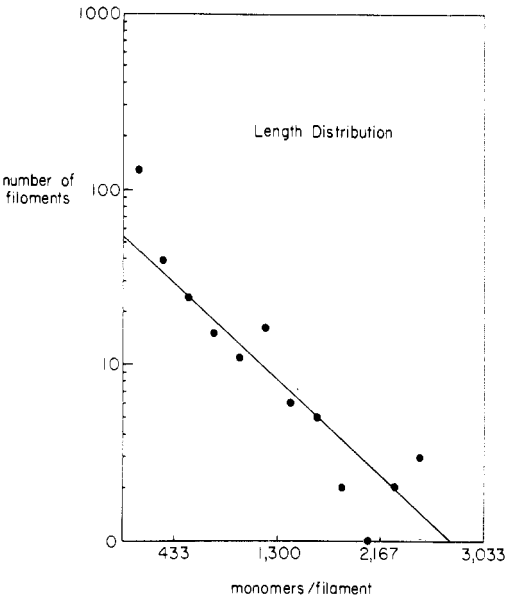

FIGURE 10: Length distribution of 2.0 μM NEM–NBD–actin 2 h after sonication. Ordinate: log filament number. Abscissa: filament length, expressed as monomers per filament. The separate population of short filaments may quite possibly be an artifact, i.e., represent an overestimation of the number of the very short filaments which are difficult to distinguish from background material. The average length of 400 monomers per filament is shorter than described in the literature, possibly because NEM–NBD–actin had not fully recovered from the effects of sonication (cf. Materials and Methods).

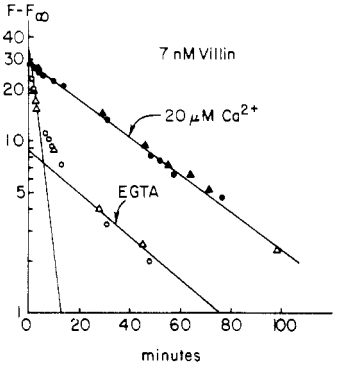

FIGURE 11: Plot of log $F - F_\infty$ of the data of Figure 8 (top).

The length distribution of NEM–NBD–actin was essentially exponential (Figure 10) in agreement with other measurements in the literature (Oosawa & Asakura, 1975; Kawamara & Maruyama, 1972; MacLean-Fletcher & Pollard, 1980; Nunnally et al., 1981).

The time course of depolymerization in the presence of villin was described by a single exponential function (Figure 11). There were only occasional exceptions to this behavior which were attributed to inhomogeneity of that particular actin preparation. In contrast, in the absence of villin the time course of depolymerization could only be described by the sum of two exponentials representing a fast and a slow phase. This occurred with all preparations under all experimental conditions described here. The rate constant of the slow phase was about $^1/_{10}$ of that of the fast phase and very similar to the rate constant in the presence of villin. The fraction of the faster phase varied for different actin preparations between 0.3 and 0.6 of the total.

The explanation for the biphasic time course is not known. However, it is not likely that the fast population represents extra short filaments formed by fragmentation during mixing

[3] Using

$$\frac{c_{\infty(V+)}}{c_{\infty(V-)}} = \frac{k_{-(V+)}/k_{+(V+)}}{k_{-(V-)}/k_{+(V-)}} = \frac{k_{-(V+)}k_{+(V-)}}{k_{-(V-)}k_{+(V+)}}$$

and substituting $k_{+(V-)}/k_{+(V+)} = 0.1$ and $k_{-(V+)}/k_{-(V-)} = 10$, one obtains $c_{\infty(V+)}/c_{\infty(V-)} = 10 \times 0.1$. V+ and V– indicate the presence and absence of villin.

since for villin-capped filaments of the same average length the time course of depolymerization was described by a single exponential (Figure 8, bottom; cf. also Figure 11).

Discussion

It has been well documented that villin in the presence of calcium alters the kinetics of actin polymerization in a very low stoichiometry, i.e., the rate of elongation of actin filaments is inhibited at a villin/actin ratio of 1/1000 (Pollard & Craig, 1982). This stoichiometry reflects that villin acts by binding to the barbed filament ends (Pollard & Craig, 1982; Glenney et al., 1981; Bonder & Mooseker, 1983). More recently it has been shown that the actin monomer concentration also is increased in the presence of villin (Wang et al., 1983); it is shown here that one molecule of villin can prevent the polymerization of more than 1000 monomers of actin.

The titration of the critical concentration of actin with increasing villin concentrations demonstrates that the effect of villin on the critical concentration is entirely explained by the capping of the high affinity end of a polymer with a different monomer binding constant at each filament end. Once all of the barbed ends of the filaments have been capped by villin, a further increase in the villin concentration is without effect. This distinguishes villin from severin (Brown et al., 1982; Yamamoto et al., 1982) whose effect on monomer concentration is not saturable.

The villin effects on actin kinetics were rapidly reversed by removal of calcium unlike the effects of severin (Yamamoto et al., 1982), indicating that the cross-linking of actin by villin which occurs in the absence of calcium does not alter the kinetic properties of the actin molecules. It is noteworthy that the variability in the critical concentrations was much greater in the presence than in the absence of villin when the values were close to 0.2 μM for most actin preparations. Possibly the stability of actin molecules may be lower at the pointed than at the barbed end.

The continuous changes in monomer concentration after the fragmentation of villin-capped filaments reflect treadmilling, i.e., uptake of monomers at the newly formed free barbed ends and release from the pointed ends. As a result of treadmilling the monomer concentration responds to changes in the ratio of free barbed and pointed filament ends, most sensitively so when more than 80% of the barbed ends have been capped. Thus, the monomer concentration rapidly decreased with the sudden increase in the ratio on sonication. The free barbed filament ends then disappeared presumably as the result of filament reannealing[4] and of recapping with villin or villin–actin dimers (Glenney & Weber, 1981) released after complete depolymerization of capped filaments. The time course of the recovery of the steady-state monomer concentration was comparable with the time course of filament depolymerization.

Binding Constant of Villin for the Barbed Ends of Actin Filaments. A minimal value for the binding constant of villin for actin filaments was calculated on the basis of the relationship between critical concentration and the extent of capping. If the ratios of the rate constants between the barbed and pointed filament ends are known, the critical concentration may be calculated for decreasing ratios of $ends_b/ends_p$ according to the expression

$$c = \frac{k_{-b}[ends_b] + k_{-p}[ends_p]}{k_{+b}[ends_b] + k_{+p}[ends_p]}$$

[4] Reannealing is defined as the association of barbed and pointed ends of filaments, i.e., primarily of nuclei and short filaments.

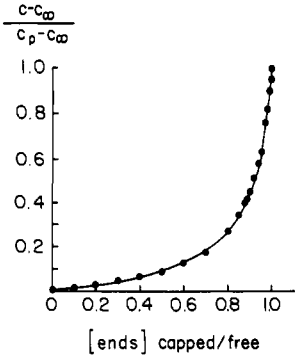

FIGURE 12: Modeled changes in critical concentration with increasing fraction of villin-capped barbed filament ends. Ordinate: $c - c_\infty$ = the increase in the critical concentration (c) over the steady-state value (c_∞) as a fraction of the maximal change in critical concentration ($c_p - c_\infty$) where c_p is the critical concentration of the pointed end. $k_{+b}/k_{+p} = 10$; $k_{-b}/k_{-p} = 1$ (b and p, barbed and pointed ends, respectively). $c_p/c_\infty = 5.6$.

where c = critical concentrations, b and p are the barbed and pointed ends, and k_+ and k_- are the rate constants of elongation and depolymerization. The average value of 10 (Table I, line 1, columns 3 and 1) was used for the ratio of the rate constants of elongation and a value of 1 for the ratio of the rate constants of depolymerization. The latter ratio based on the observed and calculated values for k_{-p} and k_{-b}, respectively (Table I), was chosen because the observed value for k_{-b} was not consistent with the effect of villin on the critical concentration of actin (Table I).

The critical concentration is not very sensitive to a small extent of capping of the barbed filament ends because the value for k_+ at the barbed filament end is much larger than that at the pointed end (Figure 12). About 95% capping of all filaments is necessary for the 70% shift toward the final value of the critical concentration produced by the action of 0.5 nM villin on pyrene–actin (Figure 3). Assuming, first, that villin binding is noncooperative and, second, that practically all of the villin remained free, a minimum value for the apparent binding constant of 1.9×10^{11} M^{-1} is obtained from the equation

$$K = \frac{[\text{capped ends}]}{[\text{free ends}][\text{free villin}]}$$

However, the binding constant may be higher; assuming an average length of 1000 monomers, the filament concentration in 0.5 μM F-actin (the concentration of polymerized actin at 0.5 nM villin in the experiment of Figure 3) would have been 0.5 nM, reducing the free villin to a fraction of the total villin.

A 70% shift of the critical concentration quite reproducibly required 10 times more villin for NEM–NBD–actin than for pyrene–actin. However, that does not necessarily indicate a 10-fold lower apparent binding constant of villin to NEM–NBD–actin. It might reflect a difference in average filament length between the two actin preparations since the concentration of free villin as a fraction of the total villin decreases with increasing number concentration of filaments.

Rate Constants. On the average the rate constants of elongation were about 10 times and the highest values 2.5 times lower than those determined by electron microscopy under otherwise similar conditions using the Bergen–Borisy (1980) extrapolation method. Since the rate constants were not significantly affected by the addition of unmodified actin, the difference most likely is due to differences in the basic actin preparations rather than the result of the modification with fluorescent probes. The most likely difference in preparation is one of age. Sometimes 10 days to 2 weeks had elapsed

between extraction of the acetone powder and the first experiments.

The rate constants of depolymerization obtained by electron microscopy were determined under conditions of net polymerization whereas our values were calculated from initial rates of net depolymerization. Therefore, differences in values between the two methods need not be attributed to differences in actin preparations but may indicate a difference in rate constants under the two experimental conditions.

In the presence of villin the rate constants of depolymerization with NEM–NBD–actin and pyrene–actin were about 3 times and the highest value about 2 times lower than the values reported for the pointed filament end by electron microscopy (Pollard & Mooseker, 1981; Table I). These are the rate constants which were consistent with the measured values for the critical concentrations.

The time course of depolymerization in the absence of villin was not exponential. It is striking that the rate constant for the slow phase of depolymerization was similar to that for the pointed end (Figure 11), suggesting that possibly half to one-third of the filaments were capped at their barbed ends even in the absence of villin either by a muscle-derived capping protein or by denatured actin molecules. The rate constant of depolymerization in the absence of villin calculated from the initial rates was slightly higher on the average, and the highest value was nearly twice as high as the sum of the rate constants obtained by electron microscopy (Table I). Thus, the rate constant of depolymerization from the barbed end is the only rate constant that was higher than the values obtained by electron microscopy. This agrees with the observation that the measured values for $k_{-b} + k_{-p}$ were higher than the calculated values (Table I) and that the effect of villin on the rate of depolymerization was not compatible with its effect on the critical concentration, as described above (Results). It should be emphasized that the latter finding was very reproducible in spite of the scatter in the absolute values of the rate constants given in Table I. This then suggests that at the barbed end the rate constant of depolymerization is higher during net depolymerization than during steady state or during net assembly. This might be explained by a difference in nucleotide content of the terminal actin molecules at the barbed end under the two conditions. Possibly, under steady-state conditions the rate of monomer addition to the barbed end exceeds the rate of phosphate release so that the terminal actin molecules at the barbed end contain ATP whereas under conditions of net depolymerization they contain ADP. A very high degree of asynchrony between phosphate release and actin assembly has been shown by Pardee & Spudich (1982) during actin polymerization in millimolar calcium. It could therefore be that a mechanism for the control of the steady-state critical concentration is through an effect on the rate constants for phosphate release during monomer binding to the filament ends.

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Registry No. Calcium, 7440-70-2.

References

Asakura, S., Taniguchi, M., & Oosawa, F. (1963) *J. Mol. Biol. 7*, 55–69.

Bergen, L. G., & Borisy, G. G. (1980) *J. Cell Biol. 84*, 141–150.

Bonder, E. M., & Mooseker, M. S. (1983) *J. Cell. Biol. 96*, 1097–1107.

Bretscher, A., & Weber, K. (1980) *Cell (Cambridge, Mass.) 20*, 839–847.

Brown, S. S., Yamamoto, K., & Spudich, J. A. (1982) *J. Cell Biol. 93*, 205–210.

Cooper, J. A., Walker, S. B., & Pollard, T. D. (1983a) *J. Muscle Res. Cell Motil. 4*, 253–262.

Cooper, J. A., Bulle, E. L., Jr., Walker, S. B., Tson, T. Y., & Pollard, T. D. (1983b) *Biochemistry 22*, 2193–2208.

Craig, S. W., & Powell, L. D. (1980) *Cell (Cambridge, Mass.) 22*, 739–746.

Craig, S. W., & Pollard, T. D. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 88–92.

Detmers, P., Weber, A., Elzinga, M., & Stephens, R. E. (1981) *J. Biol. Chem. 256*, 99–105.

Frieden, C. (1982) *J. Biol. Chem. 257*, 2882–2886.

Frieden, C., Lieberman, D., & Gilbert, H. R. (1980) *J. Biol. Chem. 255*, 8991–8993.

Glenney, J. R., & Weber, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2810–2814.

Glenney, J. R., Kaulfus, P., & Weber, K. (1981) *Cell (Cambridge, Mass.) 24*, 471–480.

Hill, T. L., & Kirschner, M. W. (1982) *Int. Rev. Cytol. 78*, 1–125.

Kawamura, M., & Maruyama, K. (1972) *J. Biochem. (Tokyo) 72*, 179–188.

Kirschner, M. W. (1980) *J. Cell. Biol. 86*, 330–334.

Korn, E. D. (1982) *Physiol. Rev. 62*, 672–737.

Kouyama, T., & Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33–38.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

MacLean-Fletcher, S., & Pollard, T. D. (1980) *Biochem. Biophys. Res. Commun. 96*, 18–27.

Matsudaira, P. T., & Burgess, D. R. (1982) *J. Cell. Biol. 92*, 648–656.

Mooseker, M. S., Graves, T. A., Wharton, K. A., Falco, N., & Howe, C. L. (1980) *J. Cell Biol. 87*, 809–822.

Mooseker, M. S., Bonder, E. M., Grimwade, B. G., Howe, C. L., Keller, T. C. S., III, Wasserman, R. H., & Wharton, K. A. (1982) *Cold Spring Harbor Symp. Quant. Biol. 46*, 855–869.

Murray, J. M., Weber, A., & Knox, M. K. (1981) *Biochemistry 20*, 641–649.

Nunnally, M. H., Powell, L. D., & Craig, S. W. (1981) *J. Biol. Chem. 256*, 2083–2086.

Oosawa, F., & Asakura, S. (1975) *Thermodynamics of the Polymerization of Protein*, pp 51–55, Academic Press, New York.

Pardee, J. D., & Spudich, J. A. (1982) *J. Cell Biol. 93*, 648–654.

Pollard, T. D., & Mooseker, M. S. (1981) *J. Cell Biol. 88*, 654–659.

Pollard, T. D., & Craig, S. W. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 55–58.

Porter, M., & Weber, A. (1979) *FEBS Lett. 105*, 259–262.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.

Tait, J. F., & Frieden, C. (1982a) *Arch. Biochem. Biophys. 216*, 133–141.
Tait, J. F., & Frieden, C. (1982b) *Biochemistry 21*, 3666–3674.
Tait, J. F., & Frieden, C. (1982c) *Biochemistry 21*, 6046–6053.
Wang, Y., Bonder, E. M., Mooseker, M. S., & Taylor, D. L. (1983) *Cell Motil. 3*, 151–165.
Weber, A., Mooseker, M. S., Walsh, T. P., Bonder, E. M., & Higgins, J. (1982) *J. Cell. Biol. 289a*.
Weber, K., & Glenney, J. R. (1982) *Cold Spring Harbor Symp. Quant. Biol. 46*, 541–552.
Weeds, A. (1982) *Nature (London) 296*, 811–816.
Wegner, A. (1976) *J. Mol. Biol. 108*, 139–150.
Wegner, A. (1982) *J. Mol. Biol. 161*, 607–615.
Yamamoto, K., Pardee, J. D., Reider, J., Stryer, L., & Spudich, J. A. (1982) *J. Cell. Biol. 95*, 711–719.

Disulfide-Bonded Aggregates of Heparan Sulfate Proteoglycans†

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ABSTRACT: Heparan sulfate proteoglycans have been isolated from Swiss mouse 3T3 cells by using two nondegradative techniques: extraction with 4 M guanidine or 2.5% 1-butanol. These proteoglycans were separated from copurifying chondroitin sulfate proteoglycans by using ion-exchange chromatography on DEAE-cellulose in the presence of 2 M urea. The purified heparan sulfate proteoglycans are substantially smaller, ca. M_r 20 000, than those isolated from these same cells with trypsin, ca. M_r 720 000 [Johnston, L. S., Keller, K. L., & Keller, J. M. (1979) *Biochim. Biophys. Acta 583*, 81–94]. However, all of the heparan sulfate proteoglycans extracted by these three methods contain similar glycosaminoglycan chains (M_r 7500) and are derived from the same pool of cell surface associated molecules. The trypsin-released heparan sulfate proteoglycan (ca. M_r 720 000) can be significantly reduced in size (ca. M_r 33 000) under strong denaturing conditions in the presence of the disulfide reducing agent dithiothreitol, which suggests that this form of the molecule is a disulfide-bonded aggregate. The heparan sulfate proteoglycan isolated from the medium also undergoes a significant size reduction in the presence of dithiothreitol, indicating that a similar aggregate is formed as part of the normal release of heparan sulfate proteoglycans into the medium. These results suggest that well-shielded disulfide bonds between individual heparan sulfate proteoglycan monomers may account for the large variation in sizes which has been reported for heparan sulfate proteoglycans isolated from a variety of cells and tissues with a variety of extraction procedures.

The presence of heparan sulfate proteoglycans (HSPGs)[1] on the surface of many cell types, in the culture medium, and in basement membranes and other extracellular matrices has been amply documented (e.g., Kraemer, 1971; Dietrich et al., 1976; Mutoh et al., 1976; Keller et al., 1978; Kanwar & Farquhar, 1979; Hassel et al., 1980; Prinz et all., 1980; Kjellén et al., 1980, 1981). Despite considerable interest in the physiological function of these molecules, detailed structural models needed for the interpretation of structure/function data such as exist for cartilage proteoglycans (Hascall & Hascall, 1981) are generally lacking [however, see Oldberg et al. (1979)]. A major reason for this deficit is the fact that the HSPGs in these various cell culture/tissue compartments have been difficult to rigorously purify and no consensus as to their size currently exists. For example, the HSPG from cell surfaces has been reported to range from ca. M_r 20 000 to ca. M_r 1 000 000 (Johnston et al., 1979; Radhakrishnamurthy et al., 1980; Vogel & Peterson, 1981; Rapraeger & Bernfield, 1983) while that from basement membranes varies from M_r 130 000 to M_r 750 000 (Hassell et al., 1980; Kanwar et al., 1981; Oohira et al., 1983). While species and tissue-specific differences may account for such observations, differences in extraction techniques alone have been reported to yield cell surface HSPGs of differing molecular weights (Oldberg et al., 1977, 1979).

The protein moiety from nonproteolytically extracted cell surface HSPG has been shown to vary from ca. M_r 6000 to ca. M_r 40 000 (Oldberg et al., 1979; Mutoh et al., 1980; Lowe-Krentz et al., 1981). Although some of this variation may be due to polymeric forms (Lowe-Krentz et al., 1981), species and tissue variations may also account for some of these differences. However, since the most commonly used test for purity of HSPG is the susceptibility of the heparan sulfate glycosaminoglycan chain to degradation with nitrous acid, the presence of tightly bound nonglycosylated proteins has generally not been excluded. Additionally, all of the present methods commonly used to remove the carbohydrate chains from the protein moiety contain steps which could result in the alteration of the HSPG core protein by either enzymatic or chemical degradation [e.g., protease contamination of chondroitin ABC lyase (Oike et al., 1980) used to remove contaminating chondroitin sulfate proteoglycans (CSPGs) or acidic conditions during nitrous acid treatment (Cifonelli, 1968; Shively & Conrad, 1976) used to remove susceptible heparan sulfate glycosaminoglycans].

During our studies on the HSPGs from Swiss mouse 3T3 cells and the development of methods to purify the HSPG core proteins, we have employed several extraction methods. These

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[1] Abbreviations: HSPG, heparan sulfate proteoglycan; CSPG, chondroitin sulfate proteoglycan; Tris, tris(hydroxymethyl)aminomethane; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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