REGULAR ARTICLES

Mechanism of Action of Cytochalasin:
Evidence That it Binds to Actin Filament Ends

SUSAN S. BROWN and JAMES A. SPUDICH
Department of Structural Biology, Sherman Fairchild Center, Stanford University School of Medicine, Stanford, California 94305. Dr. Brown's present address is the Department of Anatomy, The University of Michigan Medical School, Ann Arbor, Michigan 48109.

ABSTRACT   To test the idea that cytochalasin retards actin assembly by binding to filament ends, we have designed a new assay for cytochalasin binding in which the number of filament ends can be varied independently of the total actin concentration. Actin is reacted with polylysine-coated polystyrene beads to make filament ends (Brown and Spudich, 1979, J. Cell Biol. 80:499-504) and then reacted with [^{3}H]cytochalasin B. We have found that cytochalasin B binds to beads in the presence of actin, and that the number of cytochalasin B binding sites can be varied as a function of the number of filament ends independent of the total actin concentration by varying the bead concentration.

Recently, it has been discovered by a number of laboratories (1, 4, 5, 9, 10) that cytochalasins inhibit the rate of actin assembly. Far less than equimolar amounts of cytochalasin are required to achieve this inhibition; correspondingly, cytochalasin binds to actin filaments with a stoichiometry of one site per 500 to 10,000 actin monomers (4–6, 9), or one binding site per 1–30 μm of filament.[1] It seems likely that cytochalasin binds to the actin at the filament end where assembly is taking place, as already suggested (1, 4, 5, 9, 10, 12). The critical experiment to test this hypothesis is to show a direct relationship between the amount of cytochalasin bound and the number of filament ends. Until this report, it has not been possible to systematically vary the number of actin filament ends. One must consider the possibility that cytochalasin is not binding to actin at all, but instead is binding to some nonactin species which would only need to contaminate an actin preparation at a level of 0.01–0.2%, given the above values. Thus, it is crucial not only to vary the number of actin filament ends, but also to do so without varying the concentration of the actin or putative contaminants.

In this report, we have varied the number of filament ends while holding the total actin concentration constant, and have demonstrated that cytochalasin binding is indeed a function of the number of actin filament ends. We have been able to achieve this goal by using polylysine-coated beads (3).

MATERIALS AND METHODS

Actin from Dictyostelium discoideum was purified by the method of Uyemura et al. (14), and was disassembled as previously reported (3, 4).

Polylysine was covalently linked to itself while bound to polystyrene beads (via glutarladehyde cross-linking of the amino groups). This caused the polylysine to become permanently associated around the bead (assayed using polylysine made radioactive by reductive alkylation with [^{3}H]borohydride; reference 11).

The procedure was as follows: 1 vol of 0.11-μm polystyrene beads (Sigma Chemical Co., St. Louis, Mo.; 10% solids) or 10 vol of 1.1-μm polystyrene beads (Dow Chemical Co., Midland, Mich. 10% solids) were added to 50 vol of 5 mg/ml polylysine (Sigma Chemical Co.; type IB) while vortexing. The mixture was stirred at 4°C for 3 h to overnight. The beads were sedimented (10,000 rpm, 10 min, in an SS-34 rotor for 1.1-μm beads; 40,000 rpm, 20 min in a type 65 rotor for 0.11μm beads) and resuspended by sonication in 2,000 vol of 0.1 M potassium phosphate buffer, pH 6.2. While stirring in the hood, sodium cyanoborohydride (Sigma Chemical Co.) was added to ~10^{-3} M, and glutaraldehyde to 1.6 × 10^{-4} M immediately thereafter. Beads were then sedimented and resuspended in 200 vol of methanol to which ~0.2 M sodium borohydride was then added to reduce any remaining aldehydes. Beads were washed well with water and stored in 0.02% sodium azide.

Bead concentration is expressed as milligram per milliliter polystyrene, which was measured spectrophotometrically after dissolving the beads in dioxane (16). Number of beads per milliliter was obtained in the case of 1.1-μm beads by counting in a hemocytometer. The 0.11-μm beads are too small to be counted in the light microscope. Therefore, they were mixed with a known concentration of 1.1-μm beads and counted in the electron microscope. The number of beads per milliliter can also be calculated from the milligram per milliliter of polystyrene (density = 1.05). The calculated number agreed with the measured number for

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[1] While all the binding data agrees to the extent that a low number of cytochalasins bind per filament, the range of values obtained in different laboratories is rather broad. It is worth noting that in our hands, average filament length is a function of how the actin is purified; our present procedure yields Dictyostelium actin filaments >10 μm long, whereas a different procedure yields filaments <2 μm long (14). This variation may be the result of trace contaminants which determine length. (see also reference 6).

the 0.11-μm beads, but was 40–73% of the measured number for the 1.1-μm beads (two batches). Therefore, there is error in the measurement of polystyrene concentration and/or in the bead counting.

Monomeric (G) actin was mixed with polylysine-coated beads in 3 mM imidazole, pH 7.5, 0.2 mM dithiothreitol, 0.1 mM ATP (G buffer). Assembly to filamentous (F) actin was induced by raising the salt concentration to 0.1 M KCl and 0.1 mM MgCl₂ (F buffer).

The binding of actin to beads was followed using [³⁵S]actin labeled in vivo (12). Cytochalasin binding was followed using [³H]cytochalasin B (CB) (New England Nuclear, Boston, Mass.). Binding was assayed in 80 μl total reaction mix by sampling 10-μl aliquots and comparing total counts per minute vs. counts per minute in the supernate after centrifugation for 5 s at 100,000 g in a Beckman Airfuge (Beckman Instruments, Spinco Div., Palo Alto, Calif.) to pellet the beads. The "moles CB bound" shown in Scatchard plots (Figs. 2 and 3) are per 10 μl.

RESULTS

Actin Monomer Binds to Polylysine-coated Beads to Make Nuclei from Which Filaments Can Grow

We have previously reported that polylysine-coated beads accelerate nucleation of the assembly of actin filaments in F buffer (3). To investigate the mechanism, we examined the interaction of actin with beads in G buffer. Filaments do not assemble under these conditions, but monomers adsorb to the beads; we will refer to this as "directly bound actin."

First, we improved the beads by covalently coupling the polylysine coating around them (see Materials and Methods). This removed the complication that polylysine could slowly desorb from the beads.

Next, we mixed monomeric [³⁵S]actin with polylysine-coated beads in G buffer, and sedimented the beads (Fig. 1). We find that monomers bind to the bead and that the bead is saturated at ~4 × 10⁵ actin molecules/1.1-μm bead (two determinations gave 3.6 × 10⁵ and 5.0 × 10⁵) and 4 × 10³ actin molecules/0.11-μm bead. Thus, roughly the same number of actins are directly bound per unit surface area to the large and small beads. This amount of actin is enough to cover the bead surface approximately twice, based on an area of 25 nm² occupied per actin monomer. Because of the range of possible error in bead quantitation (see Materials and Methods), we do not wish to suggest that the beads bind exactly a bilayer of actin, but only that the actin is close-packed at the bead surface. This finding supports the idea that beads accelerate nucleation of actin assembly by bringing monomers close together at the bead surface. This would be expected to facilitate the interaction of the directly bound actin monomers to form nuclei from which filaments can grow when assembly is induced by addition of salt. According to this interpretation, actin can become associated with beads in two ways: it binds directly to the polylysine at the bead surface, even in low salt; and, upon addition of salt, the remaining free actin can form filaments by assembling onto those directly bound actin molecules that have interacted to form nuclei.

Actin Bound to Beads Binds Cytochalasin

The binding of [³H]CB to beads ± actin was assayed (Fig. 2). A Scatchard plot of the data revealed high affinity binding (K_d = 6 × 10⁻⁸ M) when actin filaments were grown onto the beads. There was no high affinity binding to beads in the absence of actin (Fig. 2).

High affinity binding to the directly bound actin alone could be observed when the remaining free actin was removed before salt was added (Fig. 3, open circles). There were slightly fewer
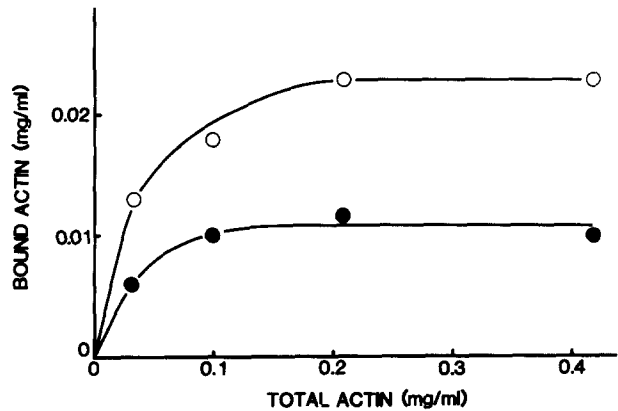

FIGURE 1 Binding of actin to polylysine-coated beads. 0.24 mg/ml (closed circles) or 0.48 mg/ml (open circles) 1.1-μm beads were mixed with 0.03-0.42 mg/ml ³⁵S-labeled actin in G buffer. The beads were sedimented after 20 min and the supernate counted to determine binding. The same curve was obtained if the beads were sedimented immediately, indicating that the binding has reached equilibrium. At saturation there is 0.05 mg actin bound per mg beads.
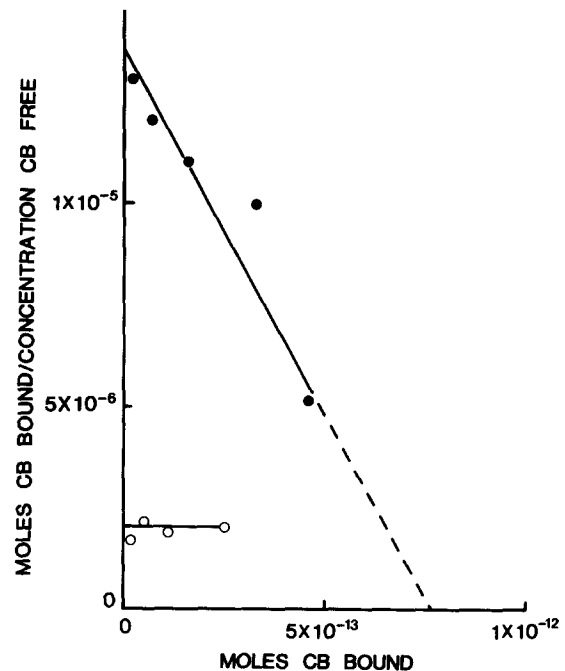

FIGURE 2 Scatchard plot of the binding of cytochalasin to beads with actin filaments. 0.4 mg/ml actin was mixed with 2 mg/ml 1.1-μm beads (closed circles) in G buffer and the salt raised to F buffer conditions to induce assembly. After 1 h at 22°C, 5 × 10⁻⁹ to 1 × 10⁻⁷ M [³H]CB was added, the beads were sedimented, and the supernate counted to determine binding. A K_d of 6 × 10⁻⁸ M was obtained. In the control experiment (open circles) actin was omitted.

binding sites under these conditions, but approximately the same K_d (4 × 10⁻⁸ M) was obtained as when filaments were assembled onto the beads (Fig. 3, compare open and closed circles). In this case there are no visible filaments, and CB is presumably binding to the nuclei formed at the bead surface, which have the conformation of actin filament ends. Salt is required either for nucleus formation or for the binding of cytochalasin, since no high affinity binding is seen in the absence of salt (Fig. 3, triangles).
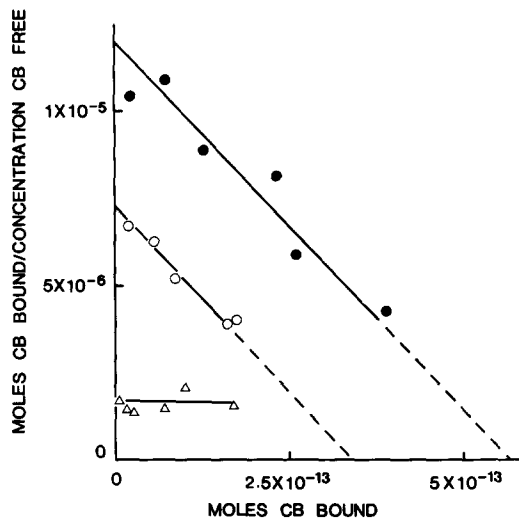
Figure 3 Scatchard plot of the binding of cytochalasin to actin directly bound to beads (open circles) vs. beads with actin filaments (closed circles). 0.4 mg/ml actin was mixed with 2 mg/ml 1.1-μm beads in G buffer and divided into three aliquots. The first aliquot was brought to F buffer conditions and assembled for 1 h, at 22°C (closed circles). The other two aliquots were centrifuged, and the beads resuspended in G buffer. One aliquot of washed beads was brought to F buffer conditions for 1 h at 22°C (open circles), and the other aliquot remained in G buffer (triangles). CB (5×10^{-9} M to 1×10^{-7} M) was added to all three aliquots for 20 min at 22°C, and the beads sedimented to determine binding. Beads in F buffer (with filaments; closed circles) and washed beads in F buffer (with directly bound actin; open circles) have a K_d of 4×10^{-8} M. Washed beads in G buffer (triangles) show no high affinity binding.

Cytochalasin Binding to Beads is a Function of the Number of Actin Filament Ends

To show that cytochalasin binding is a function of the number of filament ends, we wanted to vary the number of ends while holding the actin concentration constant. This was accomplished by varying the concentration of the beads. When filaments were assembled onto increasing concentrations of beads while the actin concentration was held constant at 0.2 mg/ml, there was apparently no change in the number of filaments/bead.[2] Thus the total number of filaments should increase linearly with bead concentration. Because the actin concentration is held constant, the filament length must get shorter; Fig. 4 confirms that this is the case.

This experiment was repeated, and beads assayed for bound actin and bound CB; the results are consistent with our expectations (Fig. 5). The open circles indicate that almost all of the actin is bound at all bead concentrations. At lower bead concentrations the number of high affinity CB-binding sites is seen to increase linearly with increasing bead concentration (Fig. 5, closed circles). However, at bead concentrations greater than ~4 mg/ml, the number of CB-binding sites starts to decrease. This decrease coincides with a slight increase (from 94 to 100%) in actin binding. A likely explanation for these changes is that there is no longer enough actin to saturate all of the bead surface. Thus the actin binding increases slightly

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[2] There were 25 ± 18 filaments per bead at 1 mg/ml beads, 26 ± 9 at 2 mg/ml beads, and 30 ± 11 at 3 mg/ml beads. It should be noted that since the standard deviation is large, and also the amount of F-actin seen is less than expected from the concentration of actin used, these numbers are consistent with but do not prove that the number of filaments/bead stays constant.

presumably because the critical concentration of monomer formerly in equilibrium with the filaments on the bead now becomes adsorbed to the bead. We suggest that the CB binding decreases as a result of the loss of close packing of actin monomers on the bead surface and therefore of the ability to make nuclei or filament ends.

To confirm that actin was indeed becoming limiting at the breakpoint in Fig. 5, we performed the experiment in a different way (Fig. 6). We again mixed actin with beads, but washed the beads to remove unbound actin before the addition of salt. As indicated earlier (Fig. 3), high affinity CB binding to this directly bound actin is seen under these conditions. Fig. 6 shows that when the assay is performed in this way, the amount of actin bound increases linearly with increasing bead concentration until all of the actin is bound. The amount of CB bound also increases at lower bead concentrations. At the point that all of the actin is bound, the amount of CB bound begins to decrease. Because the decrease coincides with the point where the bead is no longer saturated with directly bound actin, presumably there are fewer actins in close apposition which can interact and form nuclei to which CB can bind.

Ratio of Cytochalasin-Binding Sites to Actin Filament Ends

If the above arguments are correct, one would expect that the number of cytochalasin-binding sites would be equal to or a small multiple of the number of filament ends. Because we can measure the number of cytochalasin-binding sites and can obtain an approximation of the number of filaments, we are in a position to calculate an estimate of the number of binding sites per filament end. It proved difficult to obtain a precise number; however, the stoichiometry is consistent with cytochalasin binding reflecting the number of filament ends.

The number of filaments per bead was determined by electron microscopy. The smaller 0.11-μm beads were used since the amount of filament obscured by traversing the circumference of the bead and/or by the halo of stain around the bead
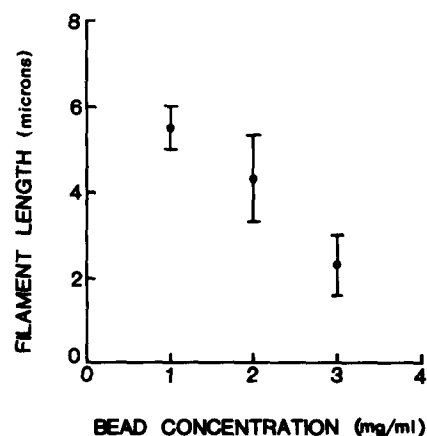

BEAD CONCENTRATION (mg/ml)

Figure 4 Actin (0.2 mg/ml) was assembled in the presence of the indicated concentrations of 1.1-μm polylysine beads. Samples were incubated 10 min at room temperature, then rinsed with 50 mM $MgCl_2$ followed by 1% aqueous uranyl acetate. The beads served as internal size standards. The bars indicate one standard deviation from the average (points). We examined six to eight beads per point. We do not wish to imply a linear relationship between bead concentration and filament length; filament length should be an exponential function (if beads are doubled, filament length should be halved) corrected for the linear increase in actin directly bound as bead concentration increases.
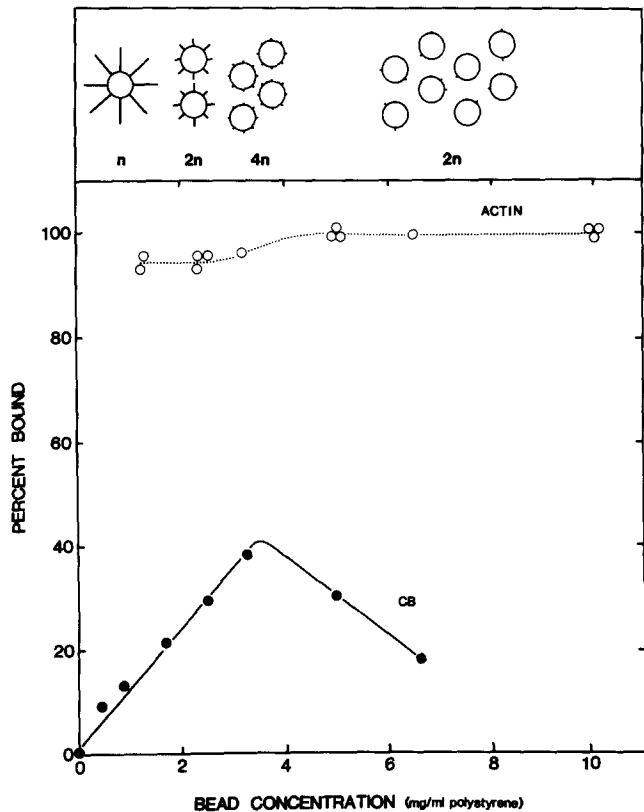
FIGURE 5 Cytochalasin binding to actin filaments as a function of bead concentration. 0.2 mg/ml ^{35}S-labeled monomeric actin was mixed with 0–10 mg/ml 1.1-μm beads in F buffer and incubated at 22°C for 1.6 h. (Assembly is complete within 5 min; reference 3). In some cases, CB was added (10^{-8} to 10^{-6} M) after assembly; this did not affect actin binding. The beads were sedimented, and actin binding (dotted line, open circles) determined by counting the supernate. In a parallel experiment, nonradioactive actin was assembled off 0–6 mg/ml beads for 0.7 h, and 3×10^{-8} M [^{3}H]CB was added. Beads were sedimented and the supernate counted to determine binding of [^{3}H]CB. Controls were run in the absence of actin, and background binding to beads was subtracted from binding in the presence of actin to give corrected [^{3}H]CB binding (closed circles). At the top is a diagrammatic representation of the predicted number of filament ends (n) as a function of bead concentration.
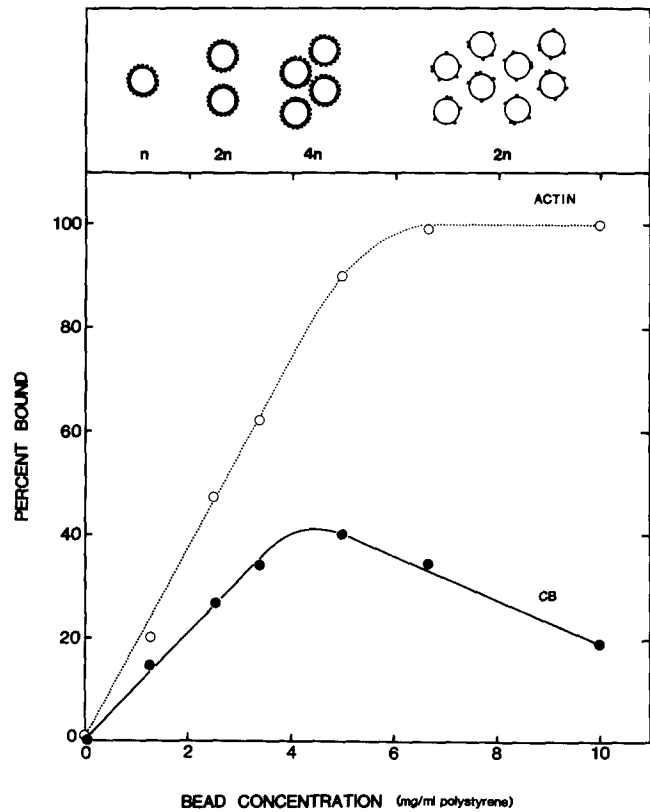

FIGURE 6 Cytochalasin binding to actin directly bound to beads as a function of bead concentration. 0.2 mg/ml ^{35}S-labeled G actin was mixed with 0–10 mg/ml 1.1-μm polylysine beads at 22°C. Beads were sedimented, and the supernate counted to determine actin binding (dotted line, open circles). The beads were then resuspended in 10^{-8} M [^{3}H]CB in F buffer and sedimented again to determine cytochalasin binding (closed circles). This curve was corrected for background binding in the presence of excess unlabeled CB (10^{-6} M), which increased linearly with bead concentration (3.2%/mg beads), and for ^{35}S cpm in the tritium channel. The top panel represents the predicted changes in number of filament ends (n) as the bead concentration is increased.
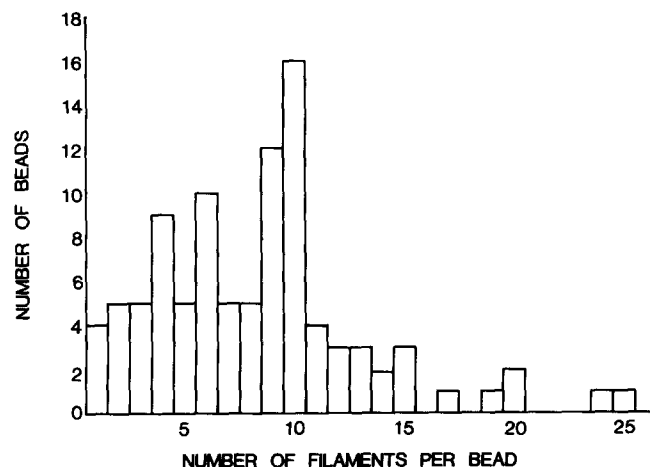
is minimal. We calculated from measuring average length and number of filaments that the amount of F-actin seen using 0.11-μm beads was approximately that expected for the concentration of actin used. However, one might still expect to miss any filament shorter than ~0.1 μm using 0.11-μm beads. Furthermore, such short filaments could not be ruled out by the quantitation of F-actin, as they would constitute a small fraction of the total actin concentration. Thus we can only obtain a minimum estimate of filament number and thus of the number of filament ends. Fig. 7 is a histogram of the number of filaments per 0.11-μm bead, which averaged 8 ± 5.

We calculate that there are 26 molecules of [^{3}H]CB bound per 0.11-μm bead.[3] If we divide this number by the number of

[3] The amount of CB bound at saturation (1.5×10^{13} molecules/ml) is determined from the x-intercept of a Scatchard plot of binding of [^{3}H]CB to 0.2 mg/ml actin, associated with 0.74 mg/ml 0.11-μm beads. We measured that 71% of the actin is directly bound under these conditions. (The slope of this plot gave a K_d of 2×10^{-8} M, about the same as seen in Figs. 2 and 3 with 1.1-μm beads). Because 0.74 mg/ml 0.11-μm beads is equal to 5.6×10^{11} beads/ml (by counting; Materials and Methods), we obtain 26 molecules of CB/bead.

FIGURE 7 Histogram of number of actin filaments per 0.1-μm bead. Average = 8 ± 5 filaments per bead (1 SD, n = 83). The data of several experiments are included. Each separate experiment gave the same distribution of filament number. 0.2 mg/ml (measured to be 71% bound) or 2 mg/ml actin was assembled off 0.74 mg/ml 0.11-μm beads. A drop was placed on an EM gird, and in some cases rinsed with 50 mM MgCl$_2$ to induce paracrystals for better visualization, before negative staining with 1% aqueous uranyl acetate.

filaments per bead, we obtain an average of 3, and a range of 2–9 CB binding sites per filament. Considering that our estimate of filament number may be low, the number of CB binding sites per filament might be less than this. Again, we emphasize that these numbers are approximations, but they fall in the range expected.

DISCUSSION

To use cytochalasin as a tool to study the functioning of actin in cells, it is first necessary to understand the details of its molecular mode of action. We provide evidence that cytochalasin retards actin assembly by binding to actin filament ends, as we can vary the number of filament ends and obtain a corresponding variation in the number of cytochalasin-binding sites. Our evidence indicates that the high affinity cytochalasin binding is not along the length of the filament, or to a possible trace contaminant, as binding can be varied while these other components are kept constant.

The ability to vary number of actin filament ends by varying the bead concentration is demonstrated directly by EM at the lower bead concentrations, where filaments are still long enough to be seen. Our argument that there is a loss of filament ends beyond saturating bead concentrations has not been independently demonstrated, but this is the most plausible explanation of the loss of cytochalasin binding and is consistent with other findings on the mode of action of the beads (3).

The data presented here, taken together with other very recent findings, provide a reasonably convincing picture of the mode of action of cytochalasin: A number of laboratories have shown that low concentrations of cytochalasin retard actin assembly (1, 4, 5, 9, 10) and bind with high affinity to F-actin (4–6, 9) but not G-actin (5), with a stoichiometry of close to one cytochalasin per filament (6, and this paper). Because the assembly is known to occur preferentially at one end of the filament (7, 8, 13, 17), we would expect cytochalasin to interfere with assembly by binding to that end. MacLean-Fletcher and Pollard (10), by assembling actin onto filament fragments decorated with heavy meromyosin, have demonstrated elegantly that cytochalasin blocks the preferred assembly end. In the absence of cytochalasin, there is a 6:1 bias in favor of growth off the barbed end; in the presence of cytochalasin, growth off the barbed end is preferentially blocked. It has been proposed (15) that the two ends of the actin filament have different equilibria with monomer, so that at steady state, monomer is continually coming off the "disassembly" (pointed) end and adding to the "assembly" (barbed) end to give rise to "treadmilling." If this is the case, and cytochalasin binds to the assembly end, depolymerization to a new equilibrium with the disassembly end would be expected. Several authors have

observed that cytochalasin D does increase the critical concentration of monomer in equilibrium with F-actin (1, and unpublished observations of Dr. P. A. Simpson in our laboratory); however, others have not observed such an effect (6, 10) perhaps because buffer conditions affect the extent of the difference, or because a small change is obscured by nonpolymerizable monomer (12). Spectrin-actin complex may be complimentary to cytochalasin by blocking the other end of the filament, as it has complimentary effects (2): it decreases the critical concentration, and tends to block the ability of cytochalasin to cause depolymerization to a higher critical concentration.

This new understanding of the mode of action of cytochalasin suggests that the reason for its profound effects on cell functioning may be because actin assembly-disassembly reactions are central to the regulation of many cellular processes.

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