Evidence for a Direct, Nucleotide-sensitive Interaction between Actin and Liver Cell Membranes

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Abstract. We have investigated the association of actin with membranes isolated from rat liver. A plasma membrane–enriched fraction prepared by homogenization in a low salt/$CaCl_2$ buffer was found to contain a substantial amount of residual actin which could be removed by treatment with 1 M Na_2CO_3/$NaHCO_3$, pH 10.5. Using a sedimentation binding assay that uses gelsolin to shorten actin filaments and render membrane binding saturable (Schwartz, M. A., and E. J. Luna. 1986. *J. Cell Biol.* 102:2067–2075), we found that membranes stripped of endogenous actin bound ^{125}I-actin in a specific and saturable manner. Scatchard plots of binding data were linear, indicating a single class of binding sites with a K_d of 1.6 μm; 66 μg actin bound/mg membrane protein at saturation. Binding of actin to liver cell membranes was negligible with unstripped membranes, was competed by excess unlabeled actin, and was greatly reduced by preheating or proteolytic digestion of the membranes. Kinetic measurements showed that binding had an initial lag phase and was strongly temperature dependent. The binding of actin to liver cell membranes was also found to be competitively inhibited by ATP and other nucleotides, including the nonhydrolyzable analogue AMP-PNP. We conclude that we have reconstituted an interaction between actin and integral membrane proteins from the rat liver. This interaction exhibits a number of distinctive features which have not been observed in other actin–membrane systems.

EVIDENCE accumulated in recent years suggests that interactions between the plasma membrane and the underlying actin–cytoskeleton play an essential role in a number of cell activities. These include the determination and maintenance of cell shape, cell motility, cell adhesion, cytokinesis, phagocytosis, and the regulation of integral membrane protein mobility and distribution (reviewed in Cohen and Smith, 1985; Geiger, 1983; Jacobson, 1983). Despite their importance in normal cell functions, little is known about actin–membrane interactions at the molecular level.

To date, there are only two mammalian systems in which the molecular nature of actin–membrane interactions has been extensively studied: the erythrocyte and the microvillus of the intestinal brush border (reviewed in Cohen and Smith, 1985; Bennett, 1989; Marchesi, 1985; Mooseker, 1985). In both systems, the binding of actin to the plasma membrane is mediated by peripheral membrane proteins. Although there have also been reports that actin may directly interact with integral membrane proteins, the interactions have not been well-characterized. For instance, integral membrane glycoproteins have been reported to co-isolate with actin following Triton X-100 extraction (Carraway et al., 1983; Carey and Todd, 1986; Carey et al., 1987), and a 70-kD laminin receptor has been found to bind actin in vitro (Brown et al., 1983).

In the cellular slime mold *Dictyostelium discoideum*, the association of actin with the plasma membrane via integral membrane proteins has been studied in detail. Using a sedimentation assay that uses either gelsolin to cut and cap actin filaments (Schwartz and Luna, 1986) or an actin derivative that does not polymerize in solution (Schwartz and Luna, 1988), saturable binding curves were obtained. It was found that only F-actin bound to *Dictyostelium discoideum* plasma membranes with measurable affinity, that membranes stabilized actin filaments at concentrations well below the normal critical concentration for polymerization, and that binding was most likely due to the multivalent association of actin monomers within a filament with binding sites that are clustered in the membrane. Further studies have since revealed that a single integral membrane protein termed ponticulin was responsible for most of the observed actin-binding activity (Wuestehube and Luna, 1988).

In this article, we describe the use of a sedimentation binding assay to study the interaction of actin with membranes from rat liver. Ultrastructural studies have shown that actin filaments are attached to the hepatocyte plasma membrane, especially in regions surrounding the bile canaliculi (French and Davies, 1975; Oda et al., 1974; Ishii et al., 1985; Oyamada and Mori, 1985). The interaction of actin with the bile canalicular membrane has thus been postulated to pro-

vide the contractile force necessary for bile flow (French and Davies, 1975; Oda et al., 1974; Phillips et al., 1983; Oshio and Phillips, 1981). This hypothesis is supported by the finding that agents that perturb actin microfilaments disrupt bile flow in treated rat livers (Phillips et al., 1983; Gabbiani et al., 1975; and Phillips et al., 1975). Here we present evidence that the binding of actin to liver cell membranes is directly mediated by integral membrane proteins and is saturable, specific, and sensitive to certain nucleotides.

Materials and Methods

Chemicals

$Na^{125}I$ in pH 7–11 sodium hydroxide was purchased from Amersham Corp. (Arlington Heights, IL). Chloramine T was purchased from Eastman Kodak Co. (Rochester, NY). Bolton-Hunter reagent was obtained from Pierce Chemical Co. (Rockford, IL). BSA, ovalbumin, DTT, PMSF, aprotinin, trypsin, leupeptin, and all nucleotides were supplied by Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Proteins

Actin, isolated from rabbit skeletal muscle by the method of Spudich and Watt (1971), was further purified by gel filtration on Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, NJ) according to the procedure of MacLean-Fletcher and Pollard (1980). The purified actin was dialyzed against depolymerization buffer (50 μM $CaCl_2$, 1 mM ATP, 1 mM DTT, 0.02% sodium azide, 2 mM Tris-HCl, pH 8.0) and stored at 4°C for 3 wk.

Gel-filtered actin was radiolabeled with $Na^{125}I$ and stored as described by Schwartz and Luna (1986).

Porcine plasma gelsolin was purified according to the simplified chromatographic procedure of Cooper et al. (1987) with one additional step. After elution from the second DEAE-Sephacel column with a 0–0.5 M NaCl gradient, fractions containing gelsolin were combined and dialyzed against 25 mM Tris-HCl, pH 7.5, 0.5 mM $CaCl_2$, 2 mM DTT overnight. The dialyzed protein was then applied to a DEAE-Sephacel column equilibrated with 25 mM Tris-HCl, pH 7.5, 0.5 mM $CaCl_2$, and eluted with the same buffer. Fractions containing purified gelsolin were pooled, EGTA was added to 5 mM, and the protein (1–2 mg/ml) was stored at −80°C until use.

Anti-hamster fibroblast actin was generously provided by Dr. Richard Hynes.

Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as the standard. 1% SDS was included in all samples.

Membrane Preparation and Extraction

Membrane Preparation 1. Unless otherwise stated, liver cell membranes were prepared according to a modification of the procedure of Dorling and LePage (1973). Briefly, the excised liver (~10 g) of an adult male Sprague-Dawley rat was placed in ice-cold low salt homogenization buffer (2 mM Tris-HCl, 0.5 mM $CaCl_2$, 0.5 mM DTT, 0.4 mM PMSF, 4 μg/ml leupeptin, 4 μg/ml aprotinin, pH 7.5). For one membrane preparation, 1 mM $Na_2B_4O_7$, 0.5 mM $CaCl_2$, 0.5 mM DTT, 1 mM PMSF, 4 μg/ml leupeptin, 4 μg/ml aprotinin, pH 7.5 was used. Although this preparation yielded similar results in our assay, nonspecific binding was higher. The tissue was washed free of blood, minced into small pieces, and dispersed with five strokes of a glass pestle in a loose fitting Dounce homogenizer (10 ml homogenization buffer per gram of tissue). The resultant homogenate was centrifuged in 50 ml conical centrifuge tubes at 150 *g* for 10 min in a refrigerated centrifuge (4°C). After decanting the supernatants, the nuclear pellets were twice resuspended in 25 ml of buffer and centrifuged as before. The supernatants were pooled, the membranes were collected by centrifugation at 2,000 *g* for 10 min, and the membrane pellet was washed with 100 ml of homogenization buffer. The final pellet was resuspended in Tris-HCl homogenization buffer and stored in 0.5 ml aliquots (5–10 mg/ml) at −80°C until use.

Membrane Preparation 2. In our initial experiments to determine if actin binds to stripped membranes, liver cell membranes were prepared as follows. Rat livers were homogenized in ice-cold Tris-HCl homogenization buffer (6 ml buffer per gram of tissue) with a Tekmar Tissumizer (Cincinnati, OH). After centrifugation of the homogenate at 2,000 *g* in a refrigerated centrifuge (4°C), the supernatant was decanted, and the pellet discarded. The supernatant was then centrifuged for 1 h at 27,000 *g*. The final membrane pellet was resuspended in homogenization buffer and stored in 0.5 ml aliquots at −80°C.

Carbonate Extraction. Membranes were resuspended in 1.0 ml carbonate extraction buffer (1 M $Na_2CO_3/NaHCO_3$, 0.5 mM DTT, 4 μg/ml aprotinin, 4 μg/ml leupeptin, pH 10.5) and incubated for 30 min at 0°C. The stripped membranes were pelleted in a Fisher microcentrifuge at 13,600 *g* for 30 min. The pellet was then washed three times, once with carbonate buffer and twice with low salt buffer (2 mM Tris-HCl, 0.5 mM DTT, 0.4 mM EDTA, 0.4 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, pH 7.6). The final pellet, referred to as stripped membranes, contained ~25% of the initial protein. It was resuspended in low salt buffer, stored overnight at 4°C, and used the next day in a binding assay.

Protease Treatment. For use as a control, stripped membranes were washed free of protease inhibitors with 2 mM Tris-HCl, pH 7.4, resuspended in 200 μg/ml pronase (Sigma Chemical Co.) and incubated for 10 min at 37°C. After centrifugation in a Fisher microcentrifuge at 13,600 *g* for 30 min, the pellet was resuspended and washed three times with low salt buffer containing protease inhibitors. The resultant membranes were then used immediately in a binding assay.

Heat Denaturation. Stripped membranes in a 500 μl polypropylene tube were heated for 30 min by immersion in a 95°C water bath.

Actin–Membrane Binding Assays

Actin-membrane binding assays were performed essentially as described by Schwartz and Luna (1986), with a few minor exceptions. Immediately before use, actin was gel-filtered on Sephadex G-25 (Pharmacia Fine Chemicals) equilibrated with 50 μM $CaCl_2$, 1 mM DTT, 0.02% sodium azide, 2 mM Tris-HCl, pH 8.0, to remove ATP. Various concentrations of ^{125}I-labeled actin and gelsolin (at a mole ratio of 15:1 actin to gelsolin) were mixed with stripped membranes (0.5 or 1.0 mg/ml) in a total volume of 30 μl of binding buffer (100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 20 mM Pipes, 100 μm $CaCl_2$, 100 μm PMSF, 0.4 μg/ml leupeptin, pH 6.8) containing 20 mg/ml ovalbumin. Unless otherwise indicated, samples were incubated at room temperature for 2.5 h, layered onto 350 μl of 10% sucrose in binding buffer in 400 μl polyethylene tubes, and centrifuged for 15–30 min at 11,600 *g* in a Microfuge 11 (Beckman Instruments, Inc., Palo Alto, CA). The tubes were then frozen, the tips containing the pellets were cut off, and the radioactivity in the pellets and supernatants was counted in a gamma counter.

For the kinetics assay, each sample was prepared on ice, and then, upon the addition of actin, it was immediately placed in the appropriate temperature water bath to initiate the binding reaction. After the indicated period of time, each sample was spun through sucrose and the radioactivity in the pellet and supernatant was counted.

For the assays in which nucleotides were used, each nucleotide was dissolved in 100 mM Pipes and adjusted to pH 7.0. The final concentration of Pipes in these assays was 40 mM. For the ATP competition experiments, the nucleotide was dissolved in water and adjusted to pH 7.0. To reduce variability in our results, we preincubated samples with nucleotides for 30 min before the addition of actin.

Electron Microscopy

Fractions containing 100 μg of either stripped or unstripped membranes were prepared for thin sectioning by centrifugation in a rotor (model SW-41; Beckman Instruments, Inc.), using BEEM hemihyperboloid polyethylene capsules (cat. no. 2323; Ladd Research Industries, Inc., Burlington, VT) in Epon centrifuge adaptors (Goodenough, 1975). The samples were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.04% picric acid cacodylate buffer with 0.04% $CaCl_2$ for 1 h, washed in cacodylate buffer, and postfixed in maleate buffered 1% osmium tetroxide for another hour. The samples were washed in H_2O, dehydrated, and embedded. Thin sections of the pellets were cut in parallel to the direction of centrifugation to ensure visualization of all elements of the fraction and stained with lead citrate and uranyl acetate.

Gel Electrophoresis and Immunoblotting

100-μg samples of stripped and unstripped membranes were run on 8% SDS polyacrylamide slab gels using the buffer system of Laemmli (1970). Gels were either stained with Coomassie brilliant blue R-250 (125 mg/liter in 50% methanol/10% acetic acid; Bio-Rad Richmond, CA) or electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Keene, NH)

overnight at 20V for immunoblotting. After transfer, the nitrocellulose was blocked with Blotto (5% wt/vol nonfat dry milk, 0.05% Tween 20, 0.01% Antifoam A, 2 mM $CaCl_2$, and 50 mM Tris, pH 8.0) as described by Johnson et al. (1984). The blot was incubated with anti-actin for 2 h, then washed four times with Blotto. To visualize the bound antibody, the blot was incubated with ^{125}I-Protein A (Amersham Corp.) for 1 h (Burnette, 1981). After several washings, the blot was dried and then autoradiographed on Kodak XAR-5 film with a Dupont Cronex Lightening Plus screen.

Results

Membrane Preparation

To measure the binding of actin to membranes, it is important that the membranes used are first stripped of their endogenous actin. This ensures that any binding measured in the assay is the result of a direct interaction of actin with the membranes rather than binding to residual endogenous actin. Early experiments in our laboratory, however, showed that rat liver cell membranes isolated by procedures that include homogenization in an isotonic sucrose buffer could not be completely extracted of actin by agents known to solubilize peripheral membrane proteins. Similar observations have also been made by others (Hubbard and Ma, 1983). The reason for this is unknown but may be the result of membrane sealing. It was therefore necessary to avoid using sucrose in our isolation procedure, which precluded use of standard methods for preparing highly purified plasma membranes. The use of discontinuous sucrose gradient centrifugation to further purify our membrane preparation after removal of peripheral membrane proteins did not result in any significant separation of membrane fractions.

Rat liver was homogenized in a low salt/$CaCl_2$ buffer, and a plasma membrane-enriched fraction was collected by a series of low speed centrifugations. This fraction was used without further treatment. When analyzed by electron microscopy for purity and structure, these membranes appeared to be substantially enriched in plasma membranes. They also showed a significant level of contamination by endoplasmic reticulum and, to a much lower extent, mitochondria, Golgi, and lysosomes. A substantial amount of amorphous material, some of which resembled filamentous networks, was also present, as were residual glycogen granules (Fig. 1). In contrast, membranes that have been subjected to extraction with carbonate buffer appear as clean, homogeneous smooth sheets and vesicles (Fig. 2).

To determine if the membranes seen in Fig. 2 are sealed or become sealed during subsequent binding assays, we preincubated stripped membranes in either binding buffer or low salt buffer for 3 h at room temperature and then digested them with 280 μg/ml trypsin in the absence or presence of 0.5% Triton X-100. Analysis by SDS-PAGE showed that the membrane proteins were essentially completely digested under all conditions tested, suggesting that little, if any, resealing occurs (data not shown).

As revealed by SDS-PAGE analysis, extraction of crude liver cell membranes with 1 M Na_2CO_3/$NaHCO_3$, pH 10.5, results in the solubilization of a number of proteins, including one that runs as a prominent band at 43 kD, the molecular mass of actin (Fig. 3 *a*). Note that a protein band that runs just slightly behind actin is not removed by extraction. Thus, to determine if actin is among the proteins removed, samples of stripped and unstripped membranes were run on an 8%

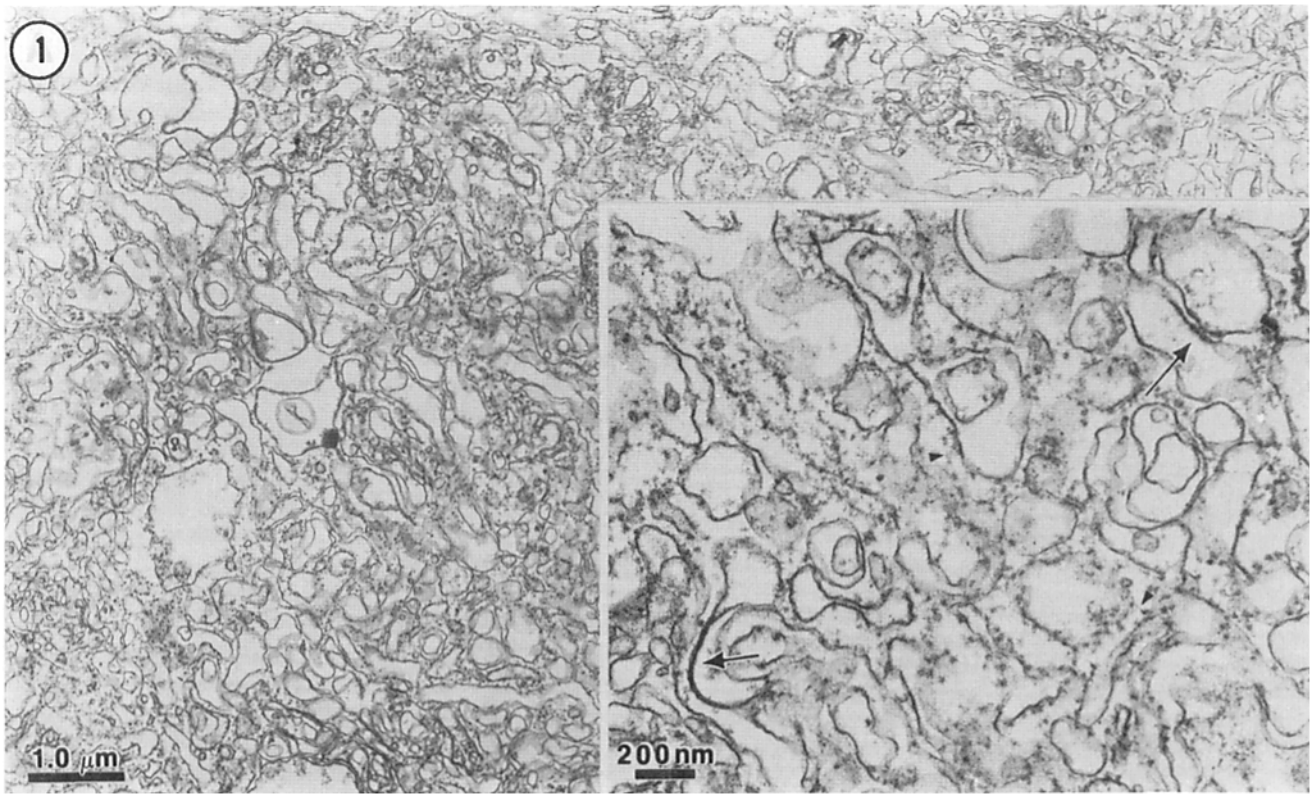

Figure 1. Electron micrograph of isolated rat liver cell membrane preparation. (*Small arrows*) Amorphous material resembling filaments; (*large arrows*) intercellular junctions.

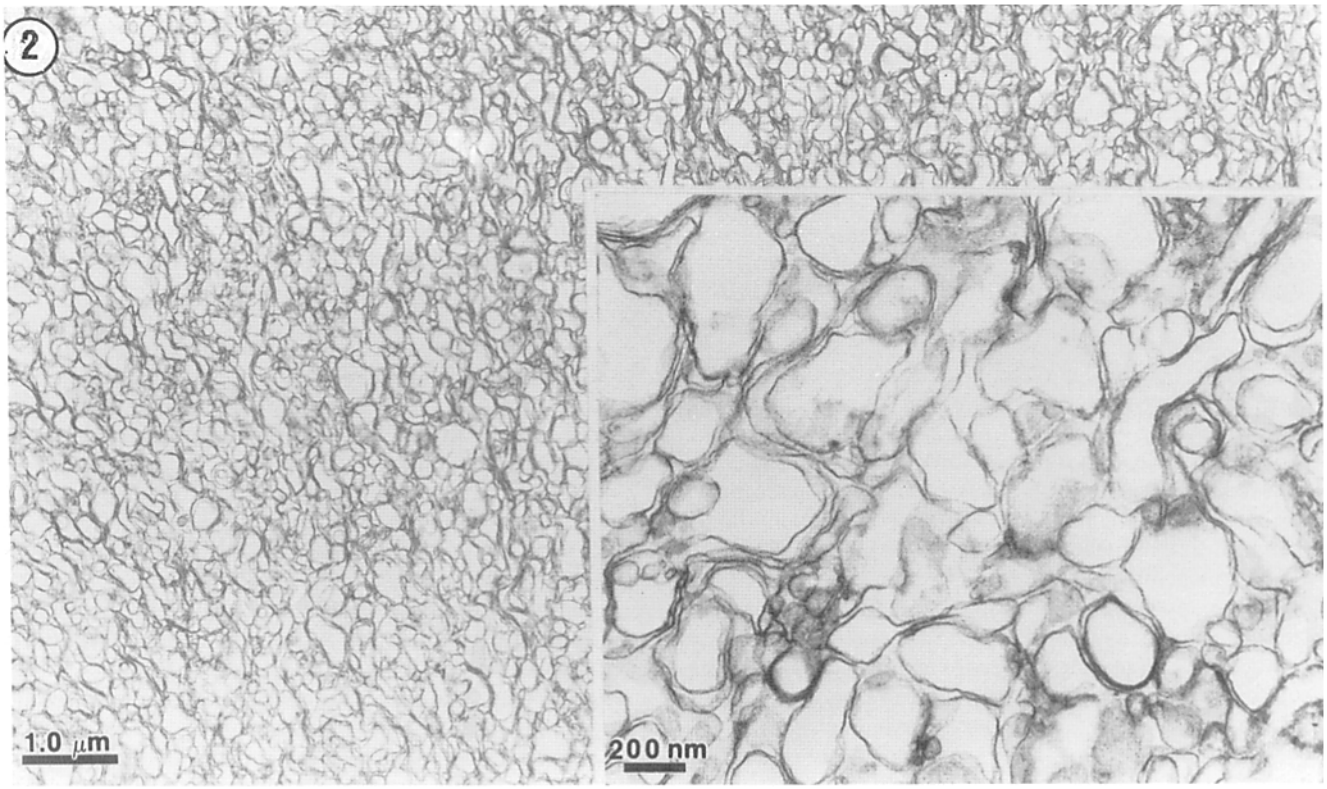

Figure 2. Electron micrograph of liver cell membranes after carbonate treatment.

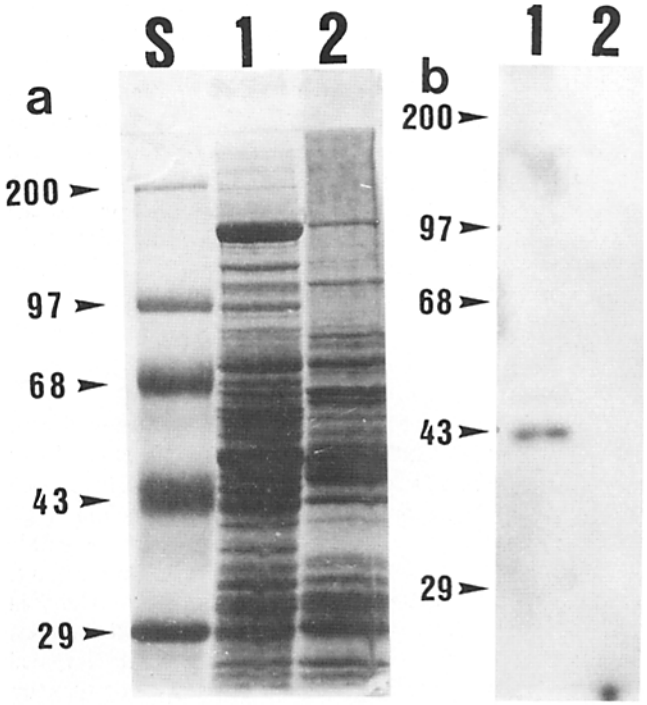

Figure 3. (*a*) Comparison of the Coomassie blue staining pattern of samples of unstripped (lane *1*) and stripped (lane *2*) liver cell membranes. Samples were run on an 8% SDS polyacrylamide gel; 100-μg samples were run in each lane. High molecular mass standards (lane *S*). (*b*) Immunoblot analysis of samples of unstripped (lane *1*) and stripped (lane *2*) liver cell membranes. 100 μg of each were run on an 8% SDS polyacrylamide gel, transferred to nitrocellulose, and probed with anti-actin followed by ^{125}I-protein A, and processed for autoradiography.

SDS polyacrylamide gel, transferred to nitrocellulose, and probed with anti-actin. As shown in Fig. 3 *b*, crude liver cell membranes contain a substantial amount of actin which is completely removed by treatment with the carbonate buffer. Assuming that all of the 43-kD band removed by the carbonate buffer was actin, then actin accounts for ~5% of the total membrane protein as determined by scanning densitometry of stripped and unstripped membranes.

Actin Binding to Extracted Liver Cell Membranes

A sedimentation binding assay (Schwartz and Luna, 1986) was used to measure the binding of ^{125}I-actin to stripped cell membranes. This assay uses the actin-capping protein gelsolin to limit actin filament length and thus render actin-membrane binding saturable. As shown in Fig. 4, extracted membranes incubated with ^{125}I-actin in the presence of gelsolin bound actin in a saturable manner. This binding appears to be specific since it was competed by excess unlabeled actin. Binding to unstripped membranes, on the other hand, was negligible.

To determine if the binding of actin to stripped liver membranes required native membrane proteins, we denatured membranes by heating and by proteolysis. In both instances, binding was greatly diminished (Fig. 4). These data, together with the fact that peripheral membrane proteins are extracted by the alkaline buffer, suggest that actin binding to liver cell membranes is mediated by integral membrane proteins.

Kinetics of binding were analyzed at three different temperatures. At each temperature, stripped membranes (0.5 mg/ml) were incubated with 75 μg/ml ^{125}I-actin for various times (0–4 h), then sedimented through sucrose. As shown

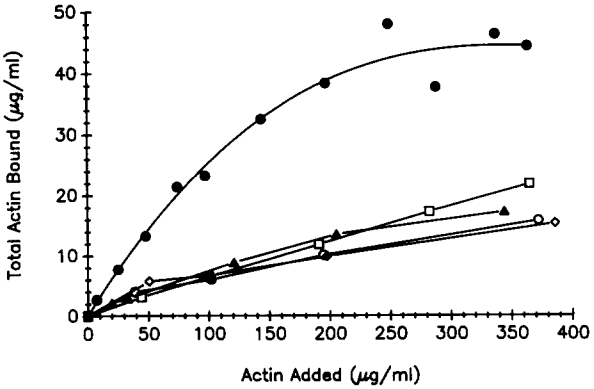

Figure 4. Total binding of ^{125}I-Bolton-Hunter-labeled actin to stripped liver membranes (1.0 mg/ml; see Materials and Methods, Membrane Preparation 2) in the presence of gelsolin at a 15:1 mole ratio of actin/gelsolin (●). (Controls) Binding to membranes preheated to 95°C for 30 min (□); binding to membranes in the presence of 2.8 mg/ml unlabeled actin (▲); binding to membranes pretreated with 200 μg/ml pronase for 10 min. at 37°C (○); and binding to unstripped membranes (◇). Samples were incubated for 1 h at room temperature.

in Fig. 5, the binding of actin to stripped membranes was strongly temperature-dependent with binding being completely inhibited at 0–4°C. Binding at room temperature was unusually slow, requiring a 2.5-h incubation to achieve a steady-state and having an initial lag phase of ~60 min. At 37°C, on the other hand, the rate of binding was substantially increased and the lag phase was reduced, but the amount of actin bound at saturation was only slightly higher. Equilibrium binding data was analyzed by the method of Scatchard (1949). Fig. 6 shows a binding curve (*a*), together with its corresponding Scatchard plot (*b*), from an assay done at room temperature. The Scatchard plot was linear with a K_d of 1.6 μM. The amount of actin bound at saturation was found to be 66 μg actin/mg membrane protein. An experiment in which actin was allowed to bind for 18 h at room temperature gave similar data.

ATP Inhibition of Actin–Membrane Binding

Normally, actin is kept in the presence of millimolar ATP.

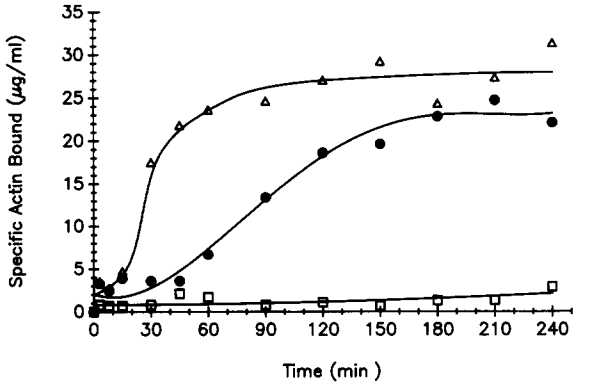

Figure 5. Time course of actin–membrane binding at three different temperatures. At each temperature, stripped membranes (0.5 mg/ml) were incubated with 75 μg/ml ^{125}I-actin for the indicated periods of time. The samples were then spun through sucrose and the radioactivity in the pellets and supernatants was counted. Binding at 0–4°C (□; 22°C (●); and 37°C (△). (Specific actin bound = total actin bound − actin bound in the presence of 25 mM ATP.)

However, we noticed that the ATP concentration had an effect on our binding curves. Therefore, we systematically investigated the role of ATP in the interaction of actin with liver plasma membranes by including different concentrations of the nucleotide in our binding assay. When stripped membranes were incubated with actin in the presence of increasing concentrations of ATP, binding was dramatically reduced (Fig. 7 *a*). In fact, we have found that 25 mM ATP inhibited actin binding as effectively as excess unlabeled actin (data not shown) and thus can be used for the assessment of nonspecific binding.

To investigate the specificity of nucleotide inhibition, we tested a number of nucleotides for their ability to inhibit actin binding. 40 μg/ml of ^{125}I-actin was incubated with stripped membranes (0.5 mg/ml) in the presence of increasing concentrations of each nucleotide. The results presented in Fig. 7 *b* show that the nucleoside triphosphates, ATP, GTP, and CTP, as well as the nucleoside diphosphate ADP, are all effective at inhibiting actin binding, with a 50% reduction in binding occurring at a nucleotide concentration of ~0.5 mM. AMP caused only a slight reduction in binding, whereas cAMP and sodium pyrophosphate had virtually no effect.

Based on the finding that ADP reduced actin binding to the same extent as the nucleoside triphosphates, it seemed unlikely that protein phosphorylation was necessary for inhibition. However, since the membranes may be capable of interconverting nucleotides, we tested the effect of adenylyl-imidodiphosphate (AMP-PNP)[1], a nonhydrolyzable analogue of ATP. AMP-PNP also substantially inhibits the binding of actin to stripped liver plasma membranes (Fig. 7, *b*, *c*, and *d*), indicating that phosphorylation is not involved.

To analyze the inhibition further, actin-binding assays were performed in the presence of different concentrations of AMP-PNP (Fig. 7 *c*). AMP-PNP was used because it would not undergo hydrolysis during the course of the experiment. A double-reciprocal plot of the results showed that inhibition was competitive with a K_i of 0.3 mM (Fig. 7 *d*). Similar results were obtained with ATP, with the K_i calculated to be 0.5 mM (data not shown).

Discussion

Although it has been shown that actin associates with liver cell membranes (French and Davies, 1975; Oda et al., 1974; Hubbard and Ma, 1983) and that perturbations of this association result in the impairment of cell function (Phillips et al., 1983; Gabbiani et al., 1975; Phillips et al., 1975), very little is known about this interaction at the molecular level. In this article, we have described the use of a sedimentation binding assay to analyze the binding of actin to liver cell membranes. We have shown that, in the presence of gelsolin, liver cell membranes bind actin in a saturable and specific manner. Since our membranes are treated with carbonate buffer before use, it does not appear that residual endogenous actin or peripheral membrane proteins are responsible for the observed binding activity. However, native membrane proteins do seem to be necessary, since binding is substantially reduced when membranes are preheated or proteolytically

1. *Abbreviation used in this paper:* AMP-PNP, adenylyl-imododiphosphate.

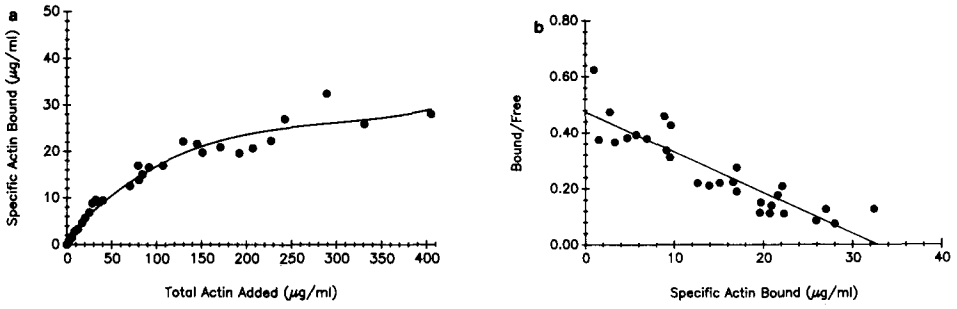

Figure 6. Analysis of equilibrium binding. (*a*) Specific binding (determined as in Fig. 5) of ^{125}I-actin to stripped liver membranes (0.5 mg/ml). Samples were incubated for 2.5 h at room temperature. (*b*) Scatchard plot of above equilibrium binding data.

digested. We conclude that integral membrane proteins most likely mediate the interaction of actin with liver cell membranes.

The membranes used in this study, although enriched in plasma membranes, are not pure. Electron micrographs show that these membranes are contaminated by endoplasmic reticulum and, to a much lesser extent, by mitochondria, Golgi, and lysosomes. Therefore, our results do not prove that the observed interaction is due to plasma membranes. Given the fact that actin has been shown by others to coisolate with plasma membranes (Hubbard and Ma, 1983; Govindan and Wieland, 1975; Yousef and Murray, 1978), we think it likely that plasma membranes are responsible. However, resolution of this point will have to await future work.

The interaction between actin and liver membranes exhibits a number of unusual features which are internally consistent and in agreement with the work of others. First, both we and others have found that membrane associated actin is very tightly bound. In addition to surviving the homogenization and centrifugation steps of the isolation procedure, membrane-bound actin is very resistant to extraction by a variety of agents. Early work in our laboratory showed that prolonged incubation in a low salt, actin-depolymerization buffer did not remove endogenous actin from our membranes, nor did high salt or 1 M KI. Hubbard and Ma (1983) made similar observations and found that membrane-bound actin was resistant to treatments that depolymerize actin in solution and was released from the membranes only by extremes of pH.

We have also observed that actin reconstituted onto stripped liver membranes was very stably bound. While we have not directly measured the dissociation rate, no dissociation of bound radiolabeled actin was detected after several hours, even in the presence of 25 mM ATP (data not shown). This, together with the fact that the binding of actin to stripped membranes was unusually slow and strongly temperature dependent, argues that binding probably does not occur through a nonspecific or simple electrostatic interaction. The strong temperature dependence and the initial lag phase also suggest that the binding event may be relatively complex, requiring some structural rearrangement.

Consistent with the slow rate of dissociation and high stability of plasma membrane–bound actin is the finding that unstripped membranes do not bind actin in our assay. This

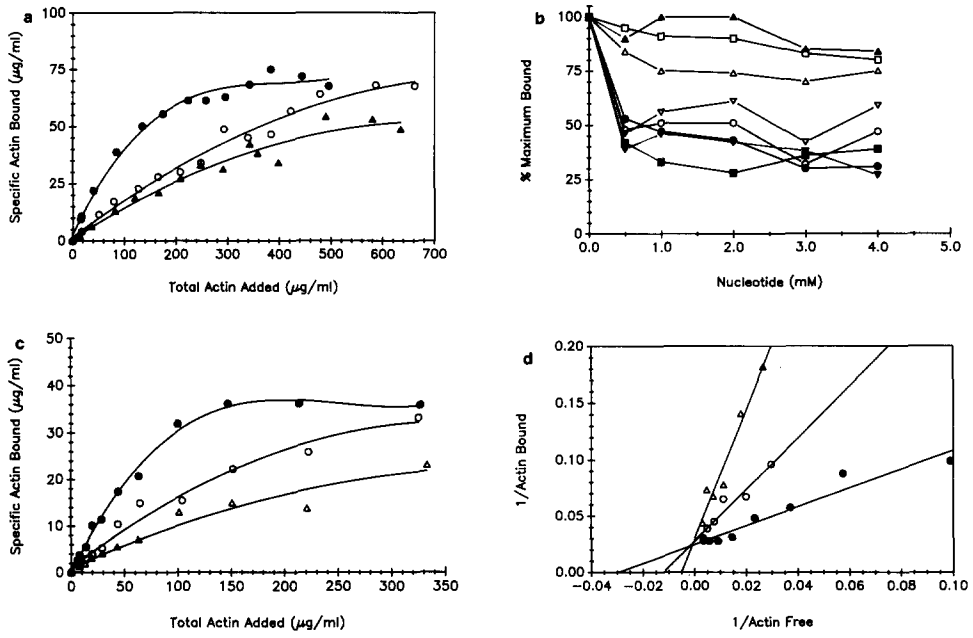

Figure 7. Effect of various nucleotides on the binding of ^{125}I-actin to stripped liver membranes. All samples were preincubated with nucleotide for 30 min before actin was added. (*a*) Specific binding of ^{125}I-actin to stripped liver membranes (1.0 mg/ml) in the presence of increasing concentrations of ATP. Binding without ATP (●); with 1.5 mM ATP (○); and with 3.0 mM ATP (▲). (Specific actin bound = total actin bound − actin bound to unstripped membranes.) (*b*) The effect of various nucleotides on actin-membrane binding. 40 μg/ml ^{125}I-actin was incubated with stripped liver membranes (0.5 mg/ml) in the presence of increasing concentrations of nucleotide. Amount of actin bound is expressed as the percent of actin bound in the absence of nucleotide. (●) ATP; (○) ADP; (▲) cAMP; (△) AMP; (■) AMP-PNP; (□) sodium pyrophosphate; (▼) GTP; (▽) CTP. (*c*) Specific binding (as determined in Fig. 5) of ^{125}I-actin to stripped liver membranes (0.5 mg/ml) in the presence of increasing concentrations of AMP-PNP, a nonhydrolyzable analogue of ATP. Binding without AMP-PMP (●); with 0.5 mM AMP-PNP (○); and with 1.5 mM AMP-PNP (△). (*d*) Double-reciprocal plot of the data shown in *c*. Without AMP-PNP (●); with 0.5 mM AMP-PNP (○); and with 1.5 mM AMP-PNP (△).

result suggests (though does not prove) that most, if not all, of the actin-binding sites are occupied in intact cells. It also argues against the possibility that the observed binding is to the extracellular side of the plasma membrane.

The binding detected in our assay appears to account for a large fraction of the initially membrane-bound actin. At saturation, actin constitutes ~6.6% of the total membrane protein. This result suggests that our assay measures a major actin-binding activity of liver plasma membranes, although other interactions may certainly exist in the intact cell that are lost after cell lysis or carbonate extraction.

Scatchard plots of our equilibrium binding data yield straight lines, indicating that the association of actin with liver cell membranes is mediated by a single class of binding sites. The finding that high concentrations of ATP can completely inhibit actin-binding supports this conclusion, since it implies that if more than one class of binding sites are present, then they all must be nucleotide sensitive. The fact that we obtained linear Scatchard plots also indicated that actin-binding to our membranes is not cooperative, which is in sharp contrast to observations with *Dictyostelium discoideum* plasma membranes in a similar assay. In that system, actin-membrane associations exhibited strong positive cooperativity (Schwartz and Luna, 1986). Because this result indicated that *Dictyostelium discoideum* membranes had a much higher affinity for actin filaments than for actin monomers, a mechanism for binding was proposed in which actin-binding is tightly coupled to actin polymerization (Schwartz and Luna, 1988). Given that our data are not consistent with this type of model, we suggest that liver cell membranes probably bind actin through some other mechanism. Interestingly, Hubbard and Ma (1983) reported (but did not show) that the endogenous actin bound to their isolated membranes did not appear to be in the filamentous form, since, despite the high level of actin present, no myosin-decorated filaments could be detected by electron microscopy.

Perhaps the most interesting property of the actin-membrane interaction in our system is that it is inhibited by ATP and other nucleotides. Although much remains to be learned about the mechanism by which nucleotides inhibit actin binding, we do know that it occurs by competition and does not require nucleotide metabolism, since AMP-PNP is as effective an inhibitor as ATP. Our interaction appears to be distinct from the binding of actin to the 110K–calmodulin complex of the intestinal microvillus, which, although nucleotide sensitive (Verner and Bretscher, 1985), exhibits different properties. For instance, the 110K–calmodulin complex is not an integral membrane protein, and the binding of actin to this complex can be rapidly dissociated by ATP.

Extrapolation of the data from our experiments with nucleotides to the situation found in intact cells must be made with caution. Although physiological concentrations of ATP were shown to significantly inhibit actin binding to liver cell membranes, we cannot conclude that actin-membrane interactions should not occur in vivo. The actin concentration in intact cells is in the range of 0.1 mM (based on the assumption that the protein concentration in mammalian cells is 180 mg/ml [Alberts et al., 1983] and that actin constitutes 1–2% of total cell protein in the mammalian liver [Korn, 1978]) and thus should be capable of effectively competing with ATP for available binding sites. As mentioned previously, binding sites also appear to be occupied in intact cells. Thus, the exact role that ATP plays in actin-membrane associations in vivo has yet to be determined. It seems likely that this effect represents part of a more complex regulatory system.

In conclusion, we have reconstituted a direct interaction between actin and liver membranes. This interaction exhibits a number of distinctive features, including slow kinetics of association and dissociation, a strong temperature dependence, and competitive inhibition by nucleotides. We hope that these results establish a new system in which direct actin–membrane interactions can be investigated at the biochemical level.

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