Abstract. Previous work has shown that actin binds specifically and saturably to liver membranes stripped of endogenous actin (Tranter, M. P., S. P. Sugrue, and M. A. Schwartz. 1989. J. Cell Biol. 109:2833-2840). Scatchard plots of equilibrium binding data were linear, indicating that binding is not cooperative, as would be expected for F- or G-actin. To determine the state of membrane-bound actin, we have analyzed the binding of F- and G-actin to liver cell membranes. G-actin in low salt depolymerization buffer and EF-actin, a derivative that polymerizes very poorly in solution, bind to liver cell membranes as well as untreated actin in polymerization buffer. Phalloidin-stabilized F-actin binds, but to a lesser extent. The binding of F- and G-actins are mutually competitive and are inhibited by ATP, suggesting that both forms of actin bind to the same sites. For untreated actin in polymerization buffer, the time course of binding is biphasic, with an initial rapid component which is followed by a plateau phase, then a second, slower component. The binding kinetics of pure F-actin and pure G-actin are both monophasic and match the fast and slower components, respectively, of untreated actin. In the reconstituted system, membrane-bound actin does not stain with rhodamine-phalloidin, nor are actin filaments detected by EM. Distinct regions of amorphous material, however, are visible, which stain with an anti-actin antibody. The exact nature of this material has yet to be determined. A model of actin binding is presented.

The actin cytoskeleton maintains the shape and structural integrity of the plasma membrane and plays a dynamic role in a number of membrane-associated events, including cell migration, cell adhesion, phagocytosis, and the regulation of integral membrane protein distribution and mobility (reviewed in Cohen and Smith, 1985; Geiger, 1983; Jacobson, 1983; Ishikawa, 1988). While these functions imply that an intimate link exists between the plasma membrane and the underlying cytoskeleton, the evidence for such an association is mostly indirect. To date, investigators have relied heavily on EM (Begg, 1978; French and Davies, 1975; Phillips et al., 1981; Goodloe-Holland and Luna, 1984; Hirokawa and Tilney, 1982; Sugrue and Hay, 1981) and coisolation studies (Luna et al., 1981; Mescher et al., 1981; Carraway et al., 1982; Wolosin et al., 1983; Hubbard and Ma, 1983; Yousef and Murray, 1978) to demonstrate that actin is physically connected to the plasma membrane. However, these methods provide only limited information regarding the molecular nature of actin-membrane associations.

In an attempt to gain a better understanding of actin-membrane interactions in mammalian cells, we measured the binding of radiolabeled actin to sedimentable liver cell membranes. The sedimentation actin-binding assay includes the actin-capping protein gelsolin, which, by limiting actin filament length, ensures that equilibrium-binding curves are saturable (Schwartz and Luna, 1986). This technique has previously been used to elucidate the mechanisms by which actin associates with plasma membranes isolated from the cellular slime mold Dictyostelium discoideum. Using this assay, Schwartz and Luna (1986) found that F- but not G-actin binds specifically and saturably to highly purified D. discoideum membranes. Upon further analysis, actin binding and assembly were shown to be tightly coupled, with D. discoideum membranes inducing actin filament formation at concentrations well below the critical concentration for actin polymerization (Schwartz and Luna, 1986; Schwartz and Luna, 1988). Based on these results, a model was proposed in which D. discoideum membranes induce the formation of stable actin trimers and thereby directly nucleate actin assembly (Schwartz and Luna, 1988). Key features of this model have recently been supported by the direct demonstration of a membrane-associated actin-nucleation activity (Shariff and Luna, 1990). Both the actin-nucleating and actin-binding activities of D. discoideum membranes now appear to be mediated by ponticulin, a 17-kD integral membrane glycoprotein (Wuestehube and Luna, 1987; Shariff and Luna, 1990).

We recently reported that direct interactions between actin and integral membrane proteins also occur in the mammalian liver (Tranter et al., 1989). Using the sedimentation-binding assay mentioned above, we found that F-actin...
binds specifically and saturably to liver cell membranes and that integral membrane proteins appear to be responsible. In addition, the association of actin with rat liver membranes exhibits a number of distinctive features not observed in other actin-membrane systems. Actin binding is strongly temperature dependent, displays unusual kinetics, and is competitively inhibited by certain nucleotides. Scatchard plots of equilibrium-binding data are linear, which both indicates the presence of a single class of binding sites and suggests that binding is not cooperative. Given that actin binding occurs over the same concentration range as actin polymerization (the critical concentration for actin in the presence of gelsolin is ~30 μg/ml; Schwartz and Luna, 1986), it was expected that the preferential binding of either F- or G-actin would result in positive or negative cooperativity, respectively. The absence of cooperativity is therefore inconsistent with known types of actin interactions.

While electron micrographs show occasional filamentous structures attached to unstripped liver cell membranes (Tranter et al., 1989), these filaments do not appear to be present in sufficient abundance to account for the level of endogenous membrane-bound actin (~5% of the total membrane protein). In addition, endogenous actin is very resistant to extraction by a variety of agents known to depolymerize actin filaments (Tranter et al., 1989; Hubbard and Ma, 1983). Interestingly, Hubbard and Ma (1983) have reported that actin bound to liver cell membranes does not bind myosin S-1. Since these observations suggest that endogenous actin may not be in the filamentous form, we have systematically explored the state of membrane-bound actin in our system.

Materials and Methods

Materials

Phalloidin was obtained from Calbiochem-Behring Corp. (San Diego, CA), rhodamine phalloidin from Molecular Probes Inc. (Junction City, OR), and diethylpyrocarbonate (DEPC)¹ from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were as described in Tranter et al. (1989). Actin was isolated from rabbit skeletal muscle by the method of Spudich and Watt (1971) and further purified by gel-filtration on Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, NJ) as described by MacLean-Fletcher and Pollard (1980). Gel-filtered actin was radiolabeled with Na¹²⁵I (Pharmacia Fine Chemicals, Piscataway, NJ) as described by MacLean-Schwartz and Luna (1986). Both radiolabeled and unlabeled actin were stored in the G-form by dialysis against depolymerization buffer (50 mM KCl, 2 mM MgCl₂, 0.2 mM CaCl₂, 0.2 mM ATP, 1 mM TTT, 20 mM Pipes, 100 μM PMSF, 0.4 μg/ml aprotinin, 4 μg/ml leupeptin, pH 7.4) for use in the following day.

Preparation of EF-actin and Phalloidin Actin

EF-actin was prepared essentially as described by Schwartz and Luna (1986). Unlabeled or radiolabeled actin (20 μg/ml) was dialyzed against 0.2 mM CaCl₂, 0.2 mM ATP, 2 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5 for 2 h, and then centrifuged gel filtered on a Sephadex G-25 (Pharmacia Fine Chemicals) minicolumn which had been equilibrated with the same buffer. DEPC dissolved in ethanol was added at a mole ratio of DEPC to actin of 12:1. The sample was then mixed thoroughly and incubated on ice for 20 min. 10-fold polymerization buffer was then added to yield a final concentration of 50 mM KCl, 2 mM MgCl₂, 20 mM Pipes, pH 7.0. The actin was polymerized for 1 h at room temperature then sedimented in an airfuge (Beckman Instruments, Palo Alto, CA) at 30 psi for 45 min. To remove excess DEPC, the supernatant containing EF-actin was centrifuged gel filtered on a Sephadex G-25 minicolumn equilibrated with 2 mM Tris-HCl, 50 μM CaCl₂, 1 mM DTT, 0.02% sodium azide, pH 7.0. EF-actin was then used immediately in a binding assay.

Unless otherwise indicated, phalloidin actin was prepared as follows. Unlabeled or radiolabeled actin was mixed with rhodamine phalloidin at a 15:1 mole ratio of actin to gelsolin for 1 h at room temperature in 50 mM KCl, 2 mM MgCl₂, 20 mM Pipes, 100 μM CaCl₂, pH 7.0, containing phalloidin at a concentration equivalent to actin. For some experiments, the polymerized actin was then loaded onto a Sepharose 6B column (Pharmacia Fine Chemicals) and eluted with 50 mM KCl, 2 mM MgCl₂, 20 mM Pipes, 100 μM CaCl₂, 10 μM phalloidin, pH 7.0. The excluded volume, containing phalloidin-stabilized F-actin, was then used in a binding assay.

Actin-membrane Binding Assays

Actin membranes—binding assays were performed essentially as described by Tranter et al. (1989). Assays were done in a total volume of 30 μl of binding buffer (100 mM KCl, 2 mM MgCl₂, 1 mM DTT, 20 mM Pipes, 100 μM CaCl₂, 100 μM PMSF, 0.4 μg/ml leupeptin, pH 6.8) containing 0.5 mg/ml stripped membranes, 20 mg/ml ovalbumin, and various amounts of actin. For some experiments, binding was measured in low salt buffer instead of binding buffer. Unless otherwise indicated, samples contained gelsolin at a 15:1 mole ratio of actin to gelsolin. 10 μM phalloidin was also included in assays using phalloidin actin. Samples were incubated for 2.5 h at room temperature, layered onto 350 μl 10% sucrose in assay buffer, and centrifuged for 20 min at 11,000 g and 4°C. (Beckman Instruments, Inc.) Total actin added and actin bound were then calculated from the amount of radioactivity in the pellets and supernatants. Specific actin bound was calculated by subtracting the actin bound in control samples from total actin bound. For controls, actin binding was measured in the presence of unlabeled excess actin or in the presence of 25 mM ATP. These controls have been found to yield essentially identical results. For all figures binding is expressed as μg/ml actin bound/0.5 mg membrane protein.

Kinetic assays were performed at room temperature (25°-27°C). Samples were assembled on ice, and then immediately transferred to a room temperature water bath upon the addition of actin. After the indicated period of time, each sample was spun through sucrose and analyzed as described above.

The binding of actin to isolated hepatocytes was measured as follows. Freshly isolated hepatocytes (~250,000 cells) were incubated in 100 μl William's E medium (Gibco Laboratories, Grand Island, NY) containing ~80 μg/ml untreated ¹²⁵I-actin and 20 mg/ml ovalbumin, with or without 10 mM AMP-PNP. After 1 h at either 37°C or 0-4°C, cells were layered onto 1.0 ml 10% sucrose and spun for 4 min at 3,000 rpm in a microfuge (Beckman Instruments, Inc.). The pellets and supernatants were then counted in a gamma counter. Alternatively, isolated hepatocytes were cultured overnight in William's E medium in a 24-well culture dish (~250,000 cells/well). The following day, the media was removed and replaced by 100 μl William's E medium containing ~80 μg/ml untreated ¹²⁵I-actin and 20 mg/ml ovalbumin, with or without 10 mM AMP-PNP. After a 1-h incubation at 37°C, the medium was removed, and the cells were rinsed several times. The cells were then dissolved in 2% SDS and counted in a gamma counter.

¹ Abbreviation used in this paper: DEPC, diethylpyrocarbonate.
**Rhodamine-phalloidin Staining and EM**

Stripped liver membranes (100 μg) were incubated with or without 100 μg/ml actin in 200 μl binding buffer containing 20 mg/ml ovalbumin. After incubation at room temperature for indicated periods of time, samples were layered onto 1.0 ml of 10% sucrose in assay buffer and centrifuged for 15 min at 13,600 g in a microcentrifuge (Fisher Scientific Co., Pittsburgh, PA). The pellets were washed once with binding buffer, and then fixed in 1.0 ml of 3.7% formaldehyde in binding buffer for 30 min at room temperature. Samples were spun for 15 min in the microcentrifuge, washed once with PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.0), and resuspended in 100 μl PBS containing 33-nM rhodamine-phalloidin. Samples were incubated for 30 min at room temperature, diluted to 1.5 ml with PBS, and centrifuged as before. After a final wash with PBS, pellets were resuspended in 10 μl PBS, mounted on microscope slides, and examined with a microscope (Photomicroscope; Zeiss, Oberkoehen, FRG) equipped with phase contrast and epifluorescence optics.

For EM, stripped membranes (500 μg/ml) were incubated with or without 500 μg/ml untreated actin in binding buffer for various lengths of time, and then processed for thin sectioning as previously described (Tranter et al., 1989).

For immunoelectron microscopy, two methods were used. For direct detection, polyclonal antibody (ICN Radiochemicals, Irvine, CA) against rabbit muscle actin was purified on protein A-Sepharose (Pharmacia Fine Chemicals) and adsorbed to 12-nm colloidal gold particles as described (Larsson, 1988). Excess ovalbumin was added to block remaining free sites, and the particles washed and collected. IgG–gold conjugates were added to the membranes to give 5 μg IgG per 100 μl sample. They were incubated 1 h at room temperature, then the membranes sedimented, rinsed 3× with PBS + 5% normal goat serum, and processed for EM as above. For indirect detection, anti-actin IgG was added to membranes to give 1.5 μg in 100 μl. The samples were vortexed vigorously and then sonicated very briefly (<10 s) in a bath sonicator, to ensure complete dispersal of the membrane pellet. Membranes were incubated 1 h at room temperature, and then sedimented and rinsed 3× with PBS + 10% normal goat serum. Then goat anti-rabbit IgG-coated 5-nm gold particles (Amenham Corp.) were added, and samples again vortexed and sonicated. They were again incubated 1 h, rinsed, and processed for EM as before.

**Results and Discussion**

In this study, we have explored the state of membrane-bound actin by examining the ability of F- and G-actin to bind to liver cell membranes. The binding of untreated 125I-actin to stripped liver membranes was first measured in the absence and presence of the actin-capping protein gelsolin. Gelsolin is routinely used in this assay (Schwartz and Luna, 1986) to prevent actin–actin associations which tend to render binding nonsaturable (Jacobson, 1980; Cohen and Foley, 1980; Schwartz and Luna, 1986). In contrast to what has been observed in other systems (Jacobson, 1980; Cohen and Foley, 1980; Schwartz and Luna, 1986), the binding of actin to liver cell membranes is saturable with or without gelsolin (Fig. 1 a). In fact, gelsolin has little, if any, effect on the extent of actin binding. Since liver cell membranes also have no consistent effect on the viscosity of F-actin solutions, as measured by low shear viscometry (Fowler et al., 1981; Luna et al., 1981), they do not appear to cross-link actin filaments (data not shown). Taken together, these results indicate that actin bound to liver cell membranes does not polymerize into the bulk solution away from the membrane surface.

**Binding of Monomeric and Filamentous Actin to Liver Cell Membranes**

Treatment of G-actin with DEPC generates an actin derivative that polymerizes very poorly in solution (Mürlád et al., 1969; Hegyi et al., 1974; Schwartz and Luna, 1988). This derivative, termed EF-actin (Schwartz and Luna, 1988), has been particularly useful since it allows the association of monomeric actin with stripped liver membranes to be measured under the same binding conditions used for untreated actin.

Experiments with EF-actin were performed in binding buffer, in the absence of gelsolin. Fig. 1 b shows that EF-actin binds to stripped liver membranes in a specific and saturable manner, and the affinity and stoichiometry of binding (EF-actin concentration at half saturation = 65 ± 5 μg/ml; 58 ± 10 μg EF-actin bound/mg membrane protein at saturation; n = 3) are very similar to those for untreated actin in the presence of gelsolin (actin concentration at half saturation = 65 ± 15 μg/ml; 78 ± 26 μg actin bound/mg membrane protein at saturation; n = 6). Actin binding measured in low salt depolymerization buffer was also similar to that observed under standard conditions, with the amount of actin bound at saturation generally the same or greater (actin concentration at half saturation = 55 ± 5 μg/ml; 90 ± 10 μg actin bound/mg membrane protein at saturation; n = 3; data not shown).

To determine if liver cell membranes also bind filamentous actin, we incubated stripped liver cell membranes with phalloidin-stabilized actin filaments. Phalloidin binds to ac-
tin filaments in a 1:1 molar ratio and stabilizes them against depolymerization (Estes et al., 1981; Coluccio and Tilney, 1984). In initial experiments, actin was polymerized in the presence of equimolar phalloidin and then added directly to our samples. Results from these experiments revealed that phalloidin substantially reduces the extent of specific actin binding but does not completely abolish it. Although phalloidin stabilizes actin filaments, it does not alter the kinetics of polymerization. As a result, it seemed possible that residual G-actin was responsible for this low level of binding. Thus, to ensure that actin was completely filamentous, we polymerized actin as before in the presence of phalloidin, then gel filtered it on a Sepharose 6B column. This procedure separates short actin filaments from actin monomers. Actin from the excluded volume was then used for binding assays. Assays performed this way yielded results essentially identical to those without gel filtration and are displayed in Fig. 2. As can be seen, the extent of binding in the presence of phalloidin is considerably less, being 20–50% of the binding of untreated actin (n = 4).

Competitive-binding assays have been carried out to determine if F- and G-actin bind to the same sites. Fig. 3 shows the effect of increasing concentrations of EF-, phalloidin-, and untreated actin on the binding of untreated \(^{125}\)I-actin to stripped liver cell membranes. All three forms of unlabeled actin clearly reduced the binding of untreated \(^{125}\)I-actin to the same extent. Curiously, both EF- and phalloidin-actin also appear to have a higher affinity for these binding sites than untreated actin. That these three forms of actin are mutually competitive suggests that they bind either to the same site or to nearby sites on the same protein. However, given that ATP effectively inhibits the binding of all three forms of actin (data not shown), the first alternative is strongly favored. This conclusion is also consistent with Scatchard analysis which indicated that the association of actin with liver cell membranes is mediated by a single class of binding sites (Tranter et al., 1989).

**Kinetics of Actin Binding**

To further characterize their association with liver cell membranes, we have measured the kinetics of binding for un-

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Figure 2. Comparison of the specific binding of phalloidin-stabilized \(^{125}\)I-F-actin (○) and untreated \(^{125}\)I-actin (●) to stripped liver membranes.

Figure 3. Binding of untreated \(^{125}\)I-actin (70–90 µg/ml) to stripped liver cell membranes (500 µg/ml) in the presence of unlabeled untreated actin (●); unlabeled phalloidin actin (○); and unlabeled EF-actin (▲).
treated, EF-, and phalloidin-actin. The same concentration of each form of $^{125}$I-labeled actin was incubated with stripped membranes for various lengths of time at room temperature, and then sedimented through sucrose. Binding was determined as before. As shown in Fig. 4, the three forms of actin bind to liver cell membranes with very different kinetics. For untreated actin, the time course of binding is biphasic (Fig. 4 a). The initial binding phase is very rapid ($t_{1/2} \sim 5$ min) beginning immediately upon mixing and reaching a plateau within 15 min of incubation. This plateau lasts for 30–60 min and is then followed by a slower second phase of binding ($t_{1/2} \sim 15$ min). Although the absolute values for the amount of actin bound (18 ± 10 µg actin bound at plateau/mg membrane protein; 44 ± 10 µg actin bound at steady state/mg membrane protein) and the length of the lag phase (30–60 min) varied somewhat between experiments, the overall pattern was highly reproducible ($n = 5$). The kinetics for EF-actin binding are monophasic (Fig. 4 b), with a half-time of $\sim 10–15$ min. Untreated G-actin under low salt depolymerization conditions exhibits essentially identical kinetics, having a half-time for binding of $\sim 15$ min (data not shown). In sharp contrast, the kinetics of phalloidin-actin binding are monophasic, but are extremely fast ($t_{1/2} \sim 2$ min) (see Fig. 6 c). The half-time for the rapid component, however, must be regarded as an approximation, since it is close to the limit of resolution for the method.

Interestingly, the kinetics of binding for untreated actin consists of two components, the first of which is similar to...
the rate and extent of binding for F-actin alone and the second of which is similar to the rate and extent of binding of EF-actin alone.

Rhodamine-Phalloidin Staining and EM
To assess the state of assembly of membrane-bound actin, we processed samples for rhodamine-phalloidin staining and for EM. To determine if membrane-bound untreated actin is in the filamentous form, we incubated stripped liver cell membranes with 100 μg/ml actin, then stained with rhodamine-phalloidin. Since rhodamine-phalloidin binds specifically to F- but not G-actin (Estes et al., 1981), it is

Figure 5. Rhodamine-phalloidin staining of membrane-bound actin. (A) Unstripped membranes; (B) stripped membranes incubated without actin; (C) stripped membranes incubated with 100 μg/ml untreated actin for 2 h.
a useful marker for the presence of actin filaments. As shown in Fig. 5, only a low level of diffuse staining was detectable on stripped membranes alone or on stripped membranes with actin bound. Unstripped membranes, on the other hand, did exhibit specific, punctate staining, indicating that freshly isolated membranes do contain some actin filaments.

Membranes incubated with or without actin were also examined by EM. In agreement with our rhodamine–phalloidin staining results, no discernible actin filaments could be detected (Fig. 6). Whereas stripped membranes alone appeared quite smooth, membranes to which actin was added had regions where amorphous material was visible. This mate-

Figure 6. Electron micrographs of liver cell membranes with or without bound actin. (A) Unstripped membranes; (B) stripped membranes; (C) stripped membranes incubated with 500 µg/ml actin for 2 h. (Small arrows) Amorphous material; (large arrows) smooth membranes; (D) higher magnification of amorphous material associated with the membranes in (C).
Figure 7. Immunoelectron microscopy. (Large arrowheads) Gold particles decorate amorphous material. (Small arrows) Areas of smooth membranes do not label. (A) Direct detection with anti-actin IgG on 12-nm gold particles. (B) Indirect detection, with soluble antiactin followed by anti-rabbit IgG on 5-nm gold particles.

Material was most often apparent on large sheets or vesicles, suggestive of plasma membranes. In any event, it was clearly localized to a subset of the preparation. We have been unable, however, to reach any further conclusions as to the structure of this material, other than it is distinct from normal F-actin.

To determine if this amorphous material was actin, we performed immunoelectron microscopy with an anti-actin antibody. Samples were labeled with antiactin directly adsorbed to 12-nm gold particles, or were labeled by incubation with free anti-actin IgG followed by addition of 5-nm gold coated with second antibody. Both methods showed no staining of smooth membranes but heavy labeling of the amorphous material (Fig. 7). Labeling appeared to be specific since negative controls with ovalbumin-gold particles showed no labeling, and positive controls with unstripped membranes showed...
Actin Binding to Intact Hepatocytes

These results argue that the binding activity is not extracellular. However, hepatocytes do bind actin in our assay (not shown). While AMP-PNP nor was it prevented by carrying out the binding in the presence of 10 mM AMP-PNP. AMP-PNP, like ATP, inhibits actin binding but is nonhydrolyzable (Tranter et al., 1989). While some actin binding that was inhibited by excess unlabeled actin was detected, it was not inhibited by AMP-PNP nor was it prevented by carrying out the binding at 0–4°C (data not shown). Essentially identical results were also obtained with hepatocytes that were cultured overnight. Thus, no binding with the appropriate characteristics was observed. Membranes prepared from isolated hepatocytes do, however, bind actin in our assay (not shown). While these results argue that the binding activity is not extracellular, it cannot be ruled out that the binding sites become occupied by actin released during cell or membrane isolation (despite the fact that membrane isolation is carried out in the cold, conditions which inhibit actin binding in vitro) and that binding sites are not regenerated in culture.

A Model for Binding

Based on the results from our equilibrium binding and kinetics experiments, we propose a model for the binding of actin to liver cell membranes (Fig. 8). This model is meant to organize the results which have been presented. We emphasize, however, that the actual states of membrane-bound actin are unknown, and the structures depicted in Fig. 8 are not intended to be interpreted literally. It should be noted that there was no evidence for the presence of normal filaments on the membranes at any time during the time course of actin binding, as assayed by EM and rhodamine-phalloidin staining (data not shown).

Recall that the time course of binding of untreated actin has two components, the first of which approximately matches the rate and extent of binding for F-actin alone and the second of which approximately matches the rate and extent of binding of G-actin alone. According to this model, F-actin, which in assay buffer exists in equilibrium with actin monomers, binds rapidly to the membrane surface and precludes the further binding of actin monomers (step 1). This step represents the first phase of binding and occurs with both untreated and phalloidin-stabilized actin filaments. During step 1, the plateau phase, membrane-bound actin undergoes a slow rearrangement which reveals or creates additional binding sites. This step is blocked by phalloidin. Together with step 1, this step accounts for the ability of phalloidin-actin to effectively compete with other forms of actin for membrane-binding sites, even though it binds to membranes to a lesser extent. Following rearrangement, a second phase of binding occurs during which monomeric actin binds to the membrane until equilibrium is reached (step 3). This step might also involve new actin–actin associations in addition to actin-membrane contacts. Under conditions where no F-actin is present, actin monomers can bind directly to the membrane via step 3, bypassing steps 1 and 2.

While it is conceivable that these complex kinetics could result from the partial sealing of membrane vesicles (i.e., rapid binding to inside-out vesicles and slow binding to partially sealed right-out vesicles), this notion is unlikely. First, previous experiments failed to detect any sealing of vesicles before or after incubation in binding buffer (Tranter et al., 1989). Second, while sealing could possibly explain the two kinetic components, it is difficult to understand how it could account for the observed lag phase.

Conclusions

The major conclusion drawn from these studies is that actin reconstituted onto stripped liver cell membranes has properties inconsistent with either F- or G-actin. This conclusion is supported by several lines of evidence. First, Scatchard plots of equilibrium-binding data obtained with untreated actin are linear, indicating that actin-membrane binding is not cooperative under conditions that promote actin polymerization (Tranter et al., 1989). Second, both F- and G-actin bind with similar affinities, as evidenced by results with EF-, phallolidin-, and untreated actin. Third, no actin filaments have been detected in the reconstituted system, either by rhodamine-phalloidin staining or EM. Distinct regions of membrane-associated amorphous material which label with colloidal gold have, however, been visualized. This amorphous material thus appears to be actin, but its structure has yet to be determined.

This work reveals a phenomenon that is clearly complex and somewhat puzzling. Its major significance is that it raises the question of whether membrane-bound actin might be nonfilamentous. While it has generally been assumed that only filamentous actin binds to cell membranes, there are many reports in the literature that are difficult to explain with this model. In fact, there are numerous instances where membrane-associated actin appears to be in a "nontraditional" form. In the echinoderm sperm, specialized areas of...
the nuclear envelope and the acrosomal vacuole membrane become tightly associated with a pool of nonfilamentous actin (Tilney, 1976). Similarly, a domain of membrane-associated actin that does not stain with rhodamine-phalloidin has been identified in unfertilized sea urchin eggs (Bonder et al., 1989). In both of these systems, nonfilamentous actin appears to be stored in regions where it can rapidly be recruited into actin filaments.

Nonfilamentous actin has also been reported to be tightly associated with microvillar membranes from ascites tumor cells (Carraway et al., 1982) and plasma membranes from murine tumor and lymphoid cells (Mescher et al., 1981). Likewise, Hubbard and Ma (1983) have isolated membranes from the rat liver which contain a substantial amount of endogenous actin which does not decorate with myosin S-1. Thus, it seems that a least a portion of the actin bound to cell membranes is in a form about which we know very little. Mescher and co-workers (Mescher et al., 1981; Appar et al., 1986; Appar and Mescher, 1986) have suggested that a nonfilamentous form of membrane-bound actin may be an integral part of a distinct membrane skeletal structure. Their results may prove to be highly relevant to the phenomenon in liver.

It is likely that more than one form of membrane-bound actin is present in intact hepatocytes, as in other systems (Mescher et al., 1981; Gruenstein et al., 1975). While 1 M, pH 10.5, carbonate buffer is required to remove all endogenous actin from liver cell membranes, a fraction of the actin is removed by milder conditions (Tranter et al., 1989). Also, actin filaments are present on freshly isolated membranes, but not in the reconstituted system (Figs. 5 and 6). Furthermore, indirect associations would not be detected by our assay, for example, those mediated by the analogues of spectrin (Amrein-Gloor and Gazzotti, 1987) and ankyrin (Bennett, 1979) that are found in rat liver.

An important question that remains to be answered is the physiological significance of this interaction. The major difficulty in answering this question is that so little is known about actin in the hepatocyte. While it is unlikely that the binding activity which we have measured represents a mechanism by which the cortical cytoplasm becomes anchored to the plasma membrane, a structural role in another type of membrane skeleton is quite possible, perhaps a membrane skeleton analogous to that described by Mescher and colleagues (Mescher et al., 1981; Appar et al., 1985; Appar and Mescher, 1986). Alternatively, while available data argue against it, we cannot absolutely rule out the possibility that the actin-binding sites are extracellular. Resolution of these questions will very likely require the identification and characterization of the proteins responsible for this unusual association.

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