Calcium Dependence of Villin-Induced Actin Depolymerization†

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ABSTRACT: "Cutting" of actin filaments by villin was evaluated from the time course of filament depolymerization. Depolymerization was initiated by diluting polymerized actin, labeled with a fluorescent probe on either lysine-374 or cysteine-375, to a concentration well below the critical into a medium containing free villin and various concentrations of calcium (in addition to potassium and magnesium). It was observed that at high calcium concentrations (200 μM) the time course of depolymerization could not be described by the single exponential that defines it at low calcium and low villin levels. Instead, at high calcium, the exponent increased with time and the rate of depolymerization became greater than that of controls in the absence of villin. This contrasts with the inhibition of depolymerization by villin at low calcium. The latter inhibition is a consequence of the capping of the barbed filament end by villin as are the inhibition of filament elongation and the elevation of the critical concentration. Evidence is presented that the effects of villin at high calcium are the result of cutting of the actin filaments by villin. It thus appears that different calcium binding sites control capping and cutting and that the calcium binding sites regulating cutting have a much lower affinity for calcium than the sites regulating capping of the barbed filament ends.

Villin, a major protein associated with the actin bundle of microvilli in the intestinal brush border [cf. Mooseker (1983) and Bretscher (1983)] is a member of a ubiquitous class of calcium-dependent actin-binding proteins that regulate the assembly of actin filaments [cf. Craig & Pollard (1982) and Korn (1982)]. Villin has three calcium binding sites and undergoes a marked conformational change on saturation of these sites with calcium (Hesterberg & Weber, 1983a,b). When the calcium concentration is very low, villin links actin filaments into bundles (Bretscher & Weber, 1980; Mooseker et al., 1980; Matsudaira & Burgess, 1982); at higher calcium concentrations villin in various ways modifies the assembly of monomers into filaments. First, villin "caps" the barbed[1] ends of actin filaments (Glenney et al., 1981a,b; Bonder & Mooseker, 1983). This is, villin blocks elongation and depolymerization from that end and, as a result, raises the critical concentration of actin to that of the pointed end (Wang et al., 1983; Walsh et al., 1984). Second, villin nucleates actin polymerization (Bretscher & Weber, 1980; Mooseker et al., 1980). Third, when villin is added to already assembled actin filaments, it causes a rapid shortening of the filaments (Bretscher & Weber, 1980; Mooseker et al., 1980; Craig & Powell, 1980), a process that has been described as the "cutting" or "severing" action of villin (Glenney et al., 1981a,b; Pollard & Craig, 1982; Bonder & Mooseker, 1983).

In preliminary experiments, using a fall in viscosity as an indication for filament shortening, it was noted (Mooseker et al., 1980) that cutting required higher calcium concentrations than did nucleation of polymerization. In the experiments described here,[2] we have used fluorescently labeled actin to compare the calcium requirements for filament capping and cutting. Our results indicate that the calcium binding sites regulating the cutting activity of villin are different from those that control capping and that the regulatory sites for cutting have a much lower calcium affinity than those for capping.

Materials and Methods

Protein Preparations. Actin, prepared according to Spudich & Watt (1971) as modified by Murray et al. (1981), was labeled with *N*-ethylmaleimide (NEM) and 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD) to obtain NEM–NBD–actin (Detmers et al., 1981) or with pyrene (Kouyama & Mihashi, 1981) as described previously (Walsh et al., 1984). The proteins were stored at 4 °C for up to 2 weeks.

Villin was prepared according to Mooseker et al. (1980). The purified protein was either used within 2 weeks or stored in liquid nitrogen after dialysis against 75 mM KCl–10 mM imidazole buffer, pH 7.2. Preparations were frozen only once.

Tropomyosin was prepared according to Eisenberg & Keilly (1974) as modified by Clarke et al. (1976).

Protein concentrations of villin and actin were determined either by Lowry's method (1951) or in the case of actin and tropomyosin by absorbance, using for actin an E_{290} (Wegner, 1976) of 24 900 M^{-1} cm^{-1} and for tropomyosin an E_{276} of 24 500 M^{-1} cm^{-1} (Wegner, 1979).

Depolymerization Measurements. For these measurements undiluted (by native actin) labeled actin [cf. Frieden (1982) and Walsh et al. (1984)] was used in order to obtain sufficient intensity of the fluorescence measurements at the very low actin concentrations necessary for these experiments (0.05 μM). The critical concentration of each actin preparation in the absence and presence of villin was determined as previously described (Walsh et al., 1984). Depolymerization was measured by following the decrease in fluorescence of the labeled actin, using a Perkin-Elmer double-beam fluorometer, Model MPF-3L, as previously described (Walsh et al., 1984). Depolymerization measurements were carried out at 14.5 °C in polymerization solution (0.5 mM ATP, 10 mM buffer as indicated, 0.1 M KCl, 2.0 mM $MgCl_2$, and calcium as indicated). Partial depolymerization was initiated by the addition of villin to polymerized actin at steady state in the presence of calcium. Complete depolymerization was initiated by dilution of actin to a concentration (0.05 μM) well below the critical concentration (0.2 μM in the absence of calcium-ac-

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[1] Barbed and pointed ends are named after the appearance of myosin-decorated actin filaments; the barbed filament end is the high-affinity (for monomers) and rapidly reacting end.

[2] A summary of this work has been presented (Walsh et al., 1984).

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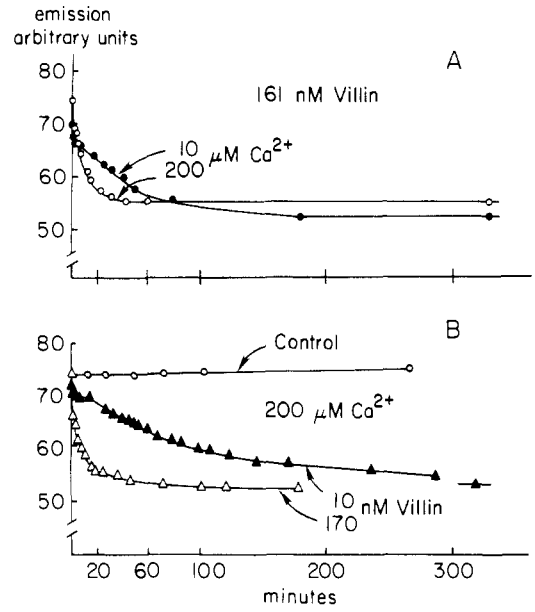

FIGURE 1: Adjustment of the monomer concentration on the addition of villin to polymerized NEM–NBD–actin. (A) Constant villin, calcium varied; (B) constant calcium, villin varied. In all cases, the fluorescence decreased by an amount corresponding to an increase of 0.3 μM in the monomer concentration. 1.3 μM NEM–NBD–actin; 10 mM Tris-HCl, pH 8.0; 15 °C.

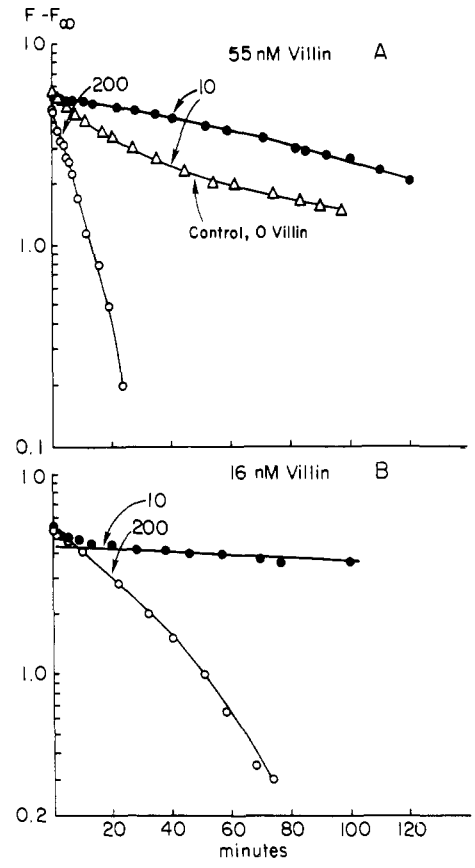

FIGURE 2: Effect of raising calcium from 10 to 200 μM on the rate of depolymerization of pyrene–actin in the presence of villin. Ordinate: log (fluorescence – fluorescence at final steady state). 1.0 μM pyrene actin was diluted to a final concentration of 0.05 μM into polymerizing solution containing 0 (control), 16, or 55 nM villin, respectively, and either 10 or 200 μM calcium (numbers on curves indicate calcium concentrations). 10 mM imidazole buffer, pH 7.4; 14.5 °C. At low calcium and low villin [(B) 10 μM calcium], the initial rate of depolymerization usually remained at control values for a relatively short period, possibly the time required for villin to cap the barbed filament ends (Walsh et al., 1984). Depolymerization in the absence of villin had not proceeded far enough to show the final slope of the slow phase, which usually equals the single slope of villin-capped actin (Walsh et al., 1984), i.e., the upper curve in Figure 2B.

tivated villin and 0.8–1.0 μM in its presence) into media containing free villin and varying concentrations of calcium. Control rates of depolymerization (not affected by villin) were measured either in the presence of villin plus EGTA,[3] when villin has no effect on actin polymerization (Walsh et al., 1984), or in the absence of villin. Since the rate of depolymerization depends on the number of filament ends, we tried to minimize filament breakage during the dilution step by adopting a very gentle stirring procedure. It consisted of 25–30 slow vertical strokes with a plastic spoon. Reproducible rates of depolymerization were obtained by this procedure (Walsh et al., 1984).

Villin-pretreated tropomyosin–actin was prepared in the following manner. Polymerized pyrene–actin (11 μM) was incubated with 100 nM villin for 24 h. Then tropomyosin was added to a final molar ratio of tropomyosin/actin of 2/7. [Tropomyosin was added in such excess as a precaution because among the wide range of values for the tropomyosin extinction coefficient found in the literature the highest was used here, based on Wegner's measurements (1979).] After another incubation period of 24 h, depolymerization was initiated by about a 100-fold dilution into polymerization medium containing 20 nM villin, 200 μM calcium, and 0.4 μM excess tropomyosin necessary to prevent dissociation of tropomysin at these low protein concentrations (Mak et al., 1983).

Results

The addition of villin to polymerized NEM–NBD–actin in the presence of calcium caused a fall in the fluorescence intensity, indicating partial depolymerization of the actin filaments (Detmers et al., 1981), and an increase in the monomer concentration to a new steady-state level (Figure 1). The new value for the critical concentration in the presence of villin was not affected either by increasing the calcium concentration from 10 to 200 μM (Figure 1A) or by increasing the villin/actin ratio from 1/130 to 1/7.5 in the presence of 200 μM calcium (Figure 1B). The rate of depolymerization, however, was considerably increased by either change. Whereas the new value for the critical concentration in the presence of villin is determined solely by the extent of capping of the barbed filament ends (Walsh et al., 1984), the rate of depolymerization also reflects the number concentration of pointed filament ends present during depolymerization.

In order to compare the effects of villin on the rate of depolymerization at different calcium concentrations, further measurements were made at below critical actin concentrations where depolymerization takes place without simultaneous reassociation of actin monomers. After it had been established that both NEM–NBD–actin and pyrene–actin gave comparable results, we used for the subsequent experiments pyrene–actin (Kouyama & Mihashi, 1981) because its fluorescence change on depolymerization is much larger than that for NEM–NBD–actin. The rate of depolymerization in the presence of villin was faster at 200 than at 10 μM calcium (Figure 2). Whereas at 10 μM calcium villin inhibited the rate of depolymerization 6–10-fold (Walsh et al., 1984), at 200 μM calcium 16 nM villin increased the rate of depolymerization above that of the control without villin.

At concentrations of free villin (i.e., not bound to barbed filament ends) at and above 1.0 nM, the time course of de-

[3] Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

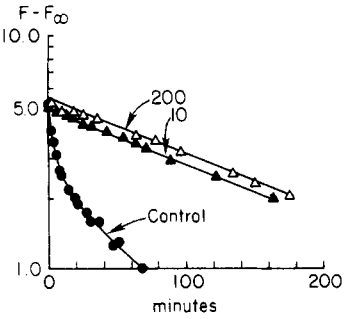

FIGURE 3: Logarithm of the fluorescence change of pyrene–actin during depolymerization after 24-h preincubation of actin filaments (4.0 μM) with villin (55 nM). The mixture was diluted 100-fold into polymerizing solution (10 mM imidazole, pH 7.4), 14.5 °C, containing either 10 μM or 200 μM calcium or 1.0 mM EGTA (control).

polymerization was significantly altered by raising the calcium concentration from 10 to 200 μM: depolymerization accelerated with time at the higher calcium concentration. Provided the distribution of filament length is exponential, the time course of depolymerization should theoretically be described by a single exponential function[4] [cf. Johnson & Borisy (1977)], with the value for the exponent given by the product of the rate constant and the number concentration of the free ends of filaments,[5] disregarding oligomers that are disassembled during the mixing period. A single exponential function was indeed observed at low calcium and low to moderate villin concentrations (Figure 2B) (Walsh et al., 1984).

A single exponential function was observed even at 200 μM calcium when virtually all villin was bound to barbed filament ends (Figure 3). This was achieved by a 24-h preincubation of 4 μM F-actin with villin prior to the initiation of depolymerization by an 80-fold dilution of the precapped actin filaments. If, however, free villin and 200 μM calcium were present together during depolymerization, then the time course of depolymerization was no longer described by a single exponential (Figure 2B). Instead, the value for the exponent showed an increase with time. An increase in the value of the exponent with time was also observed at low calcium Figure 2A), when the concentration of free villin was very high. This deviation from a single exponential in the time course of depolymerization was very different from that exhibited by control actin. In the absence of either villin or calcium, two phases, an initial fast followed by a slow phase, were observed that could be described by two exponentials (Figure 2A).

The acceleration of polymerization with time is probably the result of the "cutting" action of villin. That view is supported by the data of Figure 4 showing no acceleration of depolymerization when actin filaments were saturated with tropomyosin. It has been shown directly by electron microscopy that villin does not cut tropomyosin-containing actin filaments (Mooseker et al., 1982). For the experiment of Figure 4, villin-preshortened actin and tropomyosin–actin (for details, see Materials and Methods) were diluted into a medium containing free villin. In the presence of 200 μM calcium and 20 nM villin, the exponent describing the time course of depolymerization of the tropomyosin–actin filaments did not increase with time (Figure 4) and had a much lower value than that of actin filaments free of tropomyosin. After inactivation of the capping action of villin by removal of calcium, the rate of depolymerization of the tropomyosin–actin filaments increased considerably (Figure 4), returning to control values without villin (data not shown here).

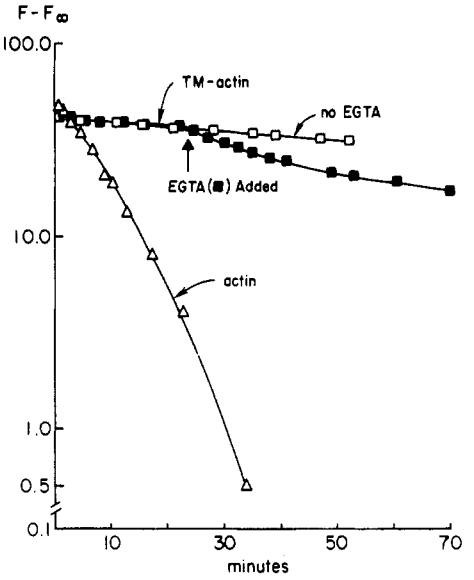

FIGURE 4: Protective action of tropomyosin against the acceleration of depolymerization by villin at high calcium. Ordinate: log (fluorescence – fluorescence at final steady state). 11 μM pyrene–actin or tropomyosin–pyrene–actin plut 100 nM villin was 100-fold diluted into polymerizing solution containing 20 nM villin and either no tropomyosin (actin) or 0.4 μM tropomyosin excess (TM–actin) and 200 μM calcium [the critical concentration of actin is not affected by the presence of tropomyosin (Walsh & Wegner, 1980)] (10 mM Tris-HCl, pH 7.6), 14.5 °C; when added, EGTA was 2.0 mM.

Discussion

These data suggest that the capping and the cutting activities of villin are regulated by different calcium binding sites. The calcium binding sites regulating the capping activity of villin apparently are saturated at 10 μM calcium. This is suggested by the observation that raising the calcium concentration from 10 to 200 μM does not alter the effects of villin on the critical concentration and on the rate of elongation (Walsh et al., 1984). It is proved by the demonstration that raising the calcium concentration from 10 to 100 μM does not affect the villin concentration, which produces half of the maximal increment of the critical concentration (Walsh et al., 1984). In this case the critical concentration responds sensitively to an increase in the concentration of the (high-affinity) calcium–villin complex, which theoretically could be achieved either by increasing the villin concentration or by increasing the calcium saturation of only partially saturated villin. Since the critical concentration was affected by an increase in villin but not by an increase in the calcium concentration, the villin must have been saturated with calcium at the site controlling capping. Preliminary calcium titrations of capping activity suggest a K_D of less than 0.1 μM. Saturation of this (these) villin calcium binding site(s) with 10 μM calcium is in fair agreement with the binding constants reported by Hesterberg & Weber (1983b) for the three calcium binding sites on villin, one slowly exchanging very tightly binding site and two rapidly exchanging sites (3–7 μM).

The villin-induced acceleration of actin depolymerization with time increased on increasing the calcium concentration from 10 to 200 μM. Such an increase in the calcium concentration has no effect on depolymerization in the absence of villin (Walsh et al., 1984). It appears very likely that this effect of villin is a manifestation of its cutting action and the ensuing increase in the concentration of filament ends for the following reasons. First, in collaboration with Clara Fran-

[4] To be published elsewhere.

[5] $dG/dt = e^{-(k_b-[a_b]+k_p-[a_p])t}$ (b and p = barbed and pointed; a = ends of polymers, i.e., $\sum M_j$, ignoring oligomers; G = monomeric actin).

zini-Armstrong, we have observed that the rate of depolymerization was directly proportional to filament number when the number was varied by polymerizing actin at different villin/actin ratios [enabling us previously to determine rate constants by controlling the filament number with villin (Walsh et al., 1984)]. Second, villin did not accelerate depolymerization when it acted on tropomyosin–actin filaments, which have been shown to be protected from the cutting action of villin (Mooseker et al., 1982). Third, even at 200 μM calcium the rate of depolymerization was very slow throughout its whole time course (described by a single exponential) when practically all of the villin was bound to the barbed ends of the actin filaments prior to the initiation of depolymerization as in the experiment of Figure 3. It is known that after prolonged incubation of actin filaments with villin a population of shortened (in proportion to the villin/actin ratio) capped filaments is created (Pollard & Craig, 1982). Most of the villin would be expected to remain bound to the filament ends and thus not be available for cutting even after the dilution to initiate depolymerization, because of the very high binding constant of villin for barbed filament ends [$>10^{11}$ M^{-1} (Walsh et al., 1984)].

The location of the low-affinity calcium binding site(s) regulating cutting has not yet been determined. Preliminary calcium titrations of the cutting activity revealed a complex response to calcium; the rate of cutting increased with increasing calcium concentrations up to 1.0 mM, the highest concentration used. Such a low calcium binding constant could not have been detected by the direct calcium binding measurements on villin by Hesterberg & Weber (1983). Furthermore, one must also consider the possibility that these sites might be on actin. Studies are under way to attempt to identify the binding site(s) and to determine their affinity for calcium.

The more gradual cutting of actin filaments at low calcium (as compared to that at high calcium) and relatively high villin concentrations reflect the fractional calcium saturation of the low-affinity sites, as suggested by the preliminary calcium titrations of cutting activity. If the cutting actin–villin complex were of low affinity, compatible with the large effect of increasing villin shown in figure 2, then increasing the concentration of total free villin should result in increased formation of the cutting villin–actin complex: either by raising the concentration of (low affinity) calcium–villin or by increasing the binding of villin to calcium–actin.

The possible physiological significance of the separate regulation of capping and cutting has been discussed elsewhere (Mooseker, 1984).

Acknowledgments

We are grateful to C. L. Fishkind for the villin preparations. We thank John M. Murray for his help with the manuscript.

Registry No. Calcium, 7440-70-2.

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