

Vitronectin at Sites of Cell-Substrate Contact in Cultures of Rat Myotubes

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Abstract. Affinity-purified antibodies to the serum glycoprotein, vitronectin, were used to study sites of cell-substrate contact in cultures of rat myotubes and fibroblasts. Cells were removed from the substrate by treatment with saponin, leaving fragments of plasma membrane attached to the glass coverslip. When stained for vitronectin by indirect immunofluorescence, large areas of the substrate were brightly labeled. The focal contacts of fibroblasts and the broad adhesion plaques of myotubes appeared black, however, indicating that the antibodies had failed to react with those areas. Contact sites within the adhesion plaque remained unlabeled after saponin-treated samples were extracted with Triton X-100, or after intact cultures were sheared with a stream of fixative. These procedures expose extracellular macromolecules at the cell-substrate interface, which can then be labeled with concanavalin A. In contrast, when samples were

sheared and then sonicated to remove all the cellular material from the coverslip, the entire substrate labeled extensively and almost uniformly with anti-vitronectin.

Extracellular molecules associated with substrate contacts were also studied after freeze-fracture, using a technique we term "post-release fracture labeling." Platinum replicas of the external membrane were removed from the glass with hydrofluoric acid to expose the extracellular material. Anti-vitronectin, bound to the replicas and visualized by a second antibody conjugated to colloidal gold, labeled the broad areas of close myotube-substrate attachment and the nearby glass equally well.

Our results are consistent with the hypothesis that vitronectin is present at all sites of cell-substrate contact, but that its antigenic sites are obscured by material deposited by both myotube and fibroblast cells.

WHEN a cell adheres to another cell, to the tissue culture substrate, or to another object, the structure it uses to stabilize the attachment is highly specialized and very compact. The distances across which attachment occurs are very small, ranging from ~ 1 nm to several tens of nanometers. It is therefore very difficult to penetrate these attachment sites with specific probes, such as antibodies, to learn how the macromolecules in the extracellular space are organized. The problem of access is also encountered in studies of cell-substrate attachment sites in tissue culture, where the membrane-to-substrate distance is quite small, usually on the order of 10 nm (13, 15, 17).

Rat myotubes in tissue culture establish broad areas of contact with the tissue culture substrate, at which acetylcholine receptors (AChR's)¹ preferentially accumulate (2, 9, 19, 27). These regions of the myotube surface are composed of two

easily recognized, interdigitating membrane domains: receptor domains, in which aggregated AChR are found, and contact domains, which are closer to the substrate and contain fewer AChR's than the receptor domains (9, 24). Additional cell-substrate contacts, in the form of focal contacts (13, 15, 17), are generated by fibroblasts present in our cultures. Contact domains of myotubes share many features of these focal contacts (9, 13, 15, 17, 20; Pumplin, D. W., manuscript in preparation). One of the interests of our laboratories has been to understand how cell-substrate contact facilitates the aggregation of receptors in nearby AChR domains. To approach this problem, we have devised a set of techniques which can be used to study the extracellular components at sites of cell-substrate contact.

The component we consider here is the serum glycoprotein, vitronectin (14), also known as "serum-spreading factor" (4-6, 28). This protein binds to glass and promotes the spreading of cells in culture (4-6, 14, 28). It is not known, however, if vitronectin is retained on the substrate once focal or close contacts are established. We report here that vitronectin is indeed retained at these sites but that overlying cellular materials must first be removed to reveal its presence.

1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; F-ConA, fluoresceinated concanavalin A; FGAM, fluoresceinated goat anti-mouse IgG; GAM G-15, goat anti-mouse IgG adsorbed to 15-nm colloidal gold particles; HF, hydrofluoric acid; R-BT, monotetramethylrhodamine- α -bungarotoxin; RGAM, rhodaminylated goat anti-mouse IgG; saponin-SAM, substrate-associated material obtained by extracting cultures with saponin; shear-SAM, substrate-associated material obtained by physically shearing cultures with a stream of buffered fixative.

Materials and Methods

Rat myotube cultures were prepared from hind limb muscle of neonatal rats, as described (7, 9). Cells were grown on glass coverslips in Dulbecco-Vogt modified Eagle's medium supplemented with either 10% fetal calf serum (Gibco, Grand Island, NY), or in 10% calf serum (Biocell Laboratories, Carson, CA) plus 5% fetal calf serum. Cultures, which contained both myotubes and fibroblasts, were used between 6 and 9 d after plating.

Antiserum showing a high titer of antibodies against vitronectin was obtained by injecting several mice with substrate-attached material. This antigenic mixture was prepared by extracting myotube cultures with saponin (see below), and then with 0.05% Triton X-100 for 10 min at 22°C (3). The material remaining bound to the coverslip was dissolved in 0.25% SDS, and concentrated by lyophilization. Mice were initially injected intraperitoneally with 20 µg of this material, emulsified in 0.5 ml complete Freund's adjuvant. 3 wk later, mice were boosted with 20 µg of the crude antigen, in 0.5 ml incomplete Freund's adjuvant. Around the time of boosting, antisera were screened by indirect immunofluorescence; one antiserum showed extensive labeling of serum-coated glass substrate, even in the absence of cells. Additional aliquots of this antiserum were collected over the next month. The evidence that this labeling was due to binding to vitronectin is presented in Results.

Anti-vitronectin antibody was purified by affinity chromatography. Coverslip glass (Eric Scientific, Portsmouth, NH) was ground into small fragments with a mortar and pestle, and incubated with a mixture containing equal volumes of undiluted fetal calf serum and cadet calf serum for 18 h at 22°C. The fragments were centrifuged briefly, and washed three times in buffered saline (10 mM NaP, 145 mM NaCl, pH 7.2) to remove the serum. The glass fragments were poured into a column (0.8 × 4 cm; Bio-Rad Laboratories, Richmond, CA) and washed extensively with buffered saline. An aliquot (0.5 ml) of the anti-vitronectin antiserum was applied to the column at a rate of 0.2 ml/min. Unbound materials were removed by washing with three column volumes of buffered saline. Bound protein was eluted in 2 ml of 100 mM glycine-HCl, pH 2.7, and collected into a tube containing 0.4 ml of 2 M Tris-HCl, pH 8. The resulting solution, containing 20 µg/ml of protein, was supplemented with 10 mM sodium azide and 1 mg/ml BSA and stored at 4°C.

Samples studied by immunofluorescence were first extracted with saponin, or subjected to shearing with fixative, to remove a large amount of cellular material. Extraction with saponin followed methods described previously (8). Briefly, cultures were washed in buffered saline containing 10 mM MgCl₂, 1 mM EGTA, and supplemented either with 10 mg/ml BSA (method I), or with 0.1 mM phenylmethylsulfonyl fluoride and 0.026 U/ml aprotinin (method II). Samples were then incubated with shaking for 20 min in the same solutions supplemented with 0.2% saponin. Samples were then fixed for 15 min in buffered saline containing paraformaldehyde (2%) or glutaraldehyde (2.5 or 5%). After fixation, some samples were extracted with 0.5% Triton X-100 in buffered saline for 2–5 min at 22°C. For shearing, coverslips with myotube cultures were exposed to a stream of fixative created by forcing a solution of buffered paraformaldehyde or glutaraldehyde through a 25-gauge needle, placed ~3 cm from the sample. These samples were fixed further, or were sonicated for 30 s and then fixed. Sonication was performed by placing the coverslip into a Branson B12 Ultrasonic Cleaner (Branson Cleaning Equipment Co., Shelton, CT), filled with buffered saline to a height of 10 cm. Any free aldehydes remaining after fixation were quenched with borohydride or glycine, followed by a 0.1% serum albumin solution, before further incubation.

In some experiments, we used coverslips which had been coated with fluorescently labeled serum proteins. Coverslips were incubated for 24 h at 37°C with medium containing 10% calf and 5% fetal calf sera; they were then washed several times with water. A fresh solution of 0.1 M NaHCO₃ containing ~1 mg/ml of fluorescein isothiocyanate was applied and allowed to incubate for 30 min at room temperature. After washing with buffered saline, coverslips were incubated with 0.1 M glycine in buffered saline, to inactivate any remaining isothiocyanates. The coverslips obtained from this procedure were uniformly labeled with fluorescein. Labeling was not obtained if coverslips were first incubated in culture medium lacking serum. Sterility of the modified coverslips was achieved by filtering all solutions through 0.2-µm Millex filters (Millipore Corp., Bedford, MA) before use.

For indirect immunofluorescence, most samples were incubated with affinity-purified anti-vitronectin (20 µg/ml), followed by either 20 µg/ml fluoresceinated goat anti-mouse IgG (FGAM; Cappel Laboratories, Cochranville, PA) plus 2 µg/ml monotetramethylrhodamine- α -bungarotoxin (R-BT), or 20 µg/ml rhodaminylated goat anti-mouse IgG (RGAM; Cappel Laboratories) plus 20 µg/ml fluoresceinated concanavalin A (F-ConA). All

incubations were done for 30–45 min in the presence of 1 mg/ml BSA. In some experiments, samples were labeled with R-BT (26) before extraction. Incubation was for 15–30 min at room temperature with 5 µg/ml R-BT diluted into buffered culture medium supplemented with 5% calf serum. Stained samples were mounted in 90% glycerol, 10% 1 M Tris-HCl, pH 8.0, and observed with a Zeiss IM-35 microscope equipped for epifluorescence. Methods for photomicrography have been reported (7, 9).

For immunoblotting, samples were applied to 10% or 12% polyacrylamide gels, and subjected to electrophoresis as described by Laemmli (18). Polypeptides were transferred electrophoretically to nitrocellulose paper (10). Non-fat dry milk solids (5% wt/vol) in the presence of 0.01% antifoam A, or a mixture of hemoglobin (10 mg/ml), gelatin (2.5 mg/ml), and BSA (10 mg/ml), were used to saturate the paper. Incubation with anti-vitronectin antiserum was followed by incubation with goat anti-mouse IgG conjugated to alkaline phosphatase (Cappel Laboratories). Bound antibody was visualized with fast red TR and naphthol AS-MX phosphate.

Prestained protein standards were from Bethesda Research Laboratories (Gaithersburg, MD). Unless otherwise noted, other reagents were from Sigma Chemical Co. (St. Louis, MO).

Post-release Fracture Label

For ultrastructural localization of vitronectin, intact or saponin-extracted myotube cultures were fixed with 5% glutaraldehyde, freeze-fractured with a complementary-replica device at -115°C and 10⁻⁶ Torr and immediately shadowed with platinum and carbon (21, 29). Deposition of platinum and carbon was controlled with a Balzers quartz crystal monitor.

When cells in tissue culture are fractured with the complementary-replica device, the fracture plane passes preferentially through the substrate-apposed membrane. Samples extracted with saponin consist almost exclusively of substrate-apposed membrane, and so fracture like intact cells. In both cases, the replica observed after post-release fracture labeling is that of the external lipid leaflet (E face) of the substrate-apposed membrane and other extracellular materials remaining attached to the coverslip. To expose the material lying between the substrate-apposed membrane and the coverslip, the replica and its underlying materials were separated from the glass by flotation for <1 min on 40 µl of 12% hydrofluoric acid (HF). The samples so released were rinsed immediately with three to four changes (3 ml each) of buffered saline. The replica was floated on the appropriate solution during all subsequent labeling and washing steps.

In the initial experiment, a portion of a replica made from intact myotubes was reacted for 3 h with 30 µl of buffered saline or buffered saline containing 20 µg/ml affinity-purified anti-vitronectin. To visualize bound antibody, the replicas were incubated overnight at room temperature on 30-µl drops of undiluted goat anti-mouse IgG adsorbed to 15-nm gold particles (GAM G-15) (Janssen Pharmaceuticals, SPI Supplies, West Chester, PA).

In subsequent experiments, the released replicas were washed by flotation on buffered saline containing 0.1% BSA, 0.15 M NaCl, and 0.05% Tween 20 (BSA-saline). To minimize background staining, replicas were incubated with 5% normal rabbit serum (Sternberger-Meyer Immunocytochemicals, Inc., Jarrettsville, MD) in BSA-saline for 30 min before incubation with the primary antibody. Portions cut from a single replica were incubated with either anti-vitronectin or nonimmune antisera overnight at 4°C. After washing, the replicas were incubated for 3 h at 22°C with GAM G-15. Replicas were washed further with BSA-saline and distilled water, and then picked up on Formvar-coated slot grids for viewing in the electron microscope.

To assess the effect of HF, additional samples were immunolabeled before exposure to this reagent. Saponin-extracted cultures were fixed with formaldehyde and labeled with anti-vitronectin and gold-adsorbed second antibody as above, then postfixed with glutaraldehyde. To visualize the gold particles, labeled cultures were either freeze-fractured as above or rinsed in distilled water and rapidly frozen (16) in a device cooled with liquid nitrogen (22). Rapidly frozen samples were etched at -95°C for 15–30 min in a Balzers 301M apparatus (Balzers, Hudson, NH), then replicated with platinum and carbon. Replicas from either method were released from the glass with HF, cleaned by flotation on three changes of distilled water, and picked up on Formvar-coated slot grids.

To determine concentration, gold particles were counted by hand in areas seen in the viewing binoculars of the electron microscope (2.6 µm² at 14,500×) or in micrographs enlarged to 31,700×. Wherever possible, counts were made on a myotube attachment site and on an adjacent cell-free area. Attachment sites from all parts of the replica were sampled.

Examination of stereo pairs of electron micrographs made at 45,000 × or

70,000× with ±6° of tilt confirmed that gold particles were exclusively on the side of the replica exposed to labeling solutions (Pumplin, D. W., manuscript in preparation).

Controls for the specificity of labeling by anti-vitronectin included incubation of replicas in the absence of first antibody, or incubation with non-immune mouse serum, or different mouse IgG or IgM preparations with specificities for proteins other than vitronectin. Little labeling was seen in immunofluorescence or in the electron microscope under any of these conditions, confirming that labeling after incubation with anti-vitronectin was not due to nonspecific interactions. The failure of GAM G-15 to label replicas which had not been previously incubated with anti-vitronectin clearly indicates that nonspecific adsorption of this reagent to the replica, which can be a serious problem (25), does not occur to any significant extent.

Results

To localize an extracellular molecule at sites of cell-substrate contact, three things are required: a specific probe for the molecule, a means of obtaining substrate-associated material, and a way to expose the molecule in the substrate-attached material to the probe.

We prepared antibodies to vitronectin to learn if this protein was associated with sites of cell-substrate contact. The antiserum reacted specifically with vitronectin, as demonstrated by immunoblotting on templates prepared from calf serum (Fig. 1 A). The results show that the only calf serum protein which reacts with the antiserum has an apparent polypeptide chain molecular weight of ~65,000. This is in good agreement with the molecular weight estimated for a purified vitronectin by Whateley and Knox (28). Similar results were obtained with fetal calf serum, but in this case, as with human serum (4, 5), an immunoreactive band at ~75,000 was also observed (not shown).

To purify anti-vitronectin antibodies, we first prepared a column of glass fragments which had been incubated with calf and fetal calf sera to coat them with protein. Two bands at ~65,000 and ~75,000 were the major serum proteins bound to the glass under the conditions used (Fig. 1 B, lane 1; see also reference 6). In immunoblots of the serum proteins that bound to the glass column, anti-vitronectin reaction was detected only with these two bands (Fig. 1 B, lane 2). An aliquot of anti-vitronectin antiserum was applied to the column. Unbound antibodies were then removed by washing, and specifically bound antibody was eluted by acidification. As expected for an anti-vitronectin antibody (5), the affinity-purified preparation completely blocked the spreading of mononucleate cells on serum-coated glass coverslips (not shown). The antibodies were also shown by indirect immunofluorescence to bind to cell-free areas of the coverslip, and to react in blots of substrate-associated material only with bands corresponding to vitronectin (see below). We conclude that they react preferentially with vitronectin.

We have used two independent techniques for isolating substrate-attached membrane. One, physically shearing the culture with a stream of fixative, leaves fragments of membrane behind on the coverslip. Not all of the remaining membrane is closely associated with the substrate, however. Fragments are also accompanied by varying but significant amounts of cytoplasmic material, as judged by immunofluorescence observations of cytoskeletal proteins present in these preparations (Bloch, R. J., manuscript in preparation) and by electron microscopy (Pumplin, D. W., manuscript in

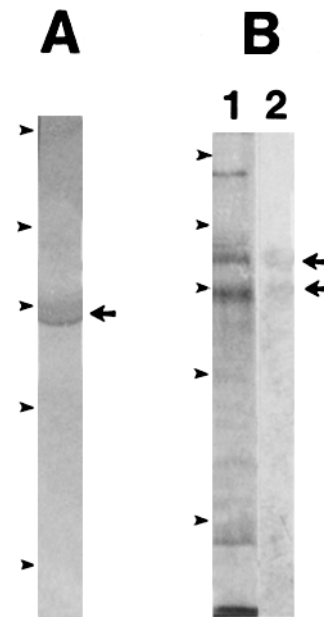
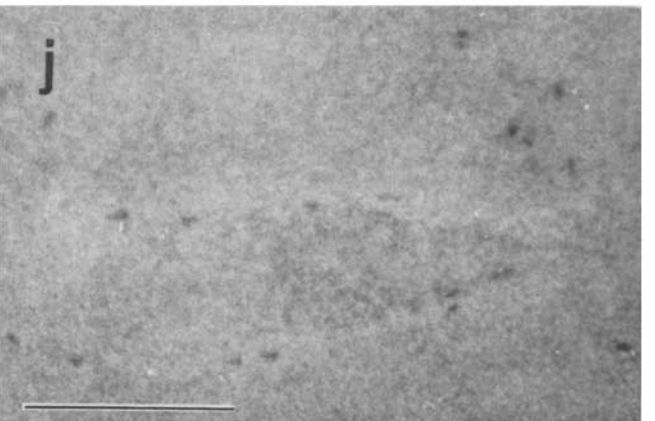
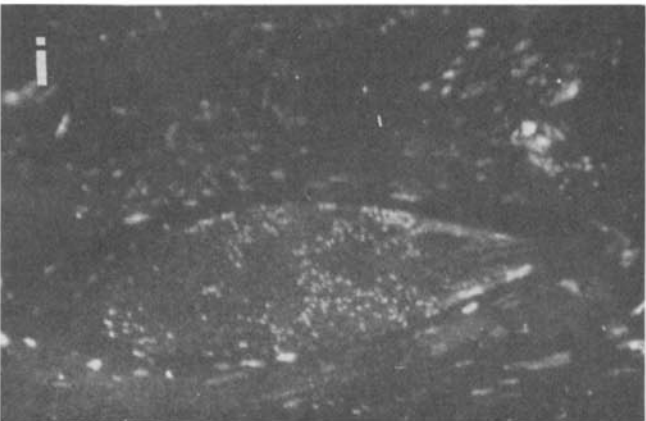
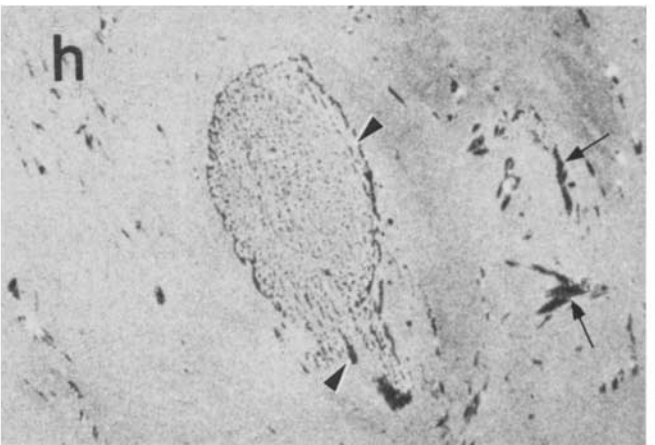
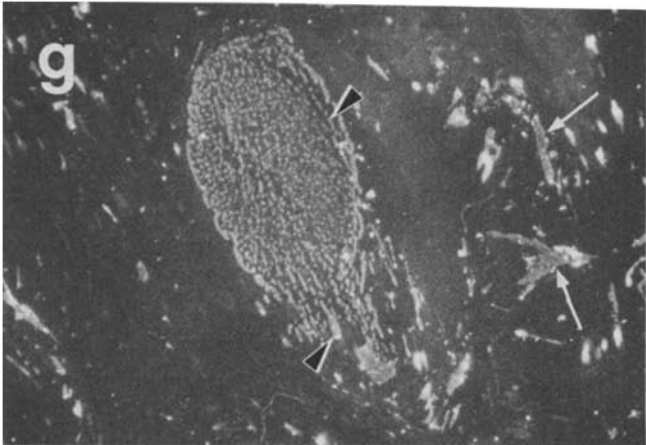
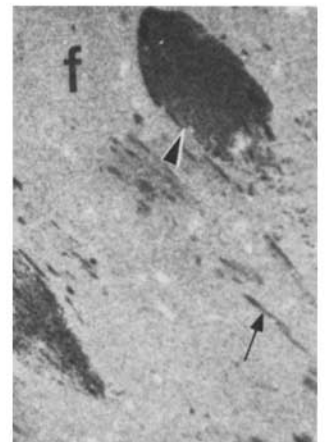
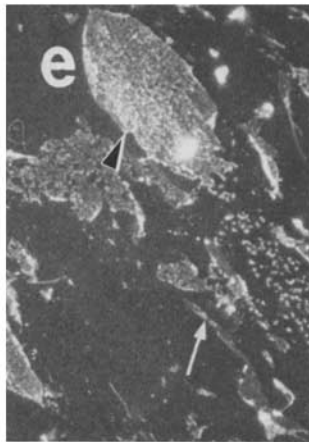
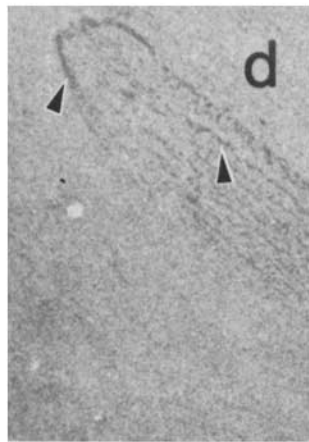
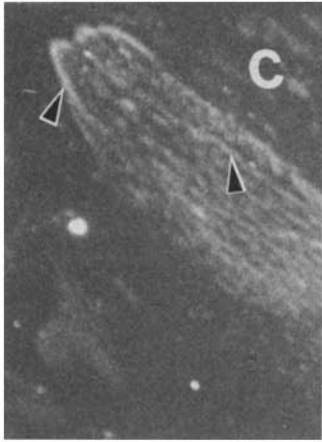
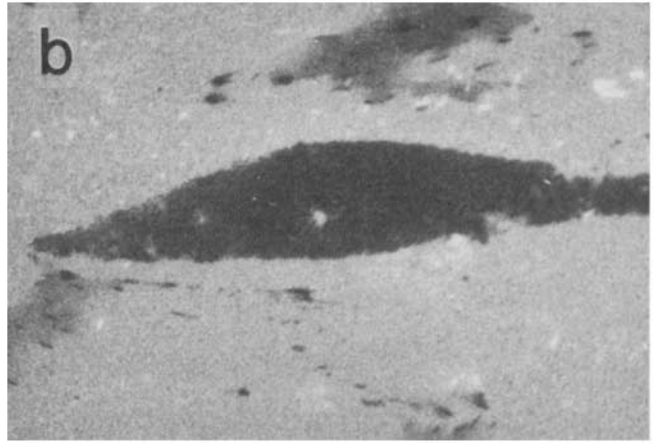
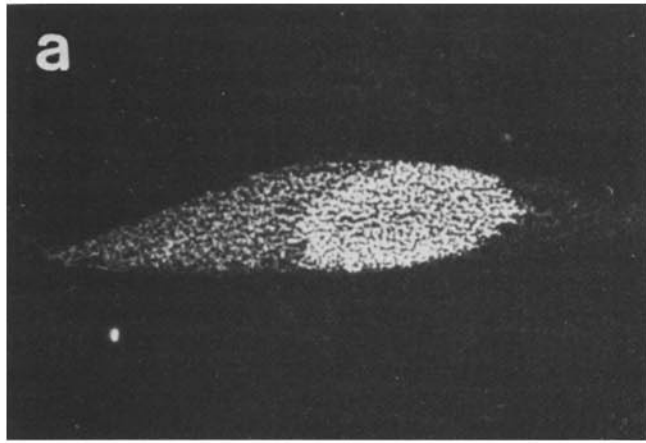


Figure 1. SDS PAGE and immunoblot of calf serum with anti-vitronectin. Aliquots of calf serum or protein bound to glass columns were subjected to electrophoresis on 10% polyacrylamide gels (18) and transferred to nitrocellulose paper (10). The blots were saturated with protein (see Materials and Methods) and incubated with anti-vitronectin antiserum (1:200) followed by an alkaline phosphatase conjugate of anti-mouse IgG (1:200). Sites of antibody binding were visualized using fast red TR and naphthol AS-MX phosphate. (A) Immunoblot of calf serum protein (15 µg), showing that antibody binds only to a band with an apparent polypeptide chain molecular

weight of ~65,000 (arrow). (B) Calf and fetal calf sera were incubated with finely ground coverslip glass, as outlined in Materials and Methods. After unbound protein was washed away with buffered saline, bound protein was eluted with dodecyl sulfate (0.25%). The eluted proteins were concentrated by lyophilization and redissolved in a small volume of water. An aliquot containing 10 µg protein was boiled in sample buffer (18) and subject to electrophoresis in a 10% polyacrylamide gel. Staining with Coomassie Brilliant Blue B revealed the two major bands with apparent molecular weights of ~65,000 and ~75,000 (arrows). (Lane 2) Immunoblot of material prepared as in lane 1, showing that the two major bands are the only ones labeled by antibody (arrows). Arrowheads mark the position of standard proteins. (Top to bottom) Myosin heavy chain (200,000); phosphorylase B (97,400); bovine serum albumin (68,000); ovalbumin (44,000); and α-chymotrypsinogen (25,700).

preparation). This material is referred to here as “shear-SAM.” The other procedure entails extracting the culture with a solution containing saponin. The substrate-associated membrane fragments obtained after such an extraction are essentially uncontaminated with cytoplasm or other membrane. This material is referred to as “saponin-SAM” (8). The immunoreactive material in saponin-SAM detected by SDS PAGE and immunoblotting is limited to two bands at ~65,000 and ~75,000 (not shown). These bands correspond to the polypeptide chain molecular weights of the vitronectins in calf and fetal calf sera.

When preparations of either shear-SAM or saponin-SAM were fixed and exposed to anti-vitronectin and counterstained with fluoresceinated goat anti-mouse antibody (FGAM), the entire substrate was labeled by the antibodies, with the exception of the areas covered by the membrane fragments retained in substrate-associated material. In saponin-SAM, the myotube-substrate adhesion plaques containing AChR clusters were visualized with R-BT (Fig. 2 a). These areas remained unlabeled by anti-vitronectin (Fig. 2 b). More extensive areas of saponin-SAM were visualized by labeling with F-ConA (3, 12; Baetscher, M., and R. J. Bloch, manuscript in preparation). In this case, membrane



fragments from fibroblasts as well as myotubes were recognized because they bound the fluorescent lectin (Fig. 2 *e*). The focal contacts also failed to label with anti-vitronectin (Fig. 2 *b* and *f*; [note, for example, small arrow in *f*]). Similar results were obtained with shear-SAM: intact adhesion plaques from myotubes, displaying AChR clusters, and focal contacts from fibroblasts were readily recognized but failed to stain with anti-vitronectin (not shown).

We considered two explanations for the observation that anti-vitronectin did not stain the substrate-attached membrane fragments from myotubes or fibroblasts. The first possibility was that these sites contained no vitronectin capable of binding antibody. As vitronectin was present wherever cell-substrate attachment sites were not apparent, the antigen may have been cleared from the substrate by the cells as they formed substrate attachment sites, as reported for fibronectin (1, 11). The second possibility was that vitronectin was indeed present at contact sites, but the antibodies were unable to gain access to it. We subjected myotube cultures to several additional treatments to test the latter possibility.

Saponin-SAM was prepared, fixed, and extracted with 0.5% Triton X-100 to render the membrane fragments permeable to antibodies. This procedure removed most of the clustered AChR of myotube adhesion plaques, suggesting that the lipid bilayer of the AChR domains had been severely disrupted. Other experiments have shown that after fixation and detergent extraction, what remains of the myotube-substrate adhesion plaques fails completely to label with a fluorescent lipid probe, which does, however, label the membrane fragments in unextracted saponin-SAM (8). This further suggests that the lipid bilayer throughout the adhesion plaque has been disrupted by treatment with Triton X-100.² Consistent with this observation, we found that there was considerably less cellular material apparent using F-ConA. Large areas of the adhesion plaques extracted with Triton X-100 were labeled with anti-vitronectin, suggesting that the intact lipid bilayer had blocked access of the antibodies to some parts of the substrate. Wherever F-ConA labeling indicated the presence of cellular material, however, vitronectin immunofluorescence was much reduced. This is illustrated

2. Ultrastructural studies of saponin-SAM after extraction with Triton X-100 showed no evidence for the presence of a bilayer in the structures remaining from adhesion plaques. Indeed, very little cellular material at all was apparent on the substrate (Pumplin, D. W., and R. J. Bloch, manuscript in preparation).

for a myotube adhesion plaque in Fig. 2, *c* and *d*. Focal contacts gave similar results (not shown).

In a second set of experiments, shear-SAM was prepared but under conditions of strong shear which removed large amounts of the cell membrane, including the AChR domains of receptor clusters. The remnants of myotube adhesion plaques and intact focal contacts of fibroblasts left in these preparations labeled with F-ConA (Fig. 2 *g*). Where this staining was apparent, however, anti-vitronectin staining was absent (Fig. 2 *h*), as also reported above for Triton-extracted saponin-SAM. These results suggest that, even when the membrane of adhesion plaques is torn away or the lipid bilayer extracted with detergent, limited areas of the underlying substrate label with concanavalin A but remain unlabeled by anti-vitronectin.

We used three independent techniques to demonstrate that cell-substrate attachment sites contained approximately as much vitronectin as neighboring cell-free substrate. In one experiment, shear-SAM was prepared, subjected to mild sonication (see Materials and Methods), and fixed. Subsequent reaction with the F-ConA revealed that the bulk of the cellular material had been removed from the substrate (Fig. 2 *i*). In the absence of concanavalin A-binding material, anti-vitronectin staining was bright over the entire substrate (Fig. 2 *j*). The shadows in immunofluorescence corresponded to limited areas of faint concanavalin A staining.

In a second experiment, we cultured cells on coverslips which had been coated with serum proteins (primarily, but not exclusively, vitronectin: see Fig. 1 *B*), and labeled with fluorescein (see Materials and Methods). This modified substrate supported normal cell growth and myotube formation, as also reported for fluorescent fibronectin (1, 11). In saponin-SAM prepared from these cultures, fluorescein was uniformly distributed over the entire substrate, even at isolated AChR clusters (compare Fig. 3, *A* and *D*). However, if these preparations were extracted with Triton X-100 and labeled with anti-vitronectin and RGAM, areas of the substrate which were brightly labeled with fluorescein were poorly labeled by the antibodies. These areas resembled the focal contacts (Fig. 3 *C*, arrows) and the linear areas of poor antibody staining within the myotube-substrate adhesion plaques (Fig. 3 *B*, arrowheads) which we observed in the experiments described above. These results rule out the possibility that cells clear away (1) or digest (11) underlying serum proteins as they attach to the substrate. Instead, they suggest that serum proteins cover the entire substrate, but that overlying

Figure 2. Labeling of areas of cell-substrate contact with anti-vitronectin. Mixed cultures of rat myotubes and fibroblasts were subjected to extraction with either saponin (to give saponin-SAM) or to shearing (to give shear-SAM). Some samples were further treated with Triton X-100 or sonication before labeling by indirect immunofluorescence with anti-vitronectin (20 µg/ml). (*a* and *b*) A myotube-substrate adhesion plaque in saponin-SAM from a culture prelabeled with R-BT before extraction with saponin and fixation. Membrane fragments containing AChR clusters (*a*) did not label with antibodies (*b*). The smaller punctate areas in *b* that failed to label probably derived from focal contacts of fibroblasts. (*c* and *d*) A myotube-substrate adhesion plaque in saponin-SAM, prepared by method II (see Materials and Methods). This sample was fixed in 5% glutaraldehyde, extracted with 0.5% Triton X-100, and then labeled. Remnants of the adhesion plaque, visualized with F-ConA (*c*), were linear, reminiscent of the contact domains of AChR clusters (3, 9, 23). These lines (e.g., panel *c*; arrowheads) failed to stain with anti-vitronectin and RGAM (*d*; arrowheads). (*e* and *f*) Saponin-SAM prepared as in *c* and *d*, but without extraction with Triton X-100. Myotube-substrate adhesion plaques (arrowheads) and focal contacts of fibroblasts (arrows) labeled with F-ConA (*e*) but not with antibodies (*f*). (*g* and *h*) Shear-SAM, prepared with strong shearing to remove the bulk of the myotube adhesion plaques. Linear remnants of contact domains, labeled with F-ConA (*g*; arrowheads) failed to label with anti-vitronectin and RGAM (*h*; arrowheads). Focal contacts also failed to label with antibodies (*g* and *h*; arrows). (*i* and *j*) Shear-SAM which was sonicated before final fixation and labeling to remove most of the cellular material remaining after shearing. F-ConA labeling of myotube-substrate adhesion plaques was much reduced (*i*), and these areas were labeled well with anti-vitronectin and RGAM (*j*). Small areas of F-ConA labeling still present (*i*) correlated with "shadows" in the antibody labeling (*j*). Bar, 10 µm.

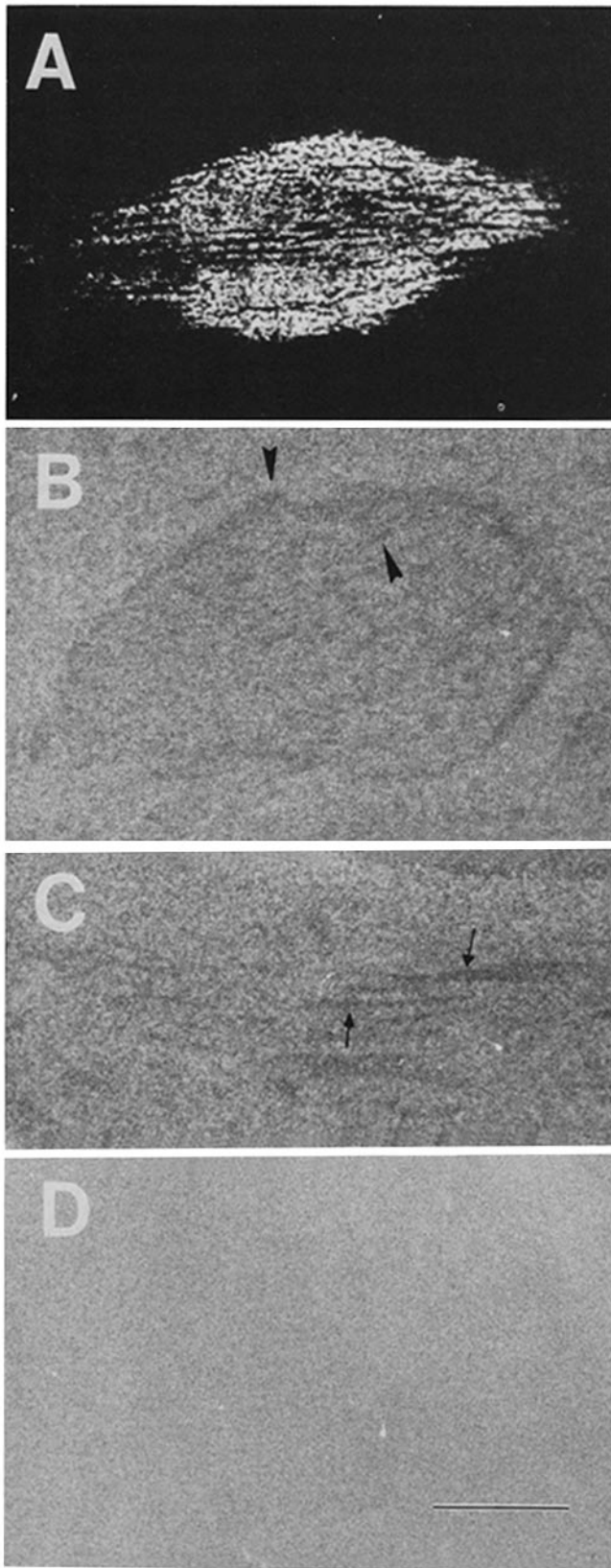


Figure 3. Labeling of areas of cell-substrate contact on fluoresceinated substrates. Glass coverslips were coated with serum proteins and labeled with fluorescein, as described in Materials and Methods. Saponin-SAM was prepared by method I (see Materials and Methods) from myotube cultures grown on these modified substrates. (A) A sample labeled with R-BT before extraction with

cellular materials prevent the labeling of the vitronectin in this protein layer by indirect immunofluorescence.

We also studied the distribution of substrate-bound vitronectin by immunocytochemical localization after freeze-fracture and replication by platinum shadowing, using a variation of procedures previously described by Pinto da Silva and Kan (23) and Rash et al. (25). In this method, the external leaflets of myotube membranes attached to the substrate were replicated. The platinum replica and underlying macromolecules were then released from the glass by treatment with hydrofluoric acid. After extensive washing, the replicas were incubated with antibodies and anti-antibodies adsorbed to colloidal gold. The advantage of this method is that, to gain access to the material underlying the replica, antibodies do not have to penetrate either a platinum layer or the normally very small space between the tissue culture substrate and the cell membrane. Furthermore, the very thin layer of material contributes little or no electron density, and thus does not obscure images of the gold particles. Appropriate controls indicate that the surface observed after following this procedure is indeed the substrate-apposed face of the external lipid leaflet (see Materials and Methods). We have termed this method "post-release fracture labeling."

In replicas processed by this method and labeled with anti-vitronectin, we found that cell-attached and cell-free areas of the replica labeled to equal extents, giving $\sim 8-12$ gold particles per μm^2 (Fig. 4; Table I). We did not observe any linear regions of the replica under the adhesion plaque that were free of label. Labeling by anti-vitronectin appeared to be specific, as significantly lower densities of gold particles were seen in samples stained with colloidal gold-adsorbed anti-mouse antibody without preincubation with anti-vitronectin, or after preincubation with non-immune serum (Table I).

One concern we had in using post-release fracture labeling was the possible effects of HF on the antigenicity of vitronectin. To evaluate this, we compared gold particle densities from post-release fracture-labeled replicas to replicas labeled with anti-vitronectin and gold-adsorbed second antibody before replication and exposure to HF. After labeling, these control samples were either fractured and replicated, or subjected to quick freezing and deep etching and then replicated. As with immunofluorescence, these methods failed to label areas of the substrate overlain by intact cells or membrane fragments (not shown). When we quantitated the density of gold labeling on the cell-free substrate, however, we found four to five times more particles in these samples than in the replicas treated with HF before exposure to antibodies (Table I). In addition, the level of nonspecific labeling in these samples was several-fold lower than in the

saponin. An AChR cluster (A) is located in an area of the substrate which is uniformly labeled with fluorescein (e.g., panel D). (B and C) Sample fixed in 2.5% glutaraldehyde, extracted with 0.5% Triton X-100 and labeled with anti-vitronectin antibodies followed by FGAM. Although large areas of the substrate are labeled by the antibodies, the sites of focal contacts (C; arrows) and linear areas within a myotube adhesion plaque (B; arrowheads) are poorly labeled. (D) The fluorescein attached directly to the substrate shows no evidence of non-uniformity. As the fluorescein label on the substrate was uniform in all samples, only a single example is given here. Bar, 20 μm .

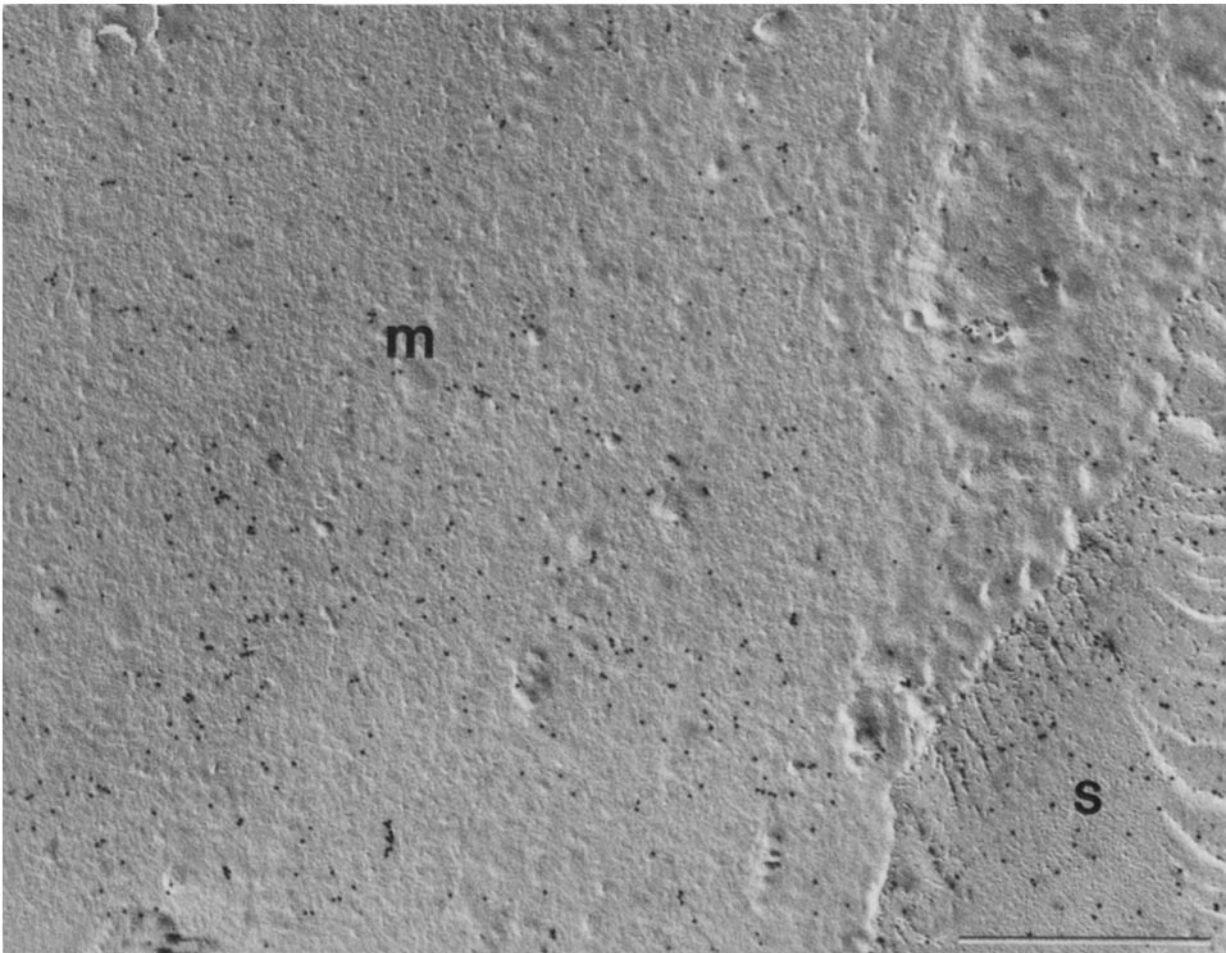


Figure 4. Post-release fracture labeling of a myotube-substrate adhesion plaque and nearby cell-free substrate. Post-release fracture labeling of myotube cultures was performed as described in Materials and Methods. Vitronectin at myotube-substrate adhesion plaques and on cell-free substrate was visualized with anti-vitronectin followed by anti-mouse IgG adsorbed to 15-nm colloidal gold particles. The myotube-substrate adhesion plaque *m* was recognized by its distinctive size and shape. The particle concentration in such regions was approximately equal to the particle concentration in nearby areas of the substrate which were free of cells (*s*) (see Table I). Bar, $\sim 1 \mu\text{m}$.

replicas obtained after post-release fracture labeling. These results suggest that treatment of samples with HF reduces the amount of antigenically active vitronectin and also increases nonspecific interactions. It is clear, nevertheless, that specific labeling by anti-vitronectin was still observed after post-release fracture label.

Another possible problem in evaluating this experiment is the high standard deviations we obtained (Table I). This probably resulted from the fact that gold particles were counted in a number of relatively small areas. If the particles were distributed randomly across the replica, such a sampling would produce a group of counts having a Poisson rather than a Gaussian distribution about their mean. The approach to a Poisson distribution is indicated by the high standard deviations, since this distribution has a standard deviation equal to the mean. Despite the high standard deviation, there was relatively good agreement between replicas prepared from different coverslips and involving intact and saponin-extracted cultures (Table I).

Discussion

Vitronectin is a serum glycoprotein that binds to glass and promotes cell spreading (4–6, 14, 28). When myotube cultures were grown on coverslips in the presence of serum, and labeled with affinity-purified antibodies against vitronectin by indirect immunofluorescence, the substrate was stained wherever cellular material was absent. We considered two alternative explanations for the absence of labeling at sites of cell-substrate contact: a failure of the antibodies to penetrate the contact regions, or an actual absence of vitronectin at these sites. The evidence we obtained strongly suggests that vitronectin is present over the entire substrate, but that the access of antibodies to the substrate is inhibited wherever cellular material is present.

We used two different strategies to expose cellular material on the substrate for subsequent study using indirect immunofluorescence. These strategies and the results obtained using them are summarized in Table II. One strategy depended on the differential stability of this material in the presence of detergent. Anti-vitronectin fails to label the cell-associated substrate of saponin-SAM, probably because the

Table I. Anti-Vitronectin Labeling of Myotube-Substrate Attachment Sites and Cell-free Substrate

Exp.	Sample	Labeling	First antibody	Region	Gold particles per μm^2	Area (#) (μm^2)
1	Intact cells	After replication and HF release	Affinity-purified anti-vitronectin	Attachment	12.6 \pm 3.7	79 (7)*
			None	Cell-free	17.0 \pm 7.8	68 (6)*
			None	Both	0.3 \pm 0.5	104 (40)‡
2	Saponin-SAM	After replication and HF release	Anti-vitronectin antiserum§	Attachment	7.8 \pm 2.8	104 (40)
				Cell-free	10.7 \pm 3.0	104 (40)
				Attachment	8.4 \pm 3.1	104 (40)
				Cell-free	10.8 \pm 3.2	104 (40)
				Both	2.4 \pm 3.0	104 (40)
3	Intact cells	After replication and HF release	Pooled non-immune serum	Attachment	13.2 \pm 6.6	70 (27)
				Cell-free	8.2 \pm 4.3	70 (27)
				Both	1.6 \pm 1.2	94 (36)
4	Saponin-SAM	Before replication and HF release¶	Affinity-purified anti-vitronectin	Cell-free	59 \pm 29	16.7 (40)
				Cell-free	46 \pm 5	12.8 (45)**
				Cell-free	0.3 \pm 0.1	234 (45)
				Cell-free	0.3 \pm 0.1	234 (45)

Cultures were freeze-fractured and replicated. In experiments 1–3, the replica attached to the glass coverslip, containing external membrane leaflets (E-faces) and underlying substrate, was released from the coverslip with hydrofluoric acid (HF) and labeled with affinity-purified anti-vitronectin antibody or other antisera followed by GAM G-15. The concentration of gold particles was quantified in areas of the substrate which were free of cellular material ("cell-free") or in nearby areas of myotube-substrate attachment ("attachment"). In experiment 4, samples were fixed and labeled with antibodies and GAM G-15 before replication, which followed fracturing or quick freezing and deep etching. The total area examined for each determination is given, followed by the number of fields examined, in parentheses. See Materials and Methods for more details.

* Cell-free and attachment areas were adjacent regions quantified from one replica in micrographs enlarged to 31,700 \times . Not significantly different from each other ($P > 0.2$; Rank sum test).

‡ These and subsequent areas are the field of view of the focusing binoculars (2.6 μm^2 at 14,500 \times).

§ Duplicate replicas from same culture were examined.

¶ Myotube-substrate attachments are not labeled under these conditions.

|| Processed by quick freezing and deep etching. Gold particles remain on the replica after this procedure.

** Processed by labeling followed by routine freeze-fracture and replication. Gold particles are removed with the specimen carrier after this procedure.

‡‡ Vinculin is an intracellular protein which appears to be completely removed during saponin extraction (8).

membrane-to-glass distance is too small to allow the antibodies to penetrate and the lipid bilayer prevents direct access to the substrate from the solution. Treatment of saponin-SAM with Triton X-100 results in the extraction of most of the AChR domains and most of the lipid bilayer, even in fixed preparations, but leaves some material which reacts with F-ConA attached to the coverslip. In these extracted structures, large areas label with anti-vitronectin antibody, presumably because access to the substrate is no longer

limited. Despite the increase in access to the antibody, the linear regions labeled by F-ConA still fail to stain with anti-vitronectin. In samples obtained from cultures grown on fluorescently labeled substrates, linear regions of poor anti-vitronectin immunofluorescence staining also appear, although the underlying substrate is uniformly labeled. As these areas of poor anti-vitronectin labeling are organized linearly even in the absence of AChR domains and are closely associated with the substrate during detergent extractions, they probably derive from the contact domains of intact adhesion plaques. They are probably cellular in origin, because they are clearly derived from cells and at least some of their components can be metabolically radiolabeled with [^{35}S]methionine (Baetscher, M., and R. J. Bloch, manuscript in preparation). These observations are consistent with the idea that cellular materials remain on the coverslip even after selective detergent extraction, and that where these materials are present, labeling of vitronectin is much reduced.

The second strategy depended on the greater resistance of substrate-associated material to physical shear forces. Mild shear removes much of the cellular material from the tissue culture substrate, but leaves adhesion plaques and focal contacts behind on the coverslip (9). Stronger shear causes the AChR domains and much of the lipid bilayer to be lost,² but material reactive with F-ConA remains bound to the substrate. In this case, too, the F-ConA staining of adhesion plaques is linear, and is probably associated with structures originally involved in myotube substrate contact (Baetscher, M., and R. J. Bloch, manuscript in preparation). As in the case of Triton-extracted saponin-SAM, the linear structures retained in shear-SAM fail to label with anti-vitronectin.

Table II. Effects of Detergent Extractions and Physical Disruption on Labeling of Adhesion Plaques and Focal Contacts

Preparation	Additional treatment	Labeling				
		Adhesion plaques			Focal contacts	
		R-BT	F-ConA	Ab	F-ConA	Ab
Saponin-SAM	—*	+	+	—	+	—
	0.5% Triton‡	—	-/+	+/-	+	—
Shear-SAM (strong shear)	—‡	—	-/+	+/-	+	—
	Sonication§	—	—	+	—	+

* F-ConA labels the entire adhesion plaque. Antibody (Ab) labels only cell-free areas of the substrate. AChR domains, labeled with R-BT, are retained.

‡ F-ConA labels only linear structures within the adhesion plaque, probably derived from sites of myotube-substrate contact (3). Antibodies label the substrate within the adhesion plaque which shows no F-ConA labeling, but fail to stain the lines and patches labeled with F-ConA. AChR domains have largely been extracted.

§ Most material which labels with F-ConA is gone. The entire substrate labels with anti-vitronectin and RGAM. The "shadows" in the antibody labeling correspond to areas which retain some material which labels with F-ConA.

When nearly all shear-SAM which labels with F-ConA is removed by sonication, coverslips label almost uniformly with anti-vitronectin, suggesting that the removal of cellular materials has exposed underlying vitronectin.

These results are not consistent with the idea that the substrate underlying cells contains no vitronectin. Instead, it seems highly likely that vitronectin present under cellular materials is inaccessible to anti-vitronectin and anti-mouse IgG. This is not a problem we have encountered with all antibodies, however. After Triton X-100 extraction of saponin-SAM, antibodies against other extracellular materials were able to label linear structures which resembled those labeled by F-ConA (Bloch, R. J., D. Schubert, and M. LaCorbiere, unpublished results). When the lipid bilayer has been disrupted, therefore, antibodies and anti-antibodies gain access to some materials at sites of cell-substrate contact. We conclude from these experiments that vitronectin covers the entire substrate, but that cellular materials³ are close enough to the vitronectin on the substrate to inhibit labeling by indirect immunofluorescence.

Our observations using indirect immunofluorescence microscopy are supported by our observations using a variation of earlier methods (23, 25) which we have called "post-release fracture labeling." This technique has the drawback that the number of anti-vitronectin binding sites preserved on cell-free substrate after replication and exposure to hydrofluoric acid is ~25% that of samples in which labeling was performed before these procedures were carried out. Considering, however, that intact vitronectin antigen must be retained on the replica after treatment with hydrofluoric acid, it is perhaps surprising that the method works at all. The success of the method is probably due in part to the fact that the samples are stabilized by fixation with glutaraldehyde and platinum replication before further treatment. We expect that, with further refinement to reduce antigen loss or denaturation during HF treatment,⁴ this technique will be useful for the study of many substrate-associated macromolecules. Post-release fracture labeling is, we believe, the only method which permits the uninhibited access of antibodies to antigens on the substrate under cells, for subsequent viewing at the ultrastructural level.

The micrographs obtained after post-release fracture labeling show almost uniform labeling of the cell-associated and cell-free substrate, with no indication of linear domains which were free of label. These results are not consistent with an alternative interpretation of the results of the experiments using sonication, namely that exposure of cell-substrate attachment sites by sonication causes vitronectin to move along the substrate and occupy the sites as cellular material is removed. Instead, the results are consistent with our hypothesis that vitronectin is present in approximately uni-

form amounts underneath the entire myotube-substrate adhesion plaque.

Although we have made all our observations on both the focal contacts of fibroblasts and the broad adhesion plaques of myotubes, there are certain advantages to working with myotubes. Unlike the focal contacts of mononucleate cells, the adhesion plaques of myotubes are usually very large, and have a distinct size, shape, and linear organization. The membrane domains which are not involved in contact are usually rich in AChR's, which are easily labeled with fluorescent derivatives of α -bungarotoxin. AChR is lost when the adhesion plaque is extracted with detergent or subjected to strong shearing, making it an excellent marker for the integrity of the lipid bilayer. These properties have greatly facilitated our interpretation of the patterns obtained from indirect immunofluorescence and postfracture labeling experiments. Although in our experiments focal contacts have generally behaved in the same way as adhesion plaques, markers for the cell membrane near focal contacts are not yet available. In samples treated with detergents or strong shearing, it is therefore more difficult to be sure that access to the substrate under focal contacts is as great as it is under adhesion plaques. Nevertheless, we propose that cellular material at focal contacts, as well as at the contact sites of adhesion plaques, lies above a layer of vitronectin and inhibits the access of anti-vitronectin antibodies to this layer.

Our results with vitronectin are in sharp contrast to results with fibronectin, which is cleared from cell-substrate contact regions under some circumstances (1, 11). One of the techniques we have used, fluorescent labeling of a substrate-associated protein, is based on similar procedures used by Avnur and Geiger (1) and Chen et al. (11) to study fibronectin. Because we coated the glass coverslips with serum before fluoresceination, other serum proteins as well as vitronectin were probably labeled and so contributed to the uniform appearance of the substrate under fluorescence optics. Vitronectin comprises more than half of the substrate-attached serum protein, however, so it is unlikely to have been selectively removed from the substrate by cells without causing a detectable change in fluorescence. Fibronectin and vitronectin therefore appear to be handled differently by cells in culture, the former being selectively removed from areas of cell-substrate attachment, the latter being retained there.

Although it gives clear results with proteins such as fibronectin and vitronectin, which can be selectively introduced onto the tissue culture substrate, the technique of coupling fluorescently labeled proteins to the substrate may be difficult to use to study other macromolecules associated with cell-substrate attachment. We have therefore developed several new techniques—selective detergent extraction, physical shearing, and post-release fracture labeling—to study such macromolecules. In the case of vitronectin, the results we obtained with each method agreed with those obtained from the other two, and with those obtained using a fluorescently labeled protein substrate. Further application of these methods should allow us to identify and localize some of the proteins of cellular origin that are associated with myotube-substrate adhesion.

We thank E. Concaugh, J. Strong, W. Resneck, and B. Boswell for their expert technical assistance, and Drs. R. Gruener, P. Luther, and J. Wade for their comments on the original version of this paper. We also thank one

3. Saponin-SAM from myotube cultures which had been metabolically radiolabeled with [³⁵S]methionine does not contain a major radiolabeled band with the mobility in SDS PAGE of either of the vitronectin polypeptides (Bloch, R. J., manuscript submitted for publication). This suggests that the material underlying the adhesion plaques which labels with anti-vitronectin is not cellular in origin.

4. Preliminary experiments suggest that the proton concentration can be reduced 10³-fold and the F⁻ concentration 12-fold without affecting the release of the replica from the substrate. Under these conditions, nonspecific labeling is reduced 10-fold, but specific labeling by anti-vitronectin remains at ~25% the level seen in samples labeled before treatment with HF (Pumpilin, D. W., unpublished results).

of the referees for his very helpful suggestions concerning post-release fracture labeling.

M. Baetscher has been the recipient of a postdoctoral fellowship from the Muscular Dystrophy Association of America. Our research has been supported by grants to D. W. Pumplin from the National Institutes of Health (NS 15513) and the Muscular Dystrophy Association, and to R. J. Bloch from the March of Dimes, the NIH (NS 17282), and the Muscular Dystrophy Association. R. J. Bloch was also supported by a McKnight Scholar's Award and a Research Career Development Award (NS 00679).

Received for publication 30 July 1985, and in revised form 11 March 1986.

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