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Actin polymerization induced by a motility-related high-affinity cytochalasin binding complex from human erythrocyte membrane

(cytochalasin B/[³H]dihydrocytochalasin B/microfilament formation and membrane attachment/control of cell motility/viscosity changes)

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ABSTRACT **A high molecular weight complex (sedimentation coefficient ≈27 S) containing high-affinity binding site(s) for [³H]dihydrocytochalasin B has been isolated from a low ionic strength extract of human erythrocyte membranes by sucrose density gradient centrifugation. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis showed that actin, spectrin, and other minor components, including two polypeptides with the electrophoretic mobility of band 4.1, were present in the complex-containing fraction. Addition of this complex to a solution of muscle monomeric actin (G-actin) in a low ionic strength medium resulted in a rapid increase in viscosity to a level comparable to that of a solution of filamentous actin (F-actin). Electron microscopy showed that the viscosity increase reflected actin filament formation. The rate of induced actin polymerization was dependent on the amount of complex added to the G-actin; in less than 1 hr, less than 1 μg of protein from the complex-containing fraction induced the conversion of 0.4 mg of G-actin to the "F" form. Binding studies indicated that, upon polymerization of the actin, the cytochalasin binding complex became associated with the actin filaments. Low concentration of cytochalasins D and E and dihydrocytochalasin B inhibited actin polymerization induced by the complex; the relative potencies of the drugs in inhibiting this process corresponded to their relative affinities for the complex, as well as their relative potencies in affecting cell motility. These results suggest that the cytochalasin binding complex functions as a regulatory site for cell motility by controlling formation and membrane attachment of actin-containing microfilaments in the cell.**

Cytochalasin B (CB), a fungal metabolite, inhibits facilitated diffusion of sugars into animal cells and affects numerous forms of cellular and intracellular motility* in eukaryotic cells (for a comprehensive review, see ref. 1). As a result of binding studies involving the use of [³H]CB, the inhibitory effects on sugar transport in the human erythrocyte can now be explained in molecular terms: the drug competes with sugars for binding to high-affinity sites located in integral membrane proteins involved in the transport process (2–6). Although electron microscopic studies have shown that exposure of various cells to CB generally results in the disappearance or disruption of actin-containing microfilaments (7), a clear picture of the molecular events leading to inhibition of cell motility is just beginning to emerge.

With the aid of ³H-labeled dihydrocytochalasin B (H_2CB), a derivative that inhibits cell motility but does not inhibit sugar transport (8, 9), we were recently able to separate a class of high-affinity CB binding sites from those related to sugar transport in the human erythrocyte (10). We proposed that the high-affinity sites, tentatively designated as motility-related H_2CB binding sites, are involved in shape changes in the erythrocyte because (*i*) they are associated with cytoskeletal-contractile proteins (i.e., actin and spectrin), and (*ii*) their relative affinities for several cytochalasins correspond to the relative potencies of these compounds in inhibiting various forms of cell motility in animal cells. In this report, we describe the isolation of a high molecular weight H_2CB binding complex from a low ionic strength extract of erythrocyte membranes and demonstrate that this complex can act as an inducer of actin polymerization *in vitro*.

EXPERIMENTAL PROCEDURES

Materials. Cytochalasins B, D, and E were purchased from Aldrich. [³H]CB, prepared by the method of Lin *et al.* (11), was obtained from New England Nuclear. ³H-labeled and unlabeled H_2CB were prepared from the corresponding forms of CB by reduction with $NaBH_4$ as described (10, 12). Actin was isolated from acetone powder of rabbit skeletal muscle according to the method of Spudich and Watt (13). Unless otherwise specified, human erythrocytes were from blood generously donated by the Baltimore Red Cross Blood Center (used within 2 weeks after the blood was drawn).

Isolation of H_2CB Binding Complex. Ghosts were prepared from human erythrocytes as described (10), with an additional final wash in ice-cold 0.3 mM sodium phosphate buffer, pH 8.0. After centrifugation at 39,000 × *g* (18,000 rpm in a Beckman JA-20 rotor) at 4°C for 15 min, the ghost pellet was incubated at 37°C for 30 min with occasional mixing, and then centrifuged at 100,000 × *g* (29,000 rpm in a Beckman type 30 rotor) at 4°C for 1 hr to separate the membrane residue from the extract (designated as low ionic strength extract). Dithiothreitol (2 mM) was added to the extract, and 0.5 ml (about 1 mg of protein) was layered on top of an 11-ml sucrose density gradient prepared with a Buchler density gradient system (5–20% sucrose in 0.1 M KCl/1 mM dithiothreitol/5 mM sodium phosphate, pH 8.0). The gradient was then centrifuged at 200,000 × *g* (40,000 rpm in a Beckman SW 41 Ti rotor) in a Beckman L5-75 ultracentrifuge equipped with ω^2t integrator, at 4°C for 6 hr. After centrifugation, the gradient was retrieved from the meniscus down to the bottom of the tube with a Buchler Auto Densi-Flow IIC apparatus, and fractionated into 0.75-ml fractions. After the protein concentration and cytochalasin binding activity of each fraction had been determined, the sedimentation coefficients of the protein peak (fractions 3 and 4) and of the H_2CB binding peak (fraction 8) were estimated, assuming a protein density of 1.4 mg/ml. Fractions 6 through 11, containing high-affinity H_2CB binding activity, were pooled, dialyzed against several changes of 0.2 mM ATP/0.2

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Abbreviations: CB, cytochalasin B; H_2CB, dihydrocytochalasin B; G-actin, monomeric actin; F-actin, filamentous actin.

* The word "motility" is used in its broadest sense to describe processes involving cell locomotion, cell shape changes, or intracellular movement of particles and organelles.

mM $CaCl_2$/0.5 mM 2-mercaptoethanol/5 mM Tris·HCl, pH 8.0, and concentrated to about 80 μg of protein per ml by ultrafiltration (Amicon XM-100A filter). This preparation will be referred to as the "complex-containing preparation" in the remainder of this paper.

Cytochalasin Binding Assay. A detailed description of the cytochalasin binding assay will be described elsewhere (unpublished results). Briefly, a sample was incubated at room temperature in 500 μl of buffer containing [^{3}H]H_2CB. After 10 min, the protein in the sample and bound [^{3}H]H_2CB were precipitated by addition of a small volume of a saturated solution of sodium phosphate (monobasic) to lower the pH of the assay medium to 5.0. The precipitated protein was separated from the supernatant fraction by centrifugation at 6000 × *g* (7000 rpm in a Beckman JA-20 rotor) at 4°C. The radioactivities of the pellet, solubilized in Protosol (New England Nuclear), and the supernatant were determined by scintillation counting; the amounts of bound and free [^{3}H]H_2CB were calculated as described (10).

Viscometry. Viscosity was determined in an Ostwald-type viscometer (flow time for water of 33 s) at 25°C. Relative viscosity (η rel) was defined as the flow-time of a sample solution divided by the flow-time of the corresponding buffer.

Electron Microscopy. A 20-μl sample was applied to a hydrophilic carbon film and the excess was drawn off after 15 s. The grid was then rinsed in water, negatively stained with freshly prepared, unbuffered 1% uranyl acetate, and examined in a Philips 200 electron microscope.

RESULTS

Isolation of the H_2CB Binding Complex. High-affinity H_2CB binding sites related to cell motility can be eluted from the erythrocyte membrane with low ionic strength media (10); CB binding sites related to sugar transport, on the other hand, remain in the membrane under these conditions (2). To separate the soluble component containing H_2CB binding activity from the rest of the eluted proteins, we fractionated a low ionic strength extract of erythrocyte ghosts by sucrose density gradient centrifugation. As shown in Fig. 1, the bulk of the protein in the extract sedimented as a slow moving peak with an average sedimentation coeffficient of about 12 S. H_2CB binding activity, however, sedimented as a faster moving peak with an average sedimentation coefficient of about 27 S. Electrophoresis in sodium dodecyl sulfate/polyacrylamide gels (Fig. 2) showed

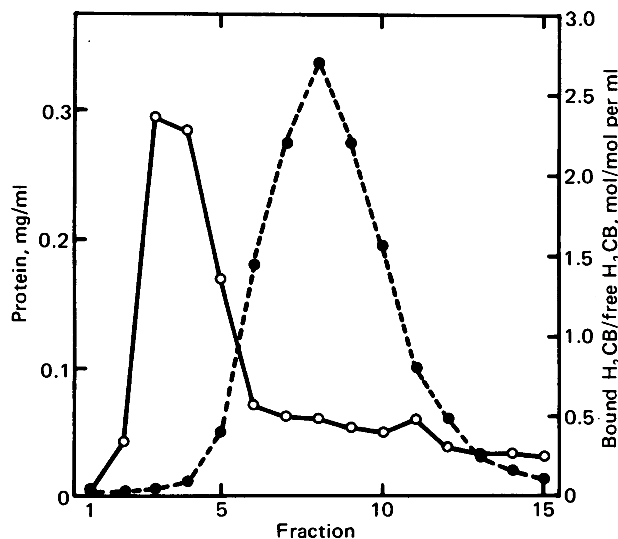

FIG. 1. Separation of the H_2CB binding complex from the bulk of the protein in a low ionic strength extract of erythrocyte ghosts by sucrose density gradient centrifugation. A sample of the extract was layered onto a 5–20% sucrose gradient, centrifuged, and fractionated. Fractions were numbered from the meniscus down to the bottom. Protein concentration (○) of each fraction was determined by the method of Hartree (14). High-affinity H_2CB binding activity (●), expressed as moles of H_2CB bound divided by the concentration of free H_2CB, was determined by assaying a 0.5 ml portion of each fraction in 12 nM [^{3}H]H_2CB.

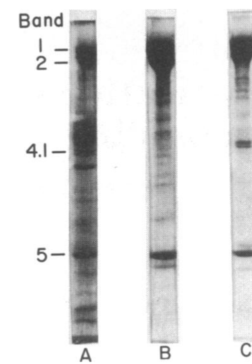

FIG. 2. Electrophoresis of ghost proteins and of sucrose density gradient fractions in sodium dodecyl sulfate/polyacrylamide gels. Samples were electrophoresed in slab gels (1.5 mm thick) and stained with Coomassie brillant blue according to the method of Laemmli (15). The gels were dried on filter paper before they were photographed. Gel A contained erythrocyte ghosts (20 μg of protein). Gel B contained material from the 12S protein peak (30 μg of protein from fraction 3). Gel C contained material from the 27S H_2CB binding activity peak (10 μg of protein from fraction 8). The gels were overloaded deliberately to show the presence of minor protein components. Bands were labeled according to the nomenclature of Steck (16). In this gel system, band 4.1 shows up as a closely spaced doublet.

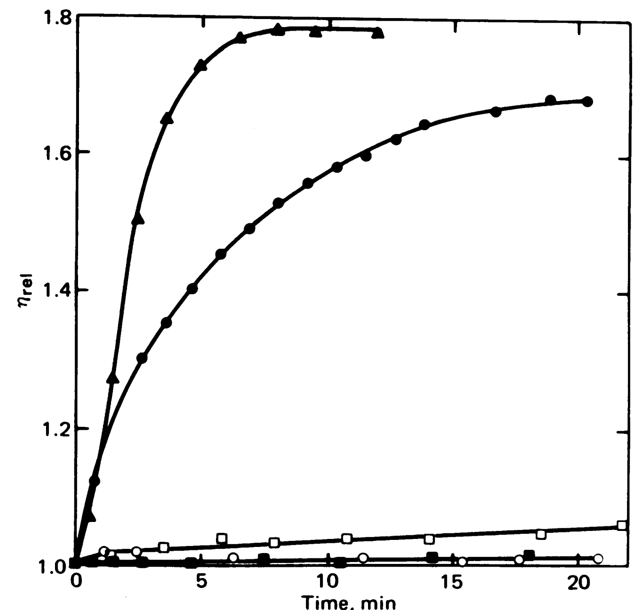

FIG. 3. Polymerization of actin induced by KCl and by the H_2CB binding complex. At zero time, 50 mM KCl (▲), 21 μg of protein from the complex-containing preparation (●), or 21 μg of protein from the 12S protein peak (fraction 3 of the sucrose density gradient dialyzed against the same buffer used to dialyze the complex-containing fractions) (□) was added to 0.4 mg of G-actin in 0.5 ml of 0.2 mM ATP/0.2 mM $CaCl_2$/0.4 mM $MgCl_2$/0.5 mM 2-mercaptoethanol/5 mM Tris·HCl, pH 8.0. Polymerization of the actin was monitored by viscometry. In the control experiments, G-actin (■) or H_2CB binding complex (○) alone was incubated in the same buffer.

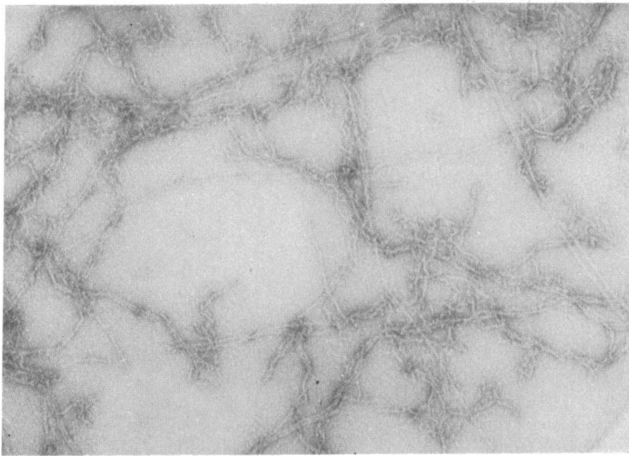

FIG. 4. Electron micrograph showing actin filaments formed by addition of H_2CB binding complex to G-actin. The sample was prepared as described in Fig. 3. (×50,000.)

that the 12S protein peak (gel B) contained most of the spectrin (bands 1 and 2) and actin (band 5) extracted from the erythrocyte membrane. The 27S H_2CB binding activity peak (gel C) also contained these two proteins and, in addition, several minor components, including two polypeptides with the electrophoretic mobility of band 4.1. The high sedimentation coefficient of the H_2CB binding activity peak indicated that the cytochalasin binding sites were located in supramolecular complexes. Whether all of the polypeptides shown in gel C were components of these complexes remains to be determined.

Induction of Actin Polymerization by the H_2CB Binding Complex. Actin is in the monomeric form (G-actin) in low ionic strength buffer. Addition of 50 mM KCl leads to polymerization of the G-actin, a process that can be conveniently monitored by measuring the increase in viscosity of the actin solution as filaments (F-actin) are formed (17).

We found that the H_2CB binding complex isolated by sucrose density gradient centrifugation has the ability to induce actin polymerization under conditions in which the protein would normally be in the monomeric form (Fig. 3). Addition of the complex-containing preparation (made from fractions 6–11) to G-actin in a low ionic strength buffer containing 0.4 mM $MgCl_2$ resulted in an increase in viscosity of the solution to a level comparable to that of a solution of F-actin formed by addition of 50 mM KCl to G-actin. In contrast, addition of a comparable amount of protein from the protein peak (fraction 3) under identical conditions produced only a relatively minor effect on viscosity. When this experiment was repeated with freshly drawn blood, combined fractions 6–11 induced actin polymerization, whereas fraction 3 caused even less polymerization than that indicated in Fig. 3 for stored blood (data not shown). Electron microscopy confirmed that the observed increase in viscosity that resulted from the addition of the complex to G-actin reflected formation of actin filaments (Fig. 4); such structures were not seen in samples containing G-actin or the complex alone (not shown).

The rate of induced viscosity increase was found to be a function of the amount of H_2CB binding complex added (Fig. 5); the final viscosity of the solution, however, was apparently dependent only on actin concentration. In less than 1 hr, as little as 0.9 μg of protein from the complex-containing preparation induced the conversion of 0.4 mg of actin from the "G" to the "F" form.

Inhibition of Actin Polymerization by Cytochalasins. Actin polymerization induced by the H_2CB binding complex was found to be inhibited by low concentrations of cytochalasins (Fig. 6*A*). H_2CB, at 0.2 and 2.0 μM, reduced the rate of viscosity increase by 65 and 85%, respectively. Cytochalasins D and E, which have higher affinities than H_2CB for motility-related binding sites (10), produced higher degrees of inhibition (over 95%) at lower concentrations (0.02 μM). In contrast to these results, 2 μM H_2CB had no appreciable effect on actin polymerization induced by 50 mM KCl (Fig. 6*B*). It appears, therefore, that the cytochalasin binding site(s) in the complex is directly, or indirectly, involved in the induction of actin polymerization.

Cosedimentation of H_2CB Binding Complex with Actin Filaments. Filaments of F-actin can be sedimented by high speed centrifugation (13). When G-actin had been induced to polymerize by addition of H_2CB binding complex and the mixture was centrifuged at high speed, most of the cytochalasin binding activity was recovered in the pellet (Fig. 7). In the control experiment, in which a solution of the complex was centrifuged before addition of G-actin, most of the cytochalasin

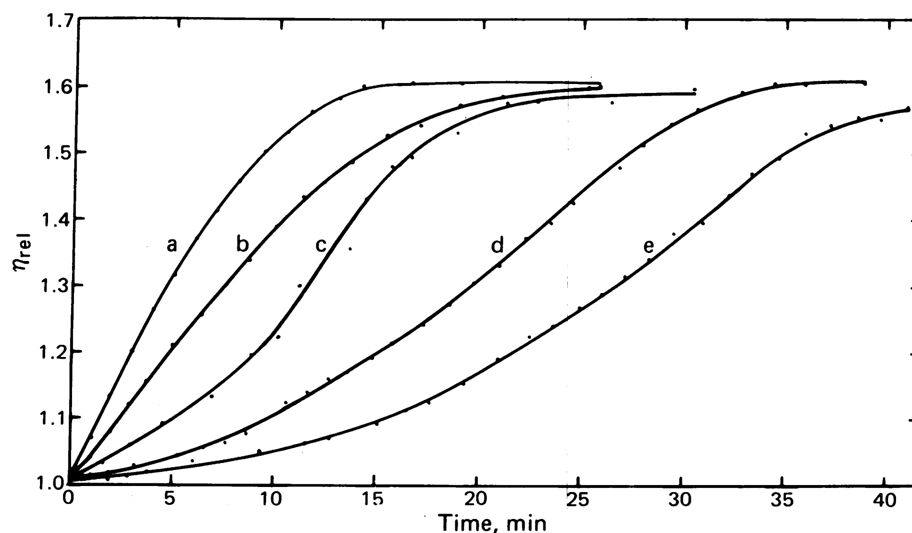

FIG. 5. Induction of actin polymerization by various amounts of H_2CB binding complex. At zero time, 14.8 μg (a), 7.4 μg (b), 3.7 μg (c), 1.9 μg (d), or 0.9 μg (e) of protein from the complex-containing fraction was added to 0.4 mg of G-actin in 0.5 ml of the buffer described in Fig. 3. Polymerization of the actin was monitored by viscometry.

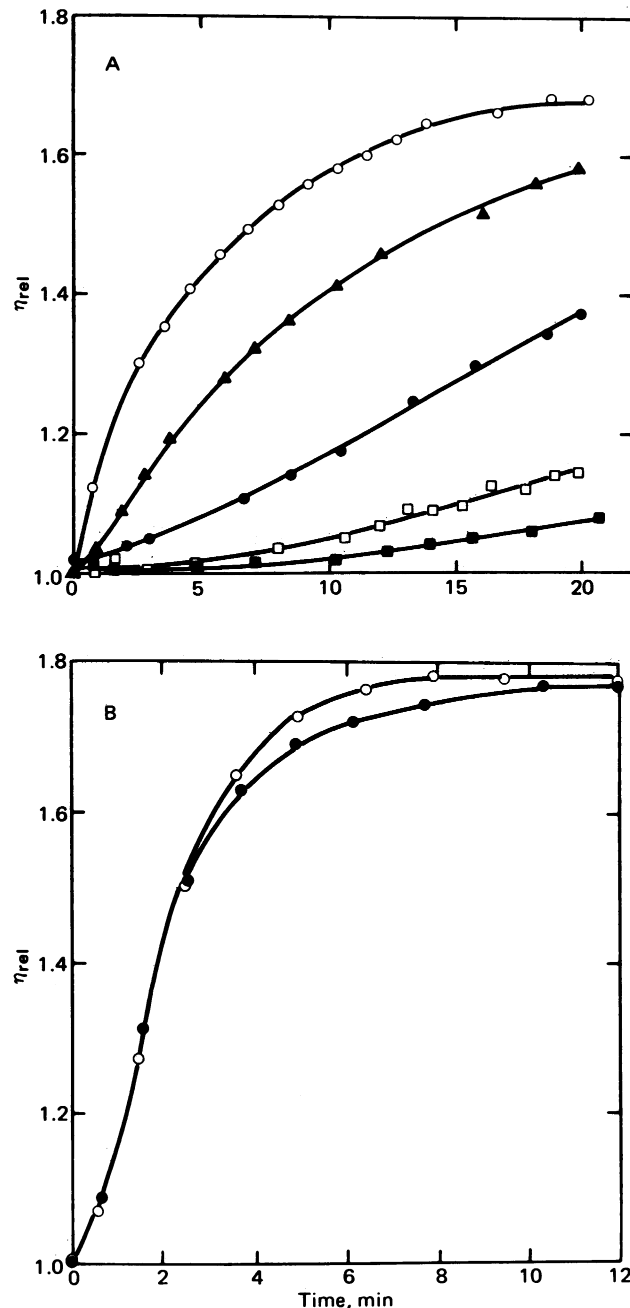

FIG. 6. Inhibition of actin polymerization by cytochalasins. (*A*) At zero time, 21 μg of protein from the preparation containing the H_2CB binding complex was added to 0.4 mg of G-actin in the buffer described in Fig. 3, in the absence (○) or presence (▲) of 0.2 μM H_2CB, or in the presence of 2 μM H_2CB (●), 0.02 μM cytochalasin D (□), or 0.02 μM cytochalasin E (■). (*B*) At zero time, G-actin (0.4 mg/0.5 ml of the same buffer used in *A* was induced to polymerize by addition of KCl (50 mM) in the absence (○) or presence (●) of 2 μM H_2CB.

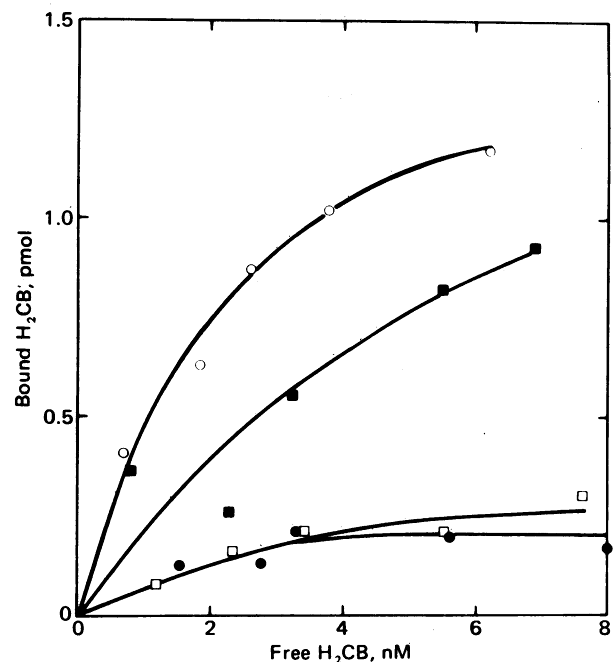

FIG. 7. Cosedimentation of H_2CB binding complex with F-actin filaments. Protein (84 μg) from the complex-containing preparation was incubated with 0.7 mg of G-actin in 1 ml of the buffer described in Fig. 3 at 25°C for 30 min. After the mixture had been centrifuged at 300,000 × g (60,000 rpm in a Beckman type 65 rotor) at 4°C for 45 min, 0.1-ml aliquots of the supernatant (□) and the pellet (resuspended in the original volume of buffer) (■) were assayed for H_2CB binding activity by using various concentrations of $[^3H]H_2CB$. In the control experiment, the complex was similarly treated, except that the actin was added to the supernatant fraction after the centrifugation step. The H_2CB binding activity of the supernatant (○) and pellet (●) fractions were determined as before.

binding activity was recovered in the supernatant. This suggests that H_2CB binding complexes became associated with actin filaments during the polymerization process.

DISCUSSION

We have shown that high-affinity H_2CB binding sites of the human erythrocyte are located in high molecular weight complexes associated with the cell membrane. These complexes have a characteristic size, as indicated by their movement as a defined peak in the sucrose gradient. The data presented here show that the complexes are powerful inducers of actin polymerization.

Pinder *et al.* (18, 19) have reported that a preparation from a low ionic strength extract of human erythrocyte ghosts, recovered in the void volume from a Sephadex G-200 column, induced actin polymerization and that the polymerization-inducing activity increased with the degree of phosphorylation of the smaller subunit of spectrin. These authors concluded that the active ingredient was spectrin, although they did not consider the degree of phosphorylation of minor components in their preparation or discuss the possible role of these components in the polymerization reaction. For example, it seems likely that their preparations included the polymerization-inducing complexes that we have been able to separate from the bulk of the spectrin; these complexes may or may not themselves contain spectrin. In our experiments the bulk of the spectrin extracted from erythrocyte ghosts was found in the 12S protein peak, but material from this peak did not cause actin to polymerize, whether the material was obtained from fresh or stored blood. In view of the findings of Pinder *et al.* (19), the possibility must be considered that the inactivity of our 12S material was due to inadequate phosphorylation of spectrin in these fractions and that the activity in the 27S fractions resulted from a higher state of phosphorylation of spectrin in the complexes. An alternative is that the ability to induce actin polymerization may in fact reside in ingredients other than spectrin, both in our preparations and in those of Pinder *et al.*

Recent experiments performed in our laboratory have shown that motility-related high-affinity cytochalasin binding sites are not unique to erythrocytes. A high molecular weight com-

plex with high-affinity cytochalasin binding activity has been identified in bovine brain (20). High-affinity binding sites for $[^3H]H_2CB$ have also been demonstrated in mouse fibroblasts (3T3 cells), chicken embryo fibroblasts, human platelets, and bovine spermatozoa (unpublished data). It is possible, therefore, that the inhibitory effects of cytochalasins on shape changes in the erythrocyte in particular, and on cell motility in general, are mediated by this class of binding sites in the following way. Machinery for cell motility in a nonmuscle cell, unlike its counterpart in muscle, is generally not a permanent structure of the cell (21, 22). Therefore, an important mechanism by which cell motility can be regulated is through the control of the assembly and disassembly of cytoskeletal-contractile structures at a particular location in the cell at a particular time (e.g., the cleavage furrow during cytokinesis and ruffling membranes during cell locomotion). Because the H_2CB binding complex has the ability to induce actin polymerization, it can play a vital role in controlling the formation of actin-containing microfilaments in the cell. Moreover, because of its association with the plasma membrane, the complex can link the filaments to the membrane to achieve a structural arrangement generally regarded as necessary for force generation (21-23). Hence, by binding with high affinity to the complex, the cytochalasins inhibit the formation of actin-containing microfilaments in the cell. This effect, either by itself or in combination with the low-affinity effect of cytochalasins on actin molecules (17, 24), will lead to inhibition of cell motility and alteration of cell morphology.

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