Detection of Vinculin-binding Proteins with an ^{125}I-Vinculin Gel Overlay Technique

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ABSTRACT Vinculin is an adhesion plaque component localized on the cytoplasmic side of the cell membrane where stress fibers end. To detect vinculin-binding proteins, we have developed an ^{125}I-vinculin gel overlay method. SDS PAGE was used to separate different protein preparations. After fixing the proteins in the gel with methanol-acetic acid, the SDS was removed with ethanol and the proteins renatured in buffer. The gel was then incubated with ^{125}I-vinculin. After extensive washing to remove nonspecifically associated label, the gel was dried and autoradiographed. Chick embryo fibroblasts, their Rous sarcoma virus transformants, and HeLa cells were found to contain two proteins (M_r 220,000 and 130,000) that bound ^{125}I-vinculin strongly and another (M_r 42,000) that bound it moderately. The 130,000-mol-wt protein was identified as vinculin itself, which suggests that it may self-associate. The 42,000-mol-wt protein was identified as actin with which vinculin is known to interact. The identity of the 220,000-mol-wt protein is not known. It is not cellular fibronectin, myosin, or filamin. When fibroblast proteins were separated into Triton X-100 soluble and insoluble fractions, most of the vinculin and the 220,000-mol-wt protein was found to be in the soluble fraction. Chicken gizzard also contained these vinculin-binding proteins along with three others of M_r 190,000, 170,000, and 100,000.

Cells in culture adhere tightly to the substrate at discrete sites called adhesion plaques and less tightly in regions of close contact (1, 22). The ends of stress fibers, bundles of actin filaments and associated proteins, are localized on the cytoplasmic side of the adhesion plaque (2). This suggests that adhesive molecules on the cell surface and elements of the actin-based cytoskeleton are physically linked.

Recently, Geiger (14) and Burridge and Feramisco (7) identified vinculin, a protein of M_r 130,000, and localized it on the cytoplasmic side of the adhesion plaque. Vinculin interacts with actin (8, 23, 30) and has been postulated to link the ends of actin filaments to the membrane (7, 14–16, 30). Since vinculin does not appear to be an integral membrane protein (14), there must be at least one transmembane connection to the substratum.

To search for such a transmembrane connection as well as other proteins that interact with vinculin and that may be adhesion plaque components, we have developed an ^{125}I-vinculin gel overlay method. In this paper, we describe this method and demonstrate that two proteins found in chick fibroblasts, chicken gizzard, and HeLa cells strongly bind ^{125}I-vinculin. The identity of one of these proteins of 220,000 mol wt is unknown; the other, of 130,000 mol wt is vinculin itself.

MATERIALS AND METHODS

Cells and Cell Preparations: Normal chick embryo fibroblasts (CEF)[1] were prepared by trypsinizing decapitated 12-d-old chick embryos with their internal organs removed. The cells were cultured in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum, 0.125 μg/ml penicillin, and 0.37 μg/ml streptomycin sulfate. The cultures were subcultured at least once and usually twice to obtain cultures consisting primarily of fibroblasts. All media and sera were from Grand Island Biological Co. (Grand Island, NY).

For infection with Rous sarcoma virus (RSV type A, American Type Culture Collection, Rockville, MD), primary confluent cultures of chick (Spafas, Inc.; Norwich, CT) fibroblasts were trypsinized and plated in DME supplemented with 2% tryptose phosphate, 1% calf serum, and 1% heat-inactivated chicken serum. After 3 h, the cells were infected with RSV-A for 1 h after which culture medium was added. After several days, the cells appeared transformed in that they were all rounded. They were trypsinized and replated in DME containing 10% tryptose phosphate, 4% calf serum, and 1% heat-inactivated chicken serum.

Cells were separated into Triton-soluble and -insoluble fractions according to the methods of Brown et al. (6). Briefly, 100-cm petri dishes of confluent CEF or RSV-CEF were washed twice at 37°C with TGMC buffer (137 mM

[1] *Abbreviations used in this paper:* CEF, chick embryo fibroblasts; RSV, Rous sarcoma virus; TGMC, 137 mM NaCl, 5 mM KCl, 5 mM dextrose, 0.5 mM $MgCl_2$, 0.025 mM $CaCl_2$, and 25 mM Tris-HCl, pH 7.4.

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NaCl, 5 mM KCl, 5 mM dextrose, 0.5 mM $MgCl_2$, 0.025 mM $CaCl_2$, and 25 mM Tris-HCl, pH 7.4). The cells were then lysed for 3 min with 1 ml of 0.5% Triton X-100 in TGMC containing 1 mM phenylmethylsulfonyl fluoride and 1 mM Na-p-tosyl-L-arginine methyl ester. The Triton X-100 supernatant was removed, made 1% in SDS, and immediately boiled. The remaining Triton-insoluble fraction was washed with 10 ml of 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na-p-tosyl-L-arginine methyl ester in TGMC. 1 ml of hot 1% SDS was added to the plate and the Triton-insoluble fraction scraped off the plate with a rubber policeman. This preparation was then boiled. For whole cell preparations, the plates were washed twice with TGMC, 2 ml of hot 1% SDS was added, and the cells scraped from the plate and boiled. HeLa cell proteins solubilized in 1% SDS were a generous gift from Dr. Claudia Kent (Purdue University).

Preparations from normal CEF enriched in adhesion plaques were made according to the methods of Avnur and Geiger (3). Cells in 100-cm plates were rinsed with 5 mM $MgCl_2$, 3 mM EGTA, and 50 mM 2(*N*-morpholino) ethanesulfonic acid, pH 6.0 and then incubated for 2 min with the same buffer containing 1 mM $ZnCl_2$. The plates were rinsed with phosphate-buffered saline and the main portion of the cells removed with a stream of phosphate-buffered saline from a squirt bottle. 1 ml of hot 1% SDS was added to the plate, the plate scraped with a rubber policeman, and the preparation immediately boiled. The material collected from 5–10 plates was precipitated with 9 vol of cold (−20°C) acetone. This mixture was centrifuged, and the pellet was solubilized in 300 μl of 2% SDS and reboiled.

Protein Preparations: Vinculin, filamin, and α-actinin were purified from frozen chicken gizzards (Pel-Freeze Biologicals, Rogers, AR) as described by Feramisco and Burridge (12) with the following modifications. Buffer B, used for the solubilization of the final ammonium sulfate pellet, as the buffer for the DE-52 column, and for vinculin storage, consisted of 10 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, and 20 mM Tris acetate, pH 7.6. The DE-52 column was eluted with a gradient of 10–250 mM NaCl. Vinculin was eluted from the column in a highly purified form and was used without further purification. Filamin and α-actinin from this column were used without further purification as standards for gel electrophoresis.

Actin was purified from acetone powders of rabbit skeletal muscle as described by MacLean-Fletcher and Pollard (26). Myosin was purified from chicken gizzard according to Barany et al. (4). Spectrin-containing human erythrocyte membrane preparations were a generous gift from Dr. Philip Low (Purdue University).

Cellular fibronectin was obtained by extracting chick embryo fibroblasts with 1 M urea in DME containing 2 mM phenylmethylsulfonyl fluoride (31). This extract was centrifuged for 15 min at 27,000 *g*. The supernatant was precipitated with 70% ammonium sulfate for 1 h and the precipitate collected by centrifugation for 15 min at 27,000 *g*. The pellet was suspended in 0.1 M NaCl, 1 mM $CaCl_2$, 0.05 M cyclohexylaminopropane sulfonic acid buffer, pH 11 and dialyzed against this buffer overnight at 4°C. Fibronectin was purified from this extract by affinity chromatography on gelatin-Sepharose 4B (11).

The concentrations of the various proteins were determined by the method of Bradford (5), using bovine serum albumin as the standard.

Iodination of Vinculin and Overlay Procedure: Vinculin was dialyzed extensively against 0.125 M sodium borate buffer, pH 8.4. 25–50 μg of the protein in 25–50 μl was incubated for 30 min at 4°C with 2 mCi ^{125}I-Bolton-Hunter reagent (>1,500 Ci/mM; ICN Pharmaceuticals, Irvine, CA) that had been dried under a gentle stream of nitrogen. The reaction was quenched for 15 min at 4°C by the addition of 0.2 M glycine in 0.125 M sodium borate, pH 8.4. Iodinated vinculin was separated from unreacted Bolton-Hunter reagent by chromatography at room temperature on G-25 Sephadex equilibrated with 0.1 M NaCl, 0.05% NaN_3, 0.25% gelatin, and 0.05 M phosphate buffer, pH 7.5. The specific activity of ^{125}I-vinculin from this procedure was 3–8 μCi/μg. After the addition of 2-mercaptoethanol to 15 mM, the ^{125}I-vinculin was stored at 4°C in the column buffer at ~12 μCi/ml. SDS PAGE (24) of a sample of the iodinated vinculin mixed with unlabeled vinculin was used to assess its purity. After electrophoresis, the gel was stained with Coomassie Brilliant Blue, destained, and then cut into 1-mm slices. The pieces were then counted in a Beckman gamma counter (Beckman Instruments, Inc., Fullerton, CA).

For the overlay, samples of various proteins were separated by SDS PAGE (24). The proteins were fixed in the gels (0.75-mm thick) with 40% methanol, 10% acetic acid in distilled water for 30 min. The gels were then rinsed three times with H_2O and incubated for at least 4 h, but usually 15 h, with 10% ethanol with one solution change. The gels were equilibrated for 24 h with Buffer C (0.5% bovine serum albumin, 0.25% gelatin, 1% Nonidet P-40, 0.01 M NaCl, 0.05% NaN_3, 1 mM EGTA, 15 mM 2-mercaptoethanol, and 20 mM HEPES, pH 7.5). After incubation with 1–2 μCi/ml ^{125}I-vinculin in Buffer C for 24 h at 37°C, the gels were washed for 3 d with at least 10 changes of Buffer C. The gels were dried and autoradiographed on Kodak X-Omat AR film (XAR-5) for 6–8 d with an intensifying screen. All overlay procedures were done at room temperature with the exception of the incubation with ^{125}I-vinculin.

RESULTS

The purity of a typical preparation of ^{125}I-vinculin is shown in Fig. 1. Most of the counts are contained in the single peak that is coincident with the Coomassie Blue–stained band containing vinculin. For comparison, a standard vinculin preparation in SDS polyacrylamide gels stained with Coomassie Blue can be seen in Fig. 2*A* (lane *e*).

The iodinated vinculin was used as a probe to detect vinculin-binding proteins in CEF with a gel overlay technique. Figure 2*A* shows a Coomassie Blue-stained gel with different fractions of CEF and several known cytoskeletal proteins. An identical gel that was used for the overlay is shown in Fig. 2*B*. Iodinated vinculin binds strongly to two protein bands in whole CEF of 220,000 mol wt and 130,000 mol wt (Fig. 2*B*, lane *d*). There is also a moderate degree of binding to a protein of 42,000 mol wt. The identity of the 220,000-mol-wt protein is not known. The 130,000-mol-wt protein is vinculin as shown by ^{125}I-vinculin binding to vinculin purified from chicken gizzards (Fig. 2*B*, lane *e*), and the 42,000-mol-wt protein is actin since the probe binds to purified actin (Fig. 2*B*, lane *g*). There is also some binding of lower intensity to other bands of various molecular weights on the gel.

The binding of the ^{125}I-vinculin to these proteins is specific since preparations of ^{125}I-vinculin boiled for 5 min do not bind to any proteins on the gel (Fig. 2*C*). Preincubation or pre- and co-incubation of the gel with unlabeled vinculin does not inhibit binding of ^{125}I-vinculin (Fig. 2*D*). In fact, preincubation with cold vinculin often leads to higher levels of ^{125}I-vinculin binding (data not shown). These results are consistent with the observation that vinculin binds to itself as shown above.

To assay the subcellular distribution of the vinculin-binding proteins, CEF were extracted with Triton X-100 in TGMC and the Triton-soluble fraction separated from the insoluble fraction that contains a portion of the cytoskeleton including

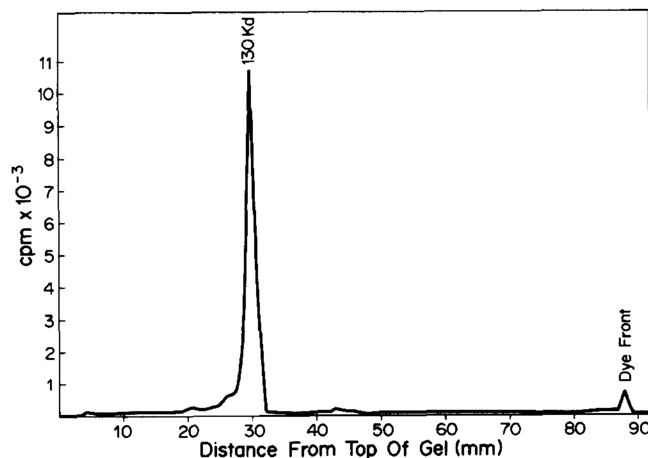

FIGURE 1 The distribution of radioactivity in a 7.5% SDS polyacrylamide gel of a sample of ^{125}I-vinculin. The labeled vinculin (30,000 cpm) was mixed with unlabeled vinculin and subjected to electrophoresis. After being stained, the gel was sliced into pieces 1 mm wide and each piece was counted in a gamma counter. Most of the radioactivity was localized in one peak corresponding to the stained vinculin band.

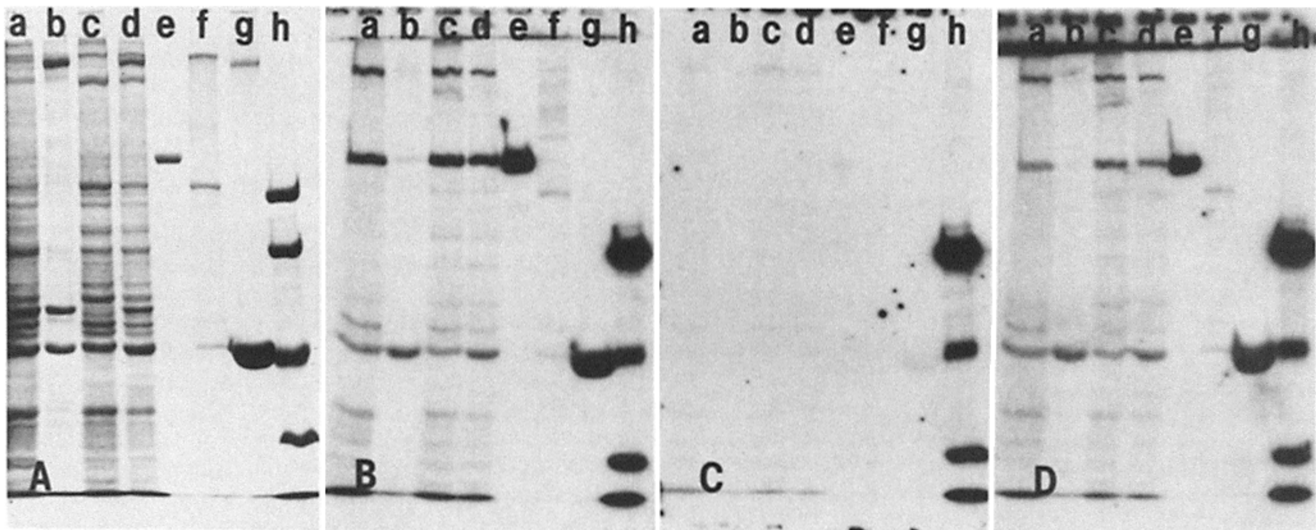

FIGURE 2 7.5% SDS polyacrylamide gels of chicken embryo fibroblast and gizzard proteins. (*A*) Coomassie Brilliant Blue-stained gel. (*B*) Autoradiograph of gel overlayed with ^{125}I-vinculin (1.5 μCi/ml). (*C*) Autoradiograph of gel overlayed with ^{125}I-vinculin (1.5 μCi/ml) that had been boiled for 5 min. (*D*) Autoradiograph of gel that was preincubated with 30 μg/ml of unlabeled vinculin and then incubated with the same concentration of unlabeled vinculin plus 1.5 μCi/ml ^{125}I-vinculin. Each gel contained: (*a*), CEF adhesion plaque preparation; (*b*), CEF Triton-insoluble proteins; (*c*), Triton-soluble CEF proteins; (*d*), total CEF proteins; (*e*), gizzard vinculin; (*f*), gizzard filamin, α-actinin, and actin; (*g*), CEF fibronectin and rabbit muscle actin; and (*h*), molecular weight standards: phosphorylase *b* (94,000), BSA (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,100). For the autoradiographs, these standards were labeled with the chloramine T method (20), with the exception of phosphorylase *b* which did not label.

adhesion plaques (3, 6) (Fig. 2*A*, lanes *b* and *c*). Each fraction was of equal volume. Under these conditions, the relative binding of ^{125}I-vinculin shows that most of the 220,000-mol-wt protein and vinculin are soluble, and that the actin is divided almost evenly between the two fractions (Fig. 2*B*, lanes *b* and *c*). The autoradiograph of the Triton-insoluble sample contains only faint bands corresponding to the 220,000-mol-wt protein and vinculin (Fig. 2*B*, lane *b*). Since vinculin is thought to be primarily cytoskeletal and in the adhesion plaques (7, 14), we have investigated this apparent discrepancy further by assaying preparations enriched in adhesion plaques from CEF (3). As shown in Fig. 2*B* (lane *a*), this fraction contains both the 220,000-mol-wt protein and vinculin, as shown by binding of the ^{125}I-vinculin. Adhesion plaque preparations such as this are concentrated from several plates of cells. Thus, these vinculin-binding proteins are also probably present in the Triton-insoluble fraction, but at insufficient levels to detect. The preparations enriched in adhesion plaques include other substrate-attached material and may also contain whole cells that are missed in the shearing process; this probably accounts for the similarity between lanes *a* and *d* of Fig. 2*A*.

We have attempted to identify the 220,000-mol-wt vinculin-binding protein by determining whether it corresponds to any known cytoskeletal proteins of similar molecular weight. It does not appear to be either fibronectin or filamin, since the ^{125}I-vinculin does not bind to these proteins from CEF or chicken gizzard, respectively (Fig. 2*B*; lanes *g* and *f*). Furthermore, ^{125}I-vinculin does not bind to spectrin in preparations of human erythrocyte membranes (Fig. 3, lane *d*). This is not due to a lack of cross-reactivity between chicken vinculin and human proteins since HeLa cells contain both the 220,000-mol-wt and 130,000-mol-wt proteins that bind ^{125}I-vinculin (Fig. 3, lane *c*). Although there seems to be some binding of the probe to gizzard myosin, this protein is of lower molecular weight than the 220,000-mol-wt protein (Fig. 3, lane *b*). Since we did not detect ^{125}I-vinculin binding to the known proteins, the identity of the 220,000-mol-wt protein remains unknown. The 220,000-mol-wt protein appears to be

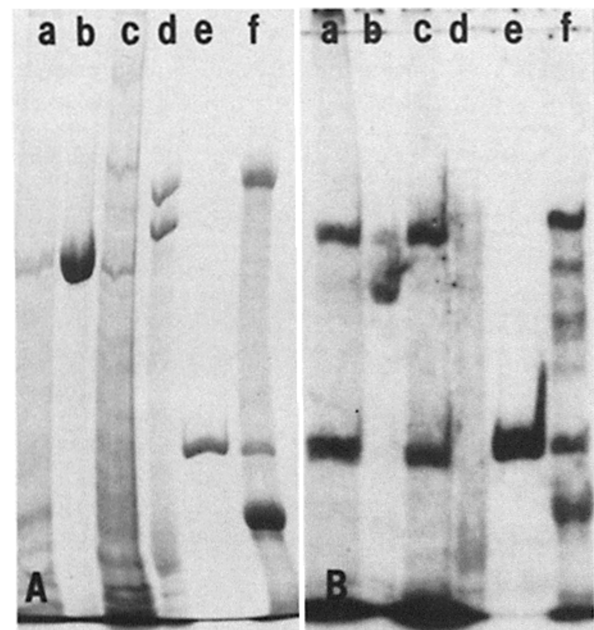

FIGURE 3 4% SDS polyacrylamide gels of preparations containing high molecular weight actin-associated proteins. (*A*) Coomassie Brilliant Blue-stained gel. (*B*) Autoradiograph of gel overlayed with ^{125}I-vinculin. Each gel contained: a, Triton-soluble CEF proteins; *b*, gizzard myosin; c, total HeLa cell proteins; *d*, spectrin-containing membrane preparations from human erythrocytes; e, gizzard vinculin; and *f*, gizzard proteins loaded onto DEAE cellulose column during purification of vinculin from low ionic strength extracts of gizzards. Because this is such a low percentage gel, many proteins from the samples of cell extracts (lanes a, c, and *f*) migrate at the dye front.

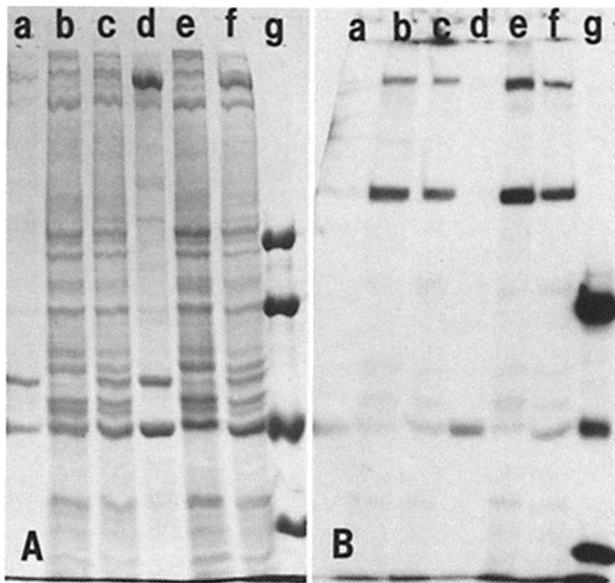

FIGURE 4 7.5% SDS polyacrylamide gels of proteins from CEF and their RSV transformants. (*A*) Coomassie Brilliant Blue-stained gel. (*B*) Autoradiograph of gel overlayed with ^{125}I-vinculin. Each gel contained: a, RSV-CEF Triton-insoluble proteins; *b*, RSV-CEF Triton-soluble proteins; *c*, total RSV-CEF proteins; *d*, CEF Triton-insoluble proteins; e, CEF Triton soluble proteins; *f*, total CEF proteins, *g*, molecular weight standards as in Fig. 2.

a relatively minor cellular component in CEF and HeLa cells, since no major Coomassie Blue-stained band corresponds to the band showing ^{125}I-vinculin binding (Fig. 3, *A* and *B*; lanes *a* and *c*).

Adult chicken gizzard also contains a major vinculin-binding protein of ~220,000 mol wt (Fig. 3, lane *f*). In 7.5% gels, this gizzard protein co-migrates with the 220,000-mol-wt protein of CEF. However, in the 4% gel shown in Fig. 3, the gizzard protein migrates slightly more slowly than the protein from CEF. Gizzard also contains several other proteins of 190,000, 170,000, and 100,000 mol wt which bind ^{125}I-vinculin. The 100,000-mol-wt protein may be α-actinin although preparations enriched in α-actinin (Fig. 2*B*, lane *f*) do not always bind the labeled vinculin. The 190,000- and 170,000-mol-wt proteins may be proteolytic fragments of the 220,000-mol-wt protein since their amount seems to increase upon storage of these preparations. Alternatively, one of them may correspond to the vinculin-like protein recently identified in smooth muscle (13, 28).

We have also examined samples of RSV-CEF for vinculin-binding proteins. Fig. 4 shows that both the 220,000-mol-wt protein and vinculin are present in RSV-CEF and that, as in the normal cells, these proteins are primarily Triton-soluble.

DISCUSSION

With the ^{125}I-vinculin gel overlay described in this paper, two proteins of 220,000- and 130,000-mol-wt (vinculin) from chick fibroblasts were discovered to bind vinculin tightly. In addition, moderate binding of the probe to actin was seen. Chicken gizzard also contained these vinculin-binding proteins as well as three others of 190,000, 170,000, and 100,000 mol wt.

The identity of the 220,000-mol-wt protein is not yet known. It does not appear to be fibronectin, filamin, or myosin. Although the ^{125}I-vinculin does not bind to erythrocyte spectrin, we have not ruled out the possibility that the 220,000-mol-wt protein may be a nonerythrocyte spectrin-like protein such as fodrin (25) or terminal web proteins 260/240 (19). A recent preliminary report by Burridge et al. (9) demonstrates that a gizzard protein of 215,000 mol wt is localized in adhesion plaques of cultured cells. Since the purification of this protein has not yet been reported, we have not tested this protein in the overlay procedure. The 220,000-mol-wt protein may, of course, not be in the adhesion plaques nor be a cytoskeletal protein; however, the similarity of its extraction properties to vinculin in Triton-lysed cells, its presence in preparations enriched in adhesion plaques, plus the observation that it binds to vinculin suggests that it may have the same subcellular distribution as vinculin. We are currently purifying the 220,000-mol-wt protein from gizzard in order to characterize its interaction with vinculin, particulary with regard to the interaction of vinculin with actin. We also intend to make an antibody against the protein to use in localization studies.

The binding of the ^{125}I-vinculin to vinculin itself suggests that this protein self-associates. This property is probably not due to disulfide bonding, since the buffer for this overlay contains 15 mM 2-mercaptoethanol. We have confirmed the self-association characteristic of vinculin in sedimentation experiments which were done as controls for examining vinculin-actin associations.[2] In brief, immediately before an experiment, stock solutions of vinculin and G-actin were centrifuged at 150,000 *g* for 30 min. Constant amounts of G-actin (0.5 mg/ml) were mixed with different concentrations of vinculin (10–75 μg/ml), and the actin was polymerized for 2 h at 25°C. The mixtures were then centrifuged for 30 min at 150,000 *g*. As controls, the same concentrations of vinculin were incubated alone under the same conditions. When the pellets were examined by SDS PAGE, no difference could be seen between the amount of vinculin pelleted in the presence or absence of actin. Thus, during the 2-h incubation, some of the vinculin alone associated sufficiently to become pelletable. These observations also suggest that vinculin self-associates and that this property is not due to the iodination of the protein.

The moderate binding of the vinculin probe to actin supports the idea that these proteins interact in some fashion, although the manner of interaction is not yet clear. Besides bundling actin filaments (21, 23), vinculin has been proposed to bind to actin filament ends and to inhibit actin polymerization (8, 30). Since we have now identified a vinculin-binding protein, it will be important to determine whether the vinculin–220,000-mol-wt protein complex interacts with actin.

The binding by ^{125}I-vinculin in this assay is considered to be specific since boiled ^{125}I-vinculin did not bind. In addition, binding to other major protein bands on the gel was not observed. Ordinarily, the additional control of eliminating binding by preincubation with the native protein is required to fulfill the requirements for a specific binding assay. In this case, the binding of the ^{125}I-vinculin is not eliminated by preincubation or pre- and co-incubation of the gel with unlabeled vinculin. This is presumably because vinculin binds to itself. Thus, after a preincubation, each of the vinculin-binding proteins in the gel will be associated with unlabeled vinculin to which ^{125}I-vinculin can bind.

Since vinculin is primarily noted for being in the adhesion

[2] Otto, J., manuscript in preparation.

plaque (7, 14), we were surprised to find that when CEF were separated into Triton-soluble and -insoluble fractions, the majority of vinculin detected by ^{125}I-vinculin binding was in the soluble fraction. The soluble vinculin may be interacting with actin filaments not tightly associated with the stress fibers that remain attached to the dish as part of the cytoskeleton under the conditions used for extraction (6). In addition to the prominent localization of vinculin in the adhesion plaques, diffuse staining and a fibrillar pattern on the dorsal cell surface also are present when cells are stained with antivinculin (7, 14); these distributions probably account for the vinculin that we observed in the soluble fraction.

Since the distribution of vinculin is altered in transformed cells (10), possibly as a result of its phosphorylation by the *src* protein kinase (27), we assayed RSV-CEF to determine both whether different vinculin-binding proteins were present and whether they were Triton-soluble or -insoluble. Our results demonstrate that the same binding proteins are present and that they are primarily Triton soluble. Transformed cells stained with antivinculin show a large amount of diffuse staining (10) so it would be expected in this case that the levels of vinculin detected by ^{125}I-vinculin binding would be higher in the soluble fraction, compared with the insoluble.

The gel overlay technique, in general, is a useful method for detecting protein-protein interactions. Although it requires that binding proteins renature after SDS treatment and gel electrophoresis, minor cellular components can often be detected as demonstrated in this paper. Neither the 220,000-mol-wt protein nor vinculin are major cellular proteins as indicated by Coomassie Blue staining of gels. This method has been used previously for cytoskeletal proteins to identify calmodulin-binding proteins in the intestinal brush border and other cells (17–19) and to identify actin-binding proteins in platelets (29). This technique will probably prove useful in identifying binding proteins for other cytoskeletal proteins as well.

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