IMMUNOLOGICAL CHARACTERIZATION OF A MAJOR TRANSFORMATION-SENSITIVE FIBROBLAST CELL SURFACE GLYCOPROTEIN

Localization, Redistribution, and Role in Cell Shape

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ABSTRACT

The major cell surface glycoprotein of chick embryo fibroblasts, cellular fibronectin (formerly known as CSP or LETS protein), was purified and used to produce monospecific antisera. After affinity purification, the anti-fibronectin was used to investigate fibronectin's localization, its transfer from intracellular to extracellular pools, its antibody-induced redistribution on the cell surface, and its role in cell shape.

Anti-fibronectin localizes to extracellular fibrils located under and between sparse cells, and to a dense matrix that surrounds confluent cells. Cellular fibronectin is also present in granular intracytoplasmic structures containing newly synthesized fibronectin before secretion. This intracellular staining disappears 2 h after treatment with cycloheximide or puromycin, and returns after removal of these protein synthesis inhibitors. In pulse-chase experiments using cycloheximide, fibronectin was sequentially transferred from the intracellular to the fibrillar extracellular forms.

Transformation of chick fibroblasts results in decreases in both extracellular and intracellular fibronectin, and in altered cell shape. Treatment of untransformed chick fibroblasts with anti-fibronectin results in rapid (30 min) alteration to a rounder cell shape resembling that of many transformed cells. These rapid shape changes are followed by a slow, antibody-induced redistribution of fibronectin to supranuclear caplike structures. This "capping" is inhibited by metabolic inhibitors.

Reconstitution of cell surface fibronectin onto transformed cells restores a more normal fibroblastic phenotype. The reconstituted fibronectin on these cells organizes into fibrillar patterns similar to those of untransformed cells. As with untransformed cells, treatment of these reconstituted cells with anti-fibronectin also results in cell rounding and "capping" of fibronectin.

KEY WORDS fibronectin · immunofluorescence · antibody-induced redistribution · secretion · adhesion

A glycoprotein of high molecular weight has been identified on the surface of a variety of fibroblastic and epithelial cells in vitro, using procedures that

label cell surface proteins by radioactive compounds or by specific antibodies (reviewed in references 15, 44, 55; see also reference 7). This glycoprotein is known as cell surface protein (CSP), large, external, transformation-sensitive (LETS) protein, or cellular fibronectin (16, 21, 58). This protein is often decreased after establishment of a permanent cell line (61), and is usually further decreased after neoplastic transformation (15, 44, 55). Cells missing this glycoprotein are generally tumorigenic (7, 12), although in vitro experiments indicate that it does not directly control growth rates (43, 60).

This glycoprotein, or the immunologically indistinguishable plasma glycoprotein "cold insoluble globulin" (plasma fibronectin) is thought to be a constituent of connective tissue and possibly of basement membranes in vivo (27, 44). Both of these proteins may mediate adhesive interactions of cells with substrata in vitro and in vivo. For example, they promote cell attachment and spreading on plastic, glass, and collagen substrate (3, 13, 23, 24, 34, 60).

We have isolated fibronectin from chick fibroblasts, and have established that it is an adhesive protein (54, 58, 59, 60). Reconstituting this protein on the surfaces of transformed cells from several species results in morphologic reversion to a more normal phenotype, apparently because of increased cell adhesion (3, 53, 54, 60).

We previously reported that an antibody directed against fibronectin inhibited its hemagglutinating activity (59) and the fibronectin-induced reversion of transformed cells (60; see also reference 3). This antibody also readily crossed species lines and stained the cell surface fibronectins of chick, human, mouse, rat, and hamster fibroblasts (61). In this paper, I describe the preparation, characterization, and a further purification of this antibody by affinity chromatography. The affinity-purified antibody is used to explore the localization, the intracellular-to-extracellular movement, the antibody-induced redistribution, and the role in cell morphology in vitro of this cell surface glycoprotein.

MATERIALS AND METHODS

Electrophoretic Purification of Cellular Fibronectin

Fibronectin was isolated from tertiary chick embryo fibroblasts cultured in roller bottles, using extraction with 1 M urea as described previously (54, 59, 60). The fibronectin was further purified either by electrophoresis or by column chromatography. For electrophoresis, the extracts were dialyzed against deionized water (3 × 4 l for 72 h), lyophilized, then resuspended in 2% sodium dodecyl sulfate (SDS) and 0.1 M dithiothreitol in 10 mM phosphate, pH 7.0. Samples were heated at 100°C for 3 min, incubated for 30 min at 37°C to complete reduction, and electrophoresed on 5% polyacrylamide slab SDS gels as described previously (40, 58), with the substitution of a single-well spacer to form the sample well of a 1-mm thick preparative slab gel.

The following precautions, adapted from Weber and Kuter (49) and Weiner et al. (50), were utilized to permit optimal recovery of antigen. (*a*) Preparative gels were stored, for 3–4 days before use, in a humidified plastic bag to permit complete polymerization. (*b*) Gels were pre-electrophoresed with 0.01% crystal violet to serve to trap possible free radicals, then further electrophoresed for twice the usual electrophoresis time (total of 3 h more) with a total of three changes of the electrode buffer. (*c*) Gels were not fixed or stained before slicing, inasmuch as these treatments were found to decrease the recovery of large proteins from SDS gels.

After electrophoresis, three vertical reference slices 3–5 mm wide were cut from the slab gel, stained for 5 min with 0.25% Coomassie Blue (ICI United States, Inc., Wilmington, Del.) in 50% TCA, and destained in 7% acetic acid; meanwhile, the remainder of the gel was stored to 4°C to decrease protein diffusion. The mobility of the CSP band was determined, and the appropriate region was sliced from the gel with a pizza cutter. The gel was triturated with a glass rod and the protein was eluted with 0.05% SDS in 5 mM ammonium bicarbonate with continuous agitation at 37°C. After 18 h, the extract was centrifuged to remove gel fragments, lyophilized, resuspended in deionized water, and dialyzed against 0.05% SDS to decrease the SDS concentration.

Purity was assessed by staining the remnants of the gel to ensure that only the fibronectin region was removed (three runs were discarded at this step), and by re-electrophoresis of the eluted antigen on analytical gels. Quantities of fibronectin were determined by densitometry of the Coomassie Blue staining of aliquots of purified fibronectin in gels that also included parallel lanes containing bovine serum albumin protein standards. 100–150 μg of fibronectin were injected at 4-wk intervals into two goats to complete Freund's adjuvant, then twice more in incomplete Freund's adjuvant. 10 days after the last injection, serum was collected. Additional serum was obtained 1 yr later from one goat after three more booster injections.

Column Chromatography

Isolated fibronectin was precipitated with 70% saturated ammonium sulfate, then resuspended at 2–5 mg/ml in buffer B (50 mM cyclohexylaminopropane sulfonic acid [CAPS], 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride [PMSF]), and the pH rapidly re-

adjusted to pH 11.0. The fibronectin was dialyzed against buffer B for 18 h with two changes, then centrifuged at 25,000 *g* for 15 min. The fibronectin was chromatographed at 4°C on a 2.5 × 100-cm upward-flow column of cross-linked Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) pre-equilibrated with buffer B. Fractions comprising the dimer peak of fibronectin described previously (56) were pooled and either used for injection as an antigen into a goat (150 μg per injection using the same schedule described for electrophoretically purified fibronectin), or covalently attached to Sepharose for affinity purification of anti-fibronectin antibodies.

Affinity Purification of Antibody

3 mg of fibronectin dimer prepared from cells prelabeled with ^{125}I (14) was reacted with 3 g of cyanogen-bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) for 2 h at room temperature in 0.1 M borate, pH 10.0, and 0.5 M NaCl. Triethylamine was added to stop the reaction, and the slurry was washed according to the manufacturer's recommendations. 83% of the fibronectin remained bound to the agarose as determined using a gamma counter. The agarose-fibronectin was poured as a 0.9-cm diameter column, and further washed before use according to the protocol used for antibody purification (see below). A second affinity column using 6 mg of fibronectin dimer was prepared in the same manner.

Antiserum was precipitated three times with 40% saturated ammonium sulfate, dialyzed against PBS, and a 10-ml aliquot containing ~50 mg/ml of protein was applied over a 2-h period to the fibronectin-agarose column. Unretained and nonspecifically adsorbed proteins were eluted by a 20-ml wash with PBS followed by three cycles of 4 column volumes each of 0.1 M sodium acetate (pH 4.8) plus 0.5 M NaCl, alternating with 0.1 M sodium borate (pH 8.5) plus 0.5 M NaCl. Antibody was eluted with 0.2 M acetic acid (pH 2.5) with 0.5 M NaCl. Each 1.0-ml fraction was neutralized immediately with NaOH, the protein peak as determined by absorbance at 280 nm was pooled, and antibody was precipitated with 50% saturated ammonium sulfate. After centrifugation, resuspension in PBS, and dialysis for 24 h against three changes of PBS, the affinity-purified antibody was adjusted to 1 mg/ml and stored at −20°C. In a typical preparation, 15 mg of antibody were recovered from 1,600 mg of globulin.

Control sera consisted of the corresponding pre-immunization sera as well as a non-immune control serum from a goat that had been injected with another unrelated, electrophoretically-purified antigen (platelet actin). The latter non-immune control serum contained no detectable antibodies by immunodiffusion or immunoprecipitation analyses of cell homogenates, and did not stain acetone-fixed cells; it serves as a control for the residual SDS and acrylamide, as well as the Freund's adjuvant with which goats are injected along with the fibronectin. The immunoglobulin G (IgG) fractions of these sera were affinity-purified using a column of 10 mg of rabbit anti-goat IgG (containing 2.0 mg of specific antibody) coupled to Sepharose, following the same procedures used for purifying the anti-fibronectin antibody.

Fab Fragments and Other Antibodies

Fab fragments of anti-fibronectin were prepared by papain cleavage (30, 35) followed by gel and affinity chromatography. The ammonium-sulfate-fractionated globulin fraction of anti-fibronectin was digested with a 1:200 (wt/wt) ratio of mercuripapain to antibody as described by Notkins et al. (30). The digest was alkylated with iodoacetic acid (30) to inactivate the papain, then dialyzed against 0.1 M potassium phosphate, pH 7.5, for 18 h. After centrifugation at 25,000 *g* for 10 min, the supernate was chromatographed on a 2.5 × 100-cm Sephadex G-100 column as described by Antoine et al. (4). The void volume containing a small amount of uncleaved antibody was discarded. Fractions from the Fab peak were pooled, precipitated with 70% ammonium sulfate, and affinity-purified on a fibronectin dimer-Sepharose column as described earlier. The final Fab preparation contained no detectable residual uncleaved heavy chain by SDS gel electrophoresis.

Anti-filamin antiserum was produced by a series of six injections of 150 μg each of chicken gizzard filamin into a goat following the protocol described above for fibronectin. The filamin was a gift from Drs. Peter Davies, Yutaka Shizuta, and Ira Pastan, and the antibody was used previously in studies of filamin by Davies et al. (8). Anti-L-cell myosin was a kind gift from Dr. Mark Willingham and Dr. Ira Pastan.

Immunofluorescence and Phase-Contrast Microscopy

Chick embryo fibroblasts were established, cultured, and passaged using 0.25% trypsin as described previously (32, 47) in Ham's F10 medium supplemented with 5% heat-inactivated calf serum and 10% tryptose phosphate broth. Chick fibroblasts in the third or fourth passage were plated at 1×10^5 cells/35-mm plastic tissue culture dish on cover slips sterilized by incubation in 95% ethanol and flaming. After 2 days at 37°C in a humidified 5% CO_2-95% air mixture, cultures were rinsed once with Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium, then fixed for 1 h in 2% formaldehyde or paraformaldehyde in PBS with Ca^{++} and Mg^{++} containing 1% sucrose to decrease cell blebbing. Fixed cultures were then rinsed three times in PBS and either extracted with acetone or used directly without permeabilization.

To extract with acetone, cells on cover slips were immersed in 1:1 (vol/vol) acetone-water at 0°C for 2 min, acetone at −20°C for 2 min, 1:1 acetone-water for

another 2 min, then placed into PBS directly without air-drying.

For indirect immunofluorescence, cultures were incubated at 22°–23°C with goat antibody in PBS for 1 h at the concentrations indicated in the text, rinsed for 30 min in four changes of PBS, then incubated for 1 h with a 1:10 dilution in PBS of fluorescein-labeled rabbit IgG directed against goat IgG heavy and light chains (fluorescein:protein molar ratio = 4.3; Miles Laboratories Inc., Elkhart, Ind.). After rinsing for 30 min in PBS, the backs of the cover slips were rinsed with deionized water and blotted dry, and the cover slip was mounted in phosphate-buffered glycerol mounting medium.

Alternatively, antibody was labeled directly with fluorescein using 1 mg of fluorescein isothiocyanate/100 mg of protein for 2 h at 23°C, followed by chromatography on Sephadex G-25 in PBS to separate labeled protein from free dye (19). The final fluorescein:protein molar ratio was 3.5. If cells were stained with this antibody, the second anti-immunoglobulin step was omitted.

Cells were examined on a Zeiss fluorescence microscope equipped with a BG12 exciter and a 500-nm barrier filter. Photographs were taken with Polaroid 107 film, ASA 3000 (Polaroid Corp., Cambridge, Mass.), and used directly for figures. Unless otherwise indicated, the fluorescence micrographs were obtained using a 40× non-phase-contrast objective of numerical aperture (NA) 1.0 with exposure times of 10–15 s. All exposure times were identical within each series in which comparisons of intensities of staining were made. Dark-field microscopy could be performed on the same cells by using a Zeiss Ultra-dark-field oil immersion condenser (NA 1.2/1.4) after partial closure of an iris diaphragm in the 40× objective.

A Zeiss Invertoscope D was utilized for the phase-contrast micrographs, using Panatomic X 35 mm film (Eastman Kodak Co.) and a green filter. Cell area of cells fixed for 1 h with glutaraldehyde-paraformaldehyde exactly as described (57) was measured as described previously (60), using a 10 × 10 eyepiece grid.

Materials

Sepharose CL-4B, Sephadex G-25 and G-100, and cyanogen-bromide-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals, Inc.); CAPS, PMSF, cycloheximide, puromycin, and triethylamine from Sigma Chemical Co., St. Louis, Mo.; crystalline trypsin and mercuripapain from Worthington Biochemical Corp., Freehold, N.J.; colchicine from Calbiochem, La Jolla, Calif.; and cytochalasin B and iodoacetic acid from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Heat-inactivated calf serum was purchased from Flow Laboratories, Inc., Rockville, Md., and tissue culture dishes from BioQuest, BBL, & Falcon Products, Oxnard, Calif. or Corning Glass Works, Corning, N.Y. Fluorescein-labeled IgG fraction of rabbit anti-goat IgG, the unlabeled IgG fraction of rabbit anti-goat IgG, incomplete and complete Freund's adjuvant, and fluorescein isothiocyanate were obtained from Miles Laboratories Inc., FA mounting medium, pH 7.2, from Difco Laboratories, Detroit, Mich., and 6-well pattern C immunodiffusion plates from Hyland Diagnostics Div., Travenol Laboratories, Inc., Costa Mesa, Calif. L-[U-^{14}C]leucine (287 and 314 mCi/mmol), L-[U-^{14}C]methionine (213 mCi/mmol), and L-[U-^{14}C]proline (261 mCi/mmol) were obtained from New England Nuclear, Boston, Mass.

RESULTS

Specificity of the Antibody

The cellular fibronectin used as immunogen was homogeneous by SDS polyacrylamide gel electrophoresis (Fig. 1). Cellular fibronectin is not soluble under the conditions usually used to evaluate antibody specificity (17, 56). We therefore first solubilized the fibronectin by elevating the pH to 11.0 (32, 56), then assayed for specificity at neutral pH. In Ouchterlony double immunodiffusion gels, confrontation of either antibody (anti-fibronectin dimer or anti-electrophoretically purified fibronectin) with either isolated fibronectin or homogenates of cultures of chick embryo fibroblasts resulted in a single immunoprecipitin line of identity (Fig. 2). If cultures of chick fibroblasts were trypsinized before homogenization to remove external fibronectin (58) or transformed by Rous sarcoma virus, the remaining precipitin line retained a junction of identity with the fibronectin of untrypsinized cultures (Fig. 2). The antibody did not form precipitin lines when confronted with calf or fetal calf serum, chicken gizzard filamin, or L-cell myosin, but did readily cross-react with chicken serum or plasma (Fig. 2: unpublished results). The reactive material in chicken serum is probably chicken plasma fibronectin (37; K. Ya-

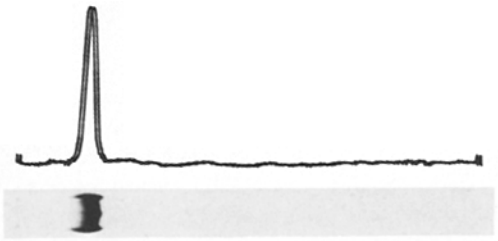


FIGURE 1 SDS-polyacrylamide gel electrophoresis of cellular fibronectin preparation used as antigen. Electrophoretically purified fibronectin analyzed on a 5% polyacrylamide SDS gel and stained for protein with Coomassie Brilliant Blue, and densitometer scan of this gel showing absorbance at 550 nm.

FIGURE 2 Double immunodiffusion analysis of anti-cellular fibronectin antibody. (*a*) Center well: 1.5 mg/ml of purified cellular fibronectin in 0.15 M NaCl, 1 mM $CaCl_2$, 50 mM CAPS buffer, pH 11.0. (*1*) Anti-fibronectin antiserum, 1:10 dilution in PBS. (*2*) Affinity-purified anti-fibronectin, 0.5 mg/ml. (*3*) Control non-immune serum, 1:10. (*4*) Antifilamin antiserum, 1:10. (*5*) Anti-L-cell myosin antiserum 1:10. Stained with Coomassie Blue. (*b*) Center well: unfractionated antiserum against electrophoretically purified cellular fibronectin, 1:5 dilution. (*1*) cellular fibronectin, 1.5 mg/ml. (*2*) Chicken serum, undiluted. (*3*) Calf serum, undiluted. (*4*) cellular fibronectin, 1.5 mg/ml. (*5*) Fetal calf serum, undiluted. (*c*) Center well: unfractionated anti-fibronectin, 1:10 dilution. (*1*) Purified cellular fibronectin, 1 mg/ml. (*2*) Homogenate of chick embryo fibroblasts, 5 mg/ml in 1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), 50 mM CAPS buffer, pH 11. (*3*) Homogenate of chick fibroblasts that had been pretreated with 10 μg/ml of trypsin for 10 min at 37°C, 5 mg/ml in Triton-CAPS. (*4*) Homogenate of Rous sarcoma virus-transformed chick fibroblasts, 8 mg/ml in Triton-CAPS. (*5*) Chicken serum, undiluted.

mada. Unpublished results). Conversely, goat antibodies against chicken gizzard filamin or mouse L-cell myosin did not react with purified cellular fibronectin (Fig. 2).

Because Keski-Oja et al. (22) had reported that their antibody against surface papain digests of chick fibroblasts formed a single line in double immunodiffusion tests against cell homogenates, yet appeared to react with at least two distinct polypeptides of mol wt 210,000 and 145,000 in immunoprecipitation experiments, the antibody was further examined for specificity by immunoprecipitation of homogenates of chick fibroblasts. Cultures of chick fibroblasts were pulse-labeled with 10 μCi/ml [^{14}C]leucine, [^{14}C]methionine, or [^{14}C]proline for 15 or 30 min each, then homogenized at pH 11 and immunoprecipitated as described previously (32). After electrophoresis of the immunoprecipitate on SDS gels, label was found almost entirely in the fibronectin band, and not in any other region of the gel other than at the front of the immunoglobulin light chain band in both control and anti-fibronectin immunoprecipitates (Fig. 3). In some immunoprecipitates, labeled for 24 h with [^{14}C]leucine, bands co-migrating with actin and myosin were found in equal amounts in gels of anti-fibronectin and control sera (Fig. 3) (32), apparently representing non-specific precipitation. A heavily labeled band of mol wt 160,000 present in cell homogenates was not precipitated by anti-fibronectin antibody.

Immunofluorescence Localization: Extracellular Fibronectin

Cells fixed with formaldehyde or paraformaldehyde exclude antibody unless extracted with acetone. When chick fibroblasts at low cell density ($<5 \times 10^4$ cells/cm^2) are fixed under these conditions and stained with affinity-purified anti-fibronectin (20 μg/ml) without acetone extraction, the fluorescence is localized to fibrils under and between cells (Fig. 4*a'*). This extracellular fibronectin does not usually extend substantially beyond

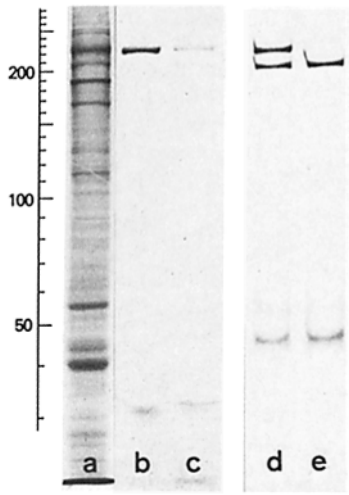


FIGURE 3 Immunoprecipitation of cell homogenates by anti-fibronectin. Cultures of chick embryo fibroblasts were labeled with [^{14}C]leucine, solubilized, and immunoprecipitated with anti-fibronectin followed by anti-antibody exactly as described previously in reference 32. Scale indicates apparent subunit mol wt $\times 10^{-3}$, using previously described protein standards (61). (*a–c*) Cells labeled with 10 μCi/ml of [^{14}C]leucine for 15 min and electrophoresed using a stacking gel system (40). (*a*) Cell homogenate. (*b*) Immunoprecipitate using 45 μg/ml of affinity-purified anti-fibronectin. (*c*) Immunoprecipitate with 45 μg/ml of control IgG. (*d–e*) Cells labeled with 1 μCi/ml of [^{14}C]leucine for 24 h and electrophoresed in a continuous gel system (61). (*d*) Immunoprecipitate using 1:20 dilution of unfractionated anti-fibronectin antiserum in the first step. (*e*) Immunoprecipitate using 1:20 dilution of non-immune goat serum in first step.

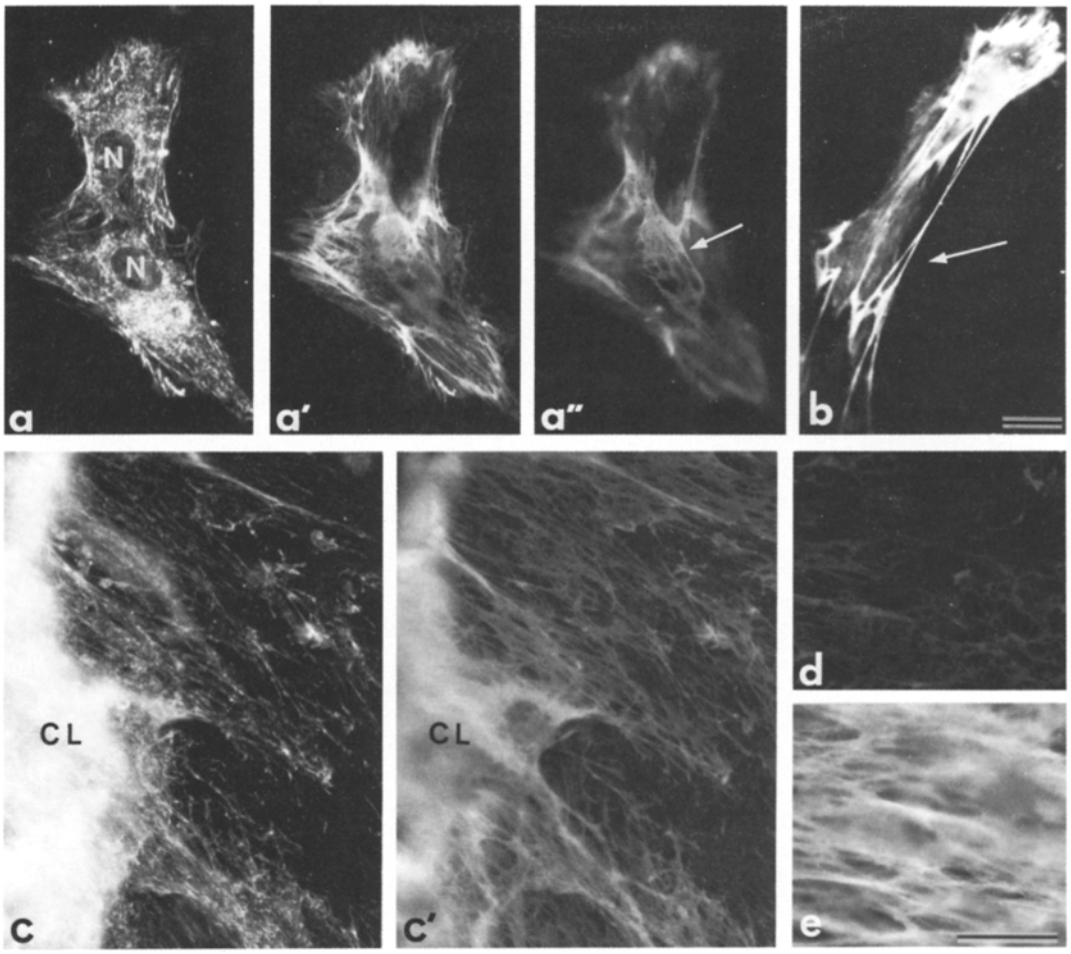


FIGURE 4 Immunofluorescence localization of extracellular fibronectin. Cells were fixed with formaldehyde, incubated with 20 μg/ml of affinity-purified anti-fibronectin, then anti-IgG labeled with fluorescein. (*a*) Two chick cells, dark-field microscopy. (*a'*) Same field by immunofluorescence microscopy, focused on the plane of the substratum. (*a''*) Immunofluorescence, focused higher on the cells to show fibronectin bridging between cells (arrow). (*b*) Cell with unusual amount of fibronectin extending to substratum at posterior of cell. (*c*) Cell layer (*CL*) retracted to the left from a wound; dark-field. (*c'*) Immunofluorescence of same field. (*d*) Fibronectin remaining on substratum after removal of cells by EDTA and pipetting. (*e*) Fibronectin on cells treated with EDTA, but not dislodged from the dish. Fig. 4*a* and *c–e* are × 520. Fig. 4*b* is a photographically reduced print, × 350. Bars, 25 μm.

the outlines of the cells, although fibrils often trail out behind cells and bridge between cells (Fig. 4*a''*). Occasionally, long fibrillar tails of fibronectin (Fig. 4*b*) or a "footprint" of a cell outline can be seen, apparently because of a sudden detachment of a cell that leaves fibronectin behind.

In denser cell cultures with increasing cell-cell contact, the fibronectin fibrils become more frequent and stain more intensely. The cells become surrounded by a longitudinally oriented, complex matrix of extracellular fibrillar material (Fig. 4*c–e* and 5*c*), as described previously (3, 7, 28, 48, 55). The affinity of this fibronectin to the cell layer vs. the affinity to the substratum was tested by the following cell detachment experiments. Cultures were wounded by scratching with a glass rod. Cells retracted laterally from the wound, and the amounts of fibronectin retracting with cells were compared to amounts remaining on the substratum. Alternatively, cells were detached

from the dish with 1 mM EDTA in phosphate-buffered saline. In both cases, most of the fibronectin was found to retract with the cell sheet, although weakly staining networks of fine fibrils remained behind on the substratum (Fig. 4*c–e*).

As previously reported by others (28), the same fibrillar, extracellular distributions of proteins are also observed if living cells are stained briefly (5 min) with anti-fibronectin, then with fluorescein-labeled anti-IgG, and then examined immediately: similar fibrillar staining patterns are also obtained using anti-fibronectin directly labeled with fluorescein or rhodamine (38: K. Yamada. Unpublished results).

Intracellular Fibronectin

Cells were extracted with acetone to permit intracellular staining. In cells that had been cultured at low cell density, this procedure resulted in staining of vaguely granular structures surrounding the nucleus, as well as the extracellular fibrils described previously (Fig. 5*a* and *b*). The perinuclear intracellular staining was excluded from large vesicles (Fig. 5*b*). Similar intracellular staining of chick cells has been reported by Stenman et al. (39).

As the cultures became more crowded, the intracellular staining became progressively harder to detect among the more heavily staining extracellular fibrils (Fig. 5*c*). Gentle trypsinization of live cells (1 μg/ml for 5 or 10 min at 37°C) removes the extracellular, fibrillar fibronectin, revealing the intracellular, granular perinuclear staining (Fig. 5*d*).

Immunofluorescence Specificity Controls

Both intracellular and extracellular staining were specific in that no staining was found when an equal amount of the affinity-purified control antibody, crude gamma globulin from this control serum, or pre-immune serum was used (Fig. 5*e* and *f*). Procedures designed to inhibit nonspecific adsorption of antibody, such as pre-incubation of cells in 25 mg/ml bovine serum albumin or 50% non-immune rabbit serum, plus addition of these proteins to all antibody staining solutions, did not affect the intracellular staining. Finally, pre-absorption of the anti-fibronectin with purified cellular fibronectin dimer (1:4 or higher wt:wt ratios of fibronectin to antibody protein) blocked the staining of cells (Fig. 5*g*).

Extracellular staining was distinguished from intracellular staining by the criteria of (*a*) sensitivity to removal by mild protease treatment of cells before fixation and staining; these conditions were previously shown to remove all extracellular fibronectin detectable by iodination (58, 61), without affecting amounts of intracellular fibronectin detected by biochemical criteria (32); and (*b*) presence on live or fixed cells before acetone extraction. Formaldehyde-fixed cells were shown to remain impermeable to intracellular diffusion of antibody by attempting to stain with antifilamin: no staining was detected unless cells were first extracted with acetone.

The intracellular staining was characteristic in that it was (*a*) not decreased in apparent intensity by external protease treatment of cells; (*b*) detected only after cells were extracted with acetone or detergents; and (*c*) absent after inhibition of protein synthesis. In addition, intracellular fibronectin could usually also be distinguished from external fibronectin by continuously changing the focal plane of the microscope to determine staining inside the plasma membrane and adjacent to the nucleus.

The immunofluorescence patterns were not due to immunological cross-reactive materials such as plasma fibronectin (cold insoluble globulin) derived by the cells from the calf serum used in culture media: (*a*) Anti-fibronectin did not react with calf serum in double immunodiffusion tests (Fig. 2); (*b*) anti-fibronectin absorbed with calf serum (100 μg/ml of affinity-purified anti-fibronectin plus 90% calf serum for 1 h at 23°C, then centrifuged at 10,000 *g* for 10 min) was as effective as unabsorbed anti-fibronectin in staining intracellular and extracellular fibronectin when used at 20 μg/ml to stain fixed cells with or without acetone extraction; and (*c*) fluorescence localization patterns were not altered if cells were plated into medium containing 5% pre-immune goat serum substituted for the calf serum, cultured for 2 days, fixed, acetone-extracted, and stained with anti-fibronectin from the same goat. If the localization had been due to a component from calf serum, this protocol should have resulted in loss of antibody localization.

Relationship of Intracellular to Extracellular Fibronectin

To evaluate whether the intracellular staining could represent newly synthesized fibronectin before secretion, cells were treated with inhibitors of protein synthesis. Cells in regular culture medium

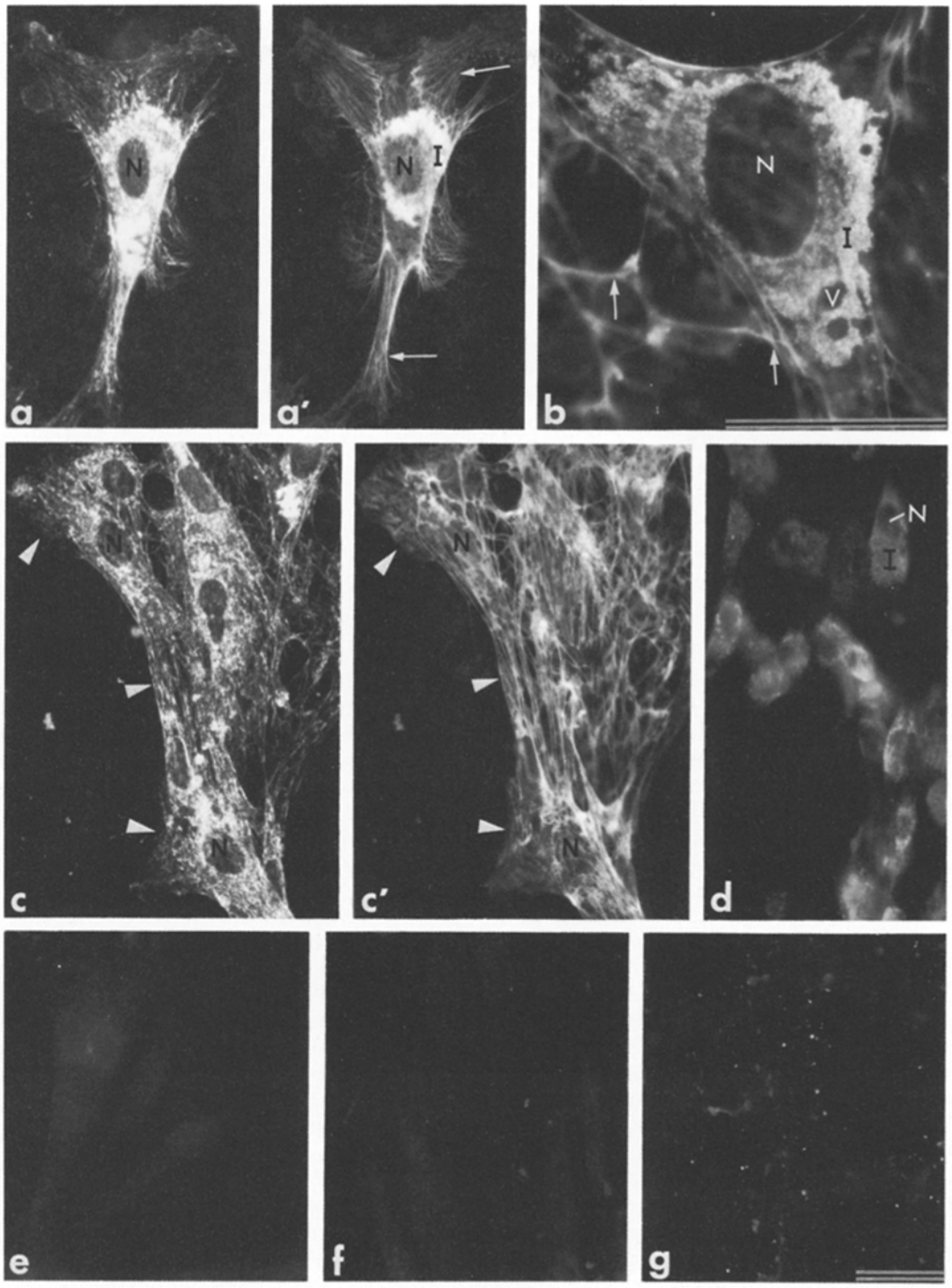


FIGURE 5 Fibronectin localization in acetone-treated cells, using 20 μg/ml of all antibodies. (*a*) Dark field. (*a'*) Immunofluorescence; compare fibrillar fibronectin (arrows) to perinuclear, granular intracellular fibronectin. (*b*) Higher magnification of another cell. (*c*) Partially confluent culture, dark field. (*c'*) Immunofluorescence; note coincidence of borders of fibronectin staining with cell margins (arrowheads). (*d*) Partially confluent culture trypsinized with 1 μg/ml of crystalline trypsin for 5 min to remove extracellular, fibrillar fibronectin. (*e*) Pre-immune IgG. (*f*) Control IgG, described in Materials and Methods. (*g*) 20 μg/ml of anti-fibronectin absorbed with 4 μg/ml of fibronectin dimer for 30 min at 23°C before incubation with fixed, acetone-extracted cells. *N* = nucleus, *I* = intracellular fibronectin, *V* = vesicle. Figs. 5*a* and *c–g* are × 520; Fig. 5*b* is × 1,300. Bars, 25 μm.

were exposed to 2 μg/ml cycloheximide, which inhibited the incorporation of [^{14}C]leucine into TCA-precipitable material by 95%, then fixed, extracted with acetone, and stained with anti-fibronectin. The intracellular staining progressively decreased in cycloheximide-treated cells, and was absent from most (>80%) of the cells by 60 min. By 120 min, <2% of the cells had any detectable intracellular staining (Fig. 6*a*, Table I). Extracellular fibronectin appeared to be unaffected by this treatment.

The inhibition of intracellular staining is reversible. Removal of cycloheximide after 120 min of treatment resulted in a progressive reappearance of the intracellular staining (Table I). Cultures from which cycloheximide was removed, then replaced immediately, showed no return of intracellular labeling. Similar results were obtained using puromycin, another protein synthesis inhibitor (Fig. 6*b*). These results suggested that the intracellular staining was due to a labile internal pool of fibronectin, such as a secretory antecedent, rather than due to ingested extracellular fibronectin. Biochemical evidence for such an intracellular pool of fibronectin has been reported previously (32).

A pulse-chase experiment testing this hypothesis is described in Fig. 7. Cells were pre-incubated for 2 h with 0.2 μg/ml of cycloheximide to deplete intracellular pools of fibronectin. The cells were then treated with trypsin at concentrations sufficient to remove extracellular fibronectin, but not to detach the cells from the substratum. Cells were then incubated in normal culture medium without cycloheximide for 2 h to permit resynthesis of fibronectin, then returned to medium containing cycloheximide to block further fibronectin synthesis. The cycloheximide pretreatment and trypsinization depleted both intracellular and extracellular fibronectin (Fig. 6*c*). Removal of cycloheximide resulted in the reappearance of intracellular staining, and a small amount of surface staining (Fig. 6*d–f*). The internal staining could be completely chased to fibrillar surface staining (Fig. 6*g* and *h*). Control cells maintained continuously in cycloheximide after trypsinization did not show either intracellular or surface fibronectin, and omitting the final incubation in cycloheximide resulted in both intracellular and extracellular staining (Fig. 6*i*).

In addition, the overall morphology of cells appeared to depend on the amount of extracellular fibronectin. Cells containing only intracellular fibronectin were generally less flattened than cells with substantial amounts of extracellular, fibrillar fibronectin, which were significantly flatter and occupied more surface area (cf. cell areas in Fig. 6*c–f* with those in Fig. 6*g–i*). However, it is conceivable that this morphological difference could also have resulted from alterations in internal structural proteins.

These results indicate that the intracellular pool of fibronectin serves as a precursor or antecedent to the fibronectin on the cell surface. This intracellular fibronectin may therefore be located in endoplasmic reticulum or Golgi apparatus. This procedure may provide a means to synchronize and to characterize the sequential biochemical alterations in fibronectin during processing and secretion.

Effects of Anti-Fibronectin on Cell Morphology

Chick fibroblasts treated with crude anti-fibronectin or with 50–100 μg/ml of the affinity-purified antibody added directly to culture media displayed rapid alterations in cell shape (Figs. 8 and 9). Within 10 min, cells began to become more rounded or cylindrical and phase-refractile, and the cell processes became blunter and less spread. The alterations appeared to be complete within 30 min (Fig. 8*a–e*). These changes in cell shape were quantitated by measuring the projected area of cells fixed with glutaraldehyde-paraformaldehyde at intervals after addition of anti-fibronectin. Mean cell area decreased by approximately one-third, and the changes were nearly complete within 20 min (Fig. 9). Equal amounts of control globulin or IgG (pre-immune or control) were without effect (Fig. 8*f*).

These alterations in shape were not accompanied by decreased cell viability as assayed by exclusion of trypan blue (Table II). In addition, the antibody treatment did not affect protein synthesis. Incorporation of [^{14}C]leucine (1 μCi/ml for 3 h) into TCA-precipitable material was not affected: untreated cultures, 66,620 cpm; treated with affinity-purified anti-fibronectin at 50 μg/ml, 70,020 cpm; and control non-immune goat IgG, 71,050 cpm (values are means of duplicate cultures in 35-mm dishes).

The morphological effects are readily reversed by rinsing cultures with fresh medium. 2–3 h after removal of antibody and incubation in medium, the cells reflattened on the substratum (Fig. 8*g*

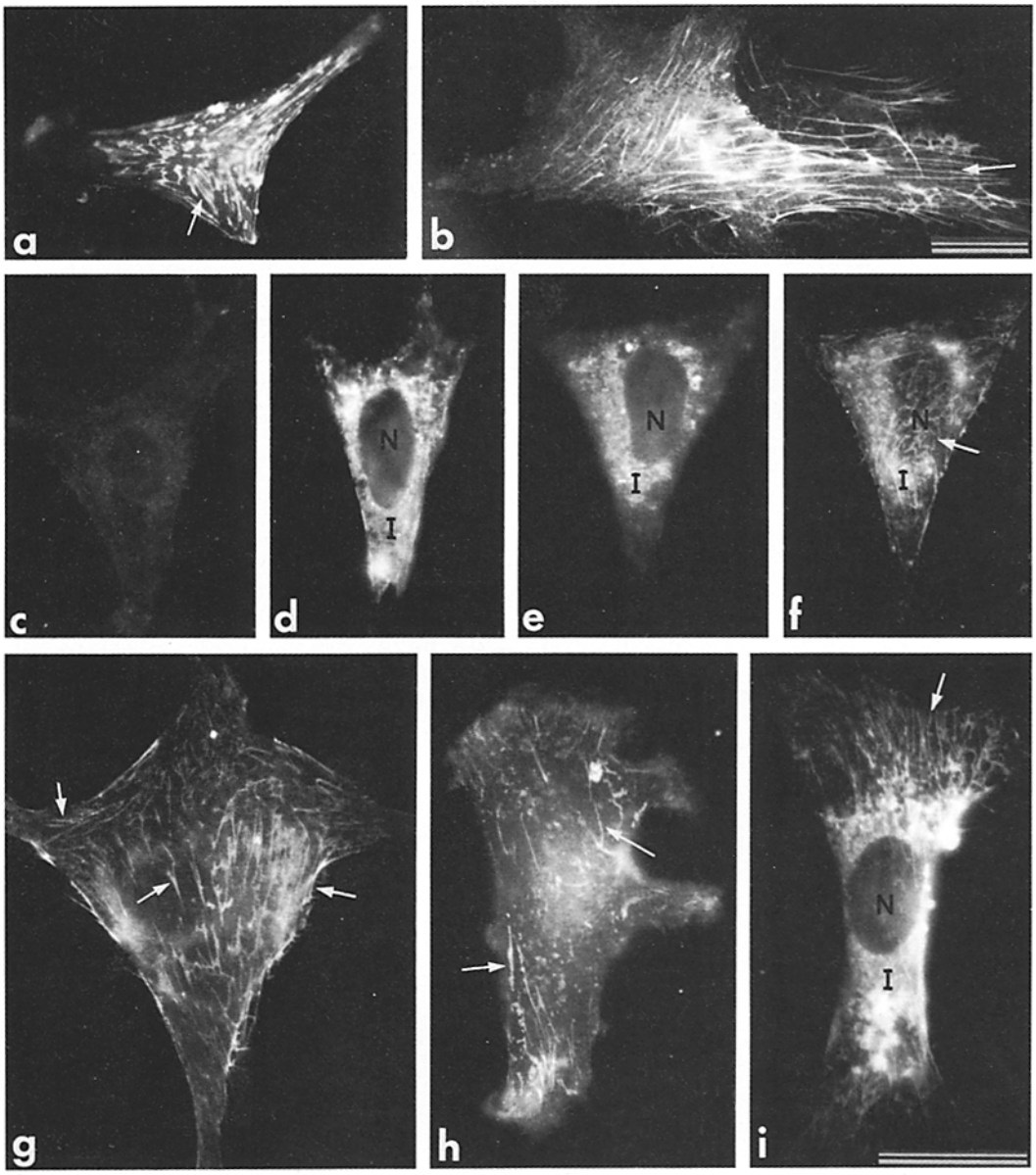


FIGURE 6 Intracellular to extracellular transfer of fibronectin. All cells were fixed, extracted with acetone, and stained for fibronectin. (*a*) Cell treated with 2 μg/ml of cycloheximide for 2 h. Note absence of intracellular, perinuclear staining. Arrow indicates fibrillar, extracellular fibronectin. (*b*) Cell treated with 2 μg/ml puromycin for 2 h. (*c*) Cell treated for 2 h with 0.2 μg/ml of cycloheximide, then trypsinized with 2 μg/ml of crystalline trypsin for 10 min at 37°C in serum-free medium. (*d*) 2 h after removal of cycloheximide. (*e*) 2 hr after removal of cycloheximide, focused on perinuclear region. (*f*) Same cell as previous picture, but focused on substratum to show tortuous fibrillar staining (arrow). (*g*) 2 h without, then a further 2 h with cycloheximide, showing fibrillar extracellular fibronectin (arrows); the diffuse staining on this cell was due to fibronectin higher on the cell that is out of focus. (*h*) 2 h without, then 2 h with cycloheximide. (*i*) 4 h without cycloheximide; note substantial amounts of both perinuclear and fibrillar staining. *N* = nucleus, *I* = intracellular fibronectin. Fig. 6*a* and *b* are × 520; Fig. 6*c–i* are × 820. Bars, 25 μm.

and *h*). Parallel cultures that were rinsed, but incubated in fresh medium containing anti-fibronectin, did not show a restoration of normal morphology (Fig. 8*i*).

Fab fragments of anti-fibronectin were also effective in producing these morphological alterations, indicating that cross-linking of antigen by multivalent antibody is not necessary for these effects on cell morphology (Fig. 8*j*). The mean cell area of cells treated for 60 min with these Fab fragments of anti-fibronectin was decreased by 37% in comparison to controls; this effect is similar in magnitude to the effects of intact anti-fibronectin.

TABLE I

Effects of Cycloheximide on Intracellular Fibronectin Staining

Experimental condition	Intracellular fluorescence present
	% of cells
Untreated	85
Cycloheximide 60 min	15
Cycloheximide 120 min	2
Reversal 60 min	77
Reversal 120 min	86

0.2 μg/ml of cycloheximide was added to low-density (2–3 × 10^4/cm^2) chick fibroblasts in regular culture medium. For testing reversibility, the cultures were incubated in cycloheximide for 2 h, washed five times, then incubated in medium without cycloheximide. 300 cells were scored for each experimental condition after fixing and staining for fibronectin using affinity-purified anti-fibronectin and indirect immunofluorescence.

The alterations in morphology produced by anti-fibronectin are similar to those observed after transformation of chick fibroblasts by the Schmidt-Ruppin strain of Rous sarcoma virus (Fig. 8*k*), with the exception of the appearance of large vacuoles in the transformed chick cells. The antibody has little or no effect on the morphology of these transformed chick cells, which are deficient in fibronectin (Fig. 8*l*). Rounded cell morphology is characteristic of many cells after malignant transformation (33, 53).

Similar antibody-induced changes in morphology of untransformed chick cells were observed using glass or plastic substrata, although cells cultured on plastic often required up to twice the concentration of antibody to produce equivalent effects; this difference may be due to increased adhesiveness of the cells to the tissue culture plastic. Similar morphological alterations were also seen if the cells were treated with the original crude antisera (1:10 dilution) after it had been heat-inactivated at 56°C for 30 min. Crude pre-immune or non-immune control antisera, anti-fibronectin preabsorbed with fibronectin, anti-myosin antiserum, or anti-chicken gizzard filamin did not result in significant morphological changes.

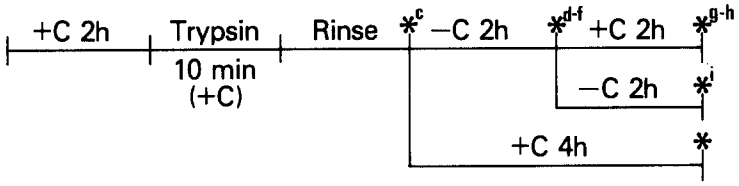
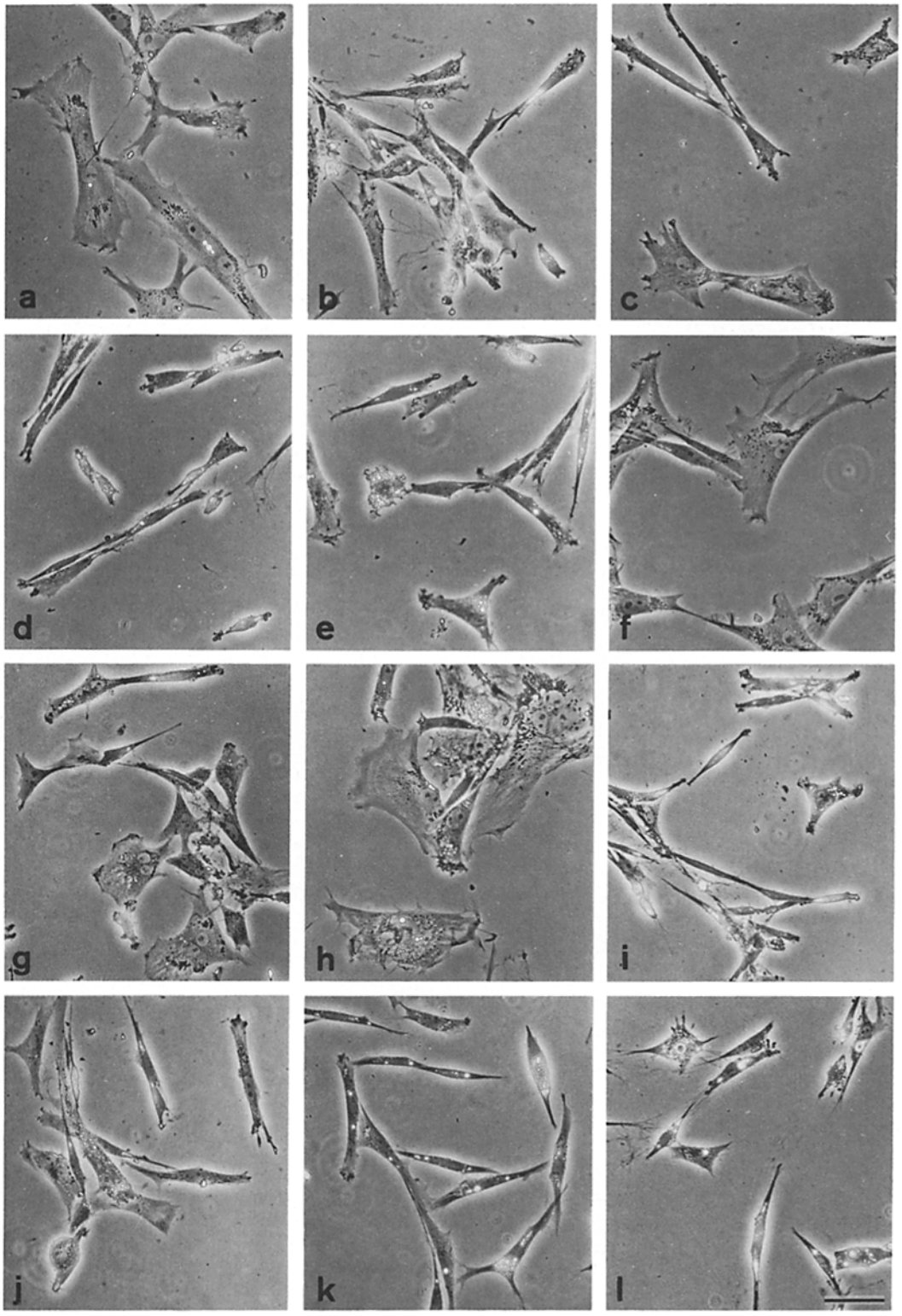


FIGURE 7 Summary of experiment analyzing intracellular fibronectin. *C* = cycloheximide, 0.2 μg/ml in culture medium. Asterisks indicate times at which cultures were fixed and stained for fibronectin; superscript letters indicate which micrographs in Fig. 6 correspond to these time-points.

FIGURE 8 Effects of anti-fibronectin on morphology of chick embryo fibroblasts. (*a*) Control chick fibroblasts. (*b*) Cells treated with crude anti-fibronectin antiserum diluted to 1:10 in regular culture medium, 30 min. (*c*) Treated with 50 μg/ml of affinity-purified anti-fibronectin for 10 min. (*d*) Treated for 20 min. (*e*) Treated for 60 min. (*f*) Treated with 50 μg/ml of affinity-purified IgG fraction of control serum for 60 min. (*g*) Reversal experiment: treated with 50 μg/ml of affinity-purified anti-fibronectin for 60 min, then rinsed five times and incubated in regular medium for 2 h. (*h*) Reversal experiment, 3 h. (*i*) Mock reversal: 50 μg/ml of affinity-purified anti-fibronectin added back to medium after rinsing five times, then incubated 3 more hours. (*j*) Treated with 100 μg/ml of affinity-purified anti-fibronectin Fab for 60 min. (*k*) Chick fibroblasts transformed by the Schmidt-Ruppin strain of Rous sarcoma virus. (*l*) Transformed chick fibroblasts treated with affinity purified anti-fibronectin, 50 μg/ml for 1 h. Phase contrast, × 190. Bar, 50 μm.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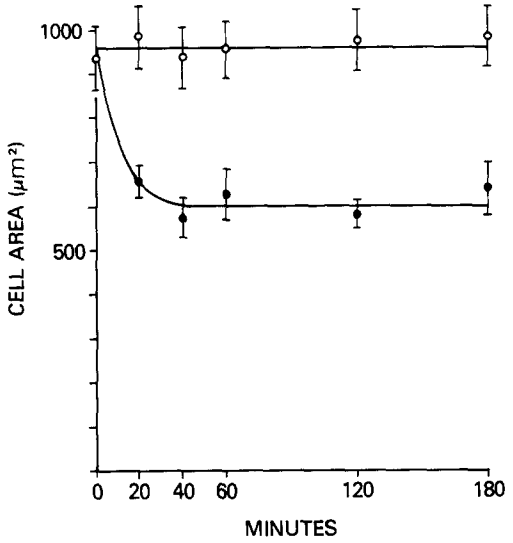

FIGURE 9 Effects of anti-fibronectin on cell area. Cells were treated with 50 μg/ml of affinity-purified anti-fibronectin or control IgG, then fixed and stained with methylene blue. Cell areas outlined on the plane of the substratum as viewed from above were determined as described in Materials and Methods. (●) treated with anti-fibronectin: (○) treated with control IgG.

Redistribution of Fibronectin by Antibody

Chick embryo fibroblasts were incubated for varying intervals of time with 50 μg/ml of affinity-purified anti-fibronectin added to the culture medium, then rinsed with PBS, fixed, and stained with fluorescein-labeled anti-antibody. After 20–30 min of incubation with anti-fibronectin, when the morphological changes described above were nearly complete, the anti-fibronectin remained in fibrillar patterns, primarily under cells. These fibrils, however, often appeared more tortuous and generally shorter (Fig. 10*a*).

After 40–60 min, the fibrillar patterns became more distorted, and the labeling of fibronectin began to redistribute to the dorsal surface of cells (the surface farthest from the substratum). Small aggregates of fibronectin were found on the lateral and dorsal surfaces of cells (Fig. 10*b*). Over the next 2 h, the fibronectin redistributed to large aggregates on the dorsal surfaces of cells, and many cells displayed "caps" of well-circumscribed, brightly fluorescing material confined to the tops of cells (Fig. 10*c* and *d*). These caplike aggregates appeared to have a fibrillar substructure, apparently because of compression of fibrillar strands of fibronectin. Such caps often extended 10–20 μm up from the upper surfaces of cells, but sloughed caps were never seen.

Although marked redistribution of fibronectin always occurred after antibody treatment of cultures of cells with minimal cell-cell contact, treatment of parallel cultures of confluent cells resulted in only minimal fibronectin redistribution even at high antibody concentrations (250 μg/ml). This resistance to redistribution was correlated with the formation of extensive extracellular fibrillar meshworks. For example, the fibronectin fibrils surrounding groups of cells that touched one another in subconfluent cultures remained relatively intact after antibody treatment, whereas marked redistribution of fibronectin occurred on neighboring groups of cells that were not in contact.

The redistribution process on single cells was quantitated by determining the numbers of cells with "caps" (>90–95% of fluorescent staining in a single, well-circumscribed mass over the cell nucleus), "redistributed" (>50% of the label on the dorsal surface of cells), or still diffuse (>50% on the ventral surface, although usually in disarray similar to or more than in Fig. 10*a*. The rate of redistribution of fibronectin is substantially slower than the rapid initial effects on cell morphology, and is minimal for 1 h (Fig. 11). After 3 h, 36% of the cells are "capped", and 68% have major redistribution of label.

"Capped" fibronectin was not ingested in significant amounts. After exposure of live chick embryo fibroblasts (CEF) to anti-fibronectin for 3 h, cells were fixed and made permeable to antibody with acetone. Staining with either fluorescein-la-

TABLE II

Effect of Anti-Fibronectin Antibodies on Cell Viability

Antibody	Concentration	Time	Viability
		h	%
Anti-fibronectin serum	1:10	3	98.2
Pre-immune serum	1:10	3	98.4
Affinity-purified anti-fibronectin	200 μg/ml	8	98.6
Affinity-purified control IgG	200 μg/ml	8	98.4
Affinity-purified Fab	100 μg/ml	3	98.0

Low-density chick embryo fibroblasts (2–3 × 10^4/cm^2) were incubated with antibody in regular culture medium as indicated, then assayed for viability by incubating for 10 min in 0.1% trypan blue and scoring a total of 500 cells per treatment.

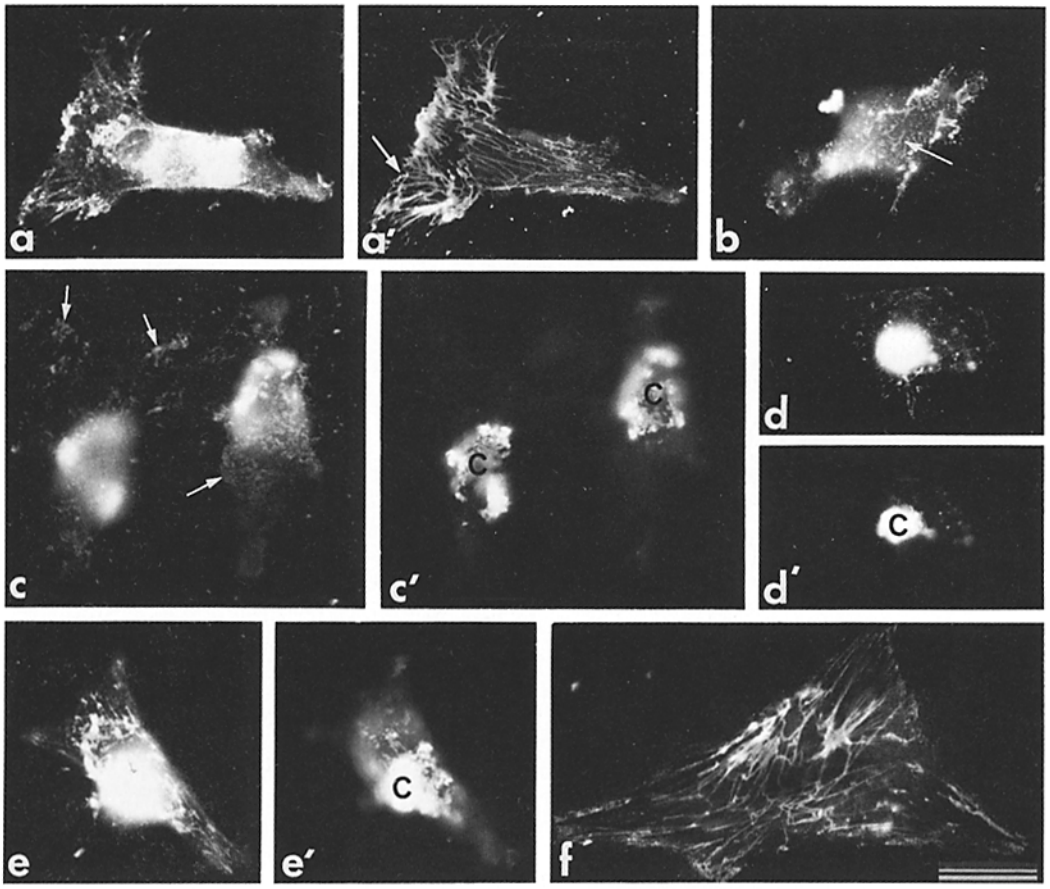

FIGURE 10 Antibody-induced redistribution of fibronectin. (*a–c*) Cells were incubated with 50 μg/ml of affinity-purified anti-fibronectin for the indicated times, fixed, and stained with anti-antibody labeled with fluorescein. (*a*) Chick fibroblast 30 min after treatment with anti-fibronectin; dark field. (*a'*) Same field, focused on substratum; immunofluorescence. (*b*) 60 min after treatment with anti-fibronectin, focused on substratum. (*c*) 120 min in anti-fibronectin; focused on substratum; note minimal amounts of fibronectin remaining under cells on substratum (arrows). (*c'*) Same field, focused on caplike structures. "*c*" on dorsal surfaces of cells. (*d*) 3 h in anti-fibronectin globulin directly coupled to fluorescein as described in Materials and Methods; focused on substratum. (*d'*) Same field, focused on caplike structure on dorsum of cell. (*e*) Cell incubated with 50 μg/ml of affinity-purified anti-fibronectin for 3 h, then extracted with acetone and incubated with fresh 50 μg/ml of anti-fibronectin, then with anti-antibody coupled to fluorescein; focused on substratum. (*e'*) Same field, focused on dorsal "cap". (*f*) Cell in 2×10^{-3} M KCN while treated with 50 μg/ml of affinity-purified anti-fibronectin, focused on substratum. Note retention of fibrillar pattern and absence of aggregates or redistribution. *c* = caplike structure. × 520. Bar, 25 μm.

beled rabbit anti-goat IgG or a sequence of anti-fibronectin, then with the second antibody, did not reveal increased intracellular fibronectin (Fig. 10*e*). Moreover, anti-fibronectin labeled directly with fluorescein is not detectable intracellularly after treatment of living chick cells for 3 h (Fig. 10*d*). Positive controls for these experiments consisted of treatment of chick cells with 100 μg/ml of fluorescein-labeled concanavalin A, which was readily ingested into intracellular vesicles.

The redistribution is inhibited by metabolic inhibitors, such as low temperature, cyanide, and partially by azide (Figs. 10*f* and 12). The experiments with cold were complicated by marked rounding of the cells, which resulted in many examples of cells that had retracted away from fibronectin, that remained on the substratum. These cells were not included in the determinations of redistribution, inasmuch as they would have resulted in an even higher apparent inhibi-

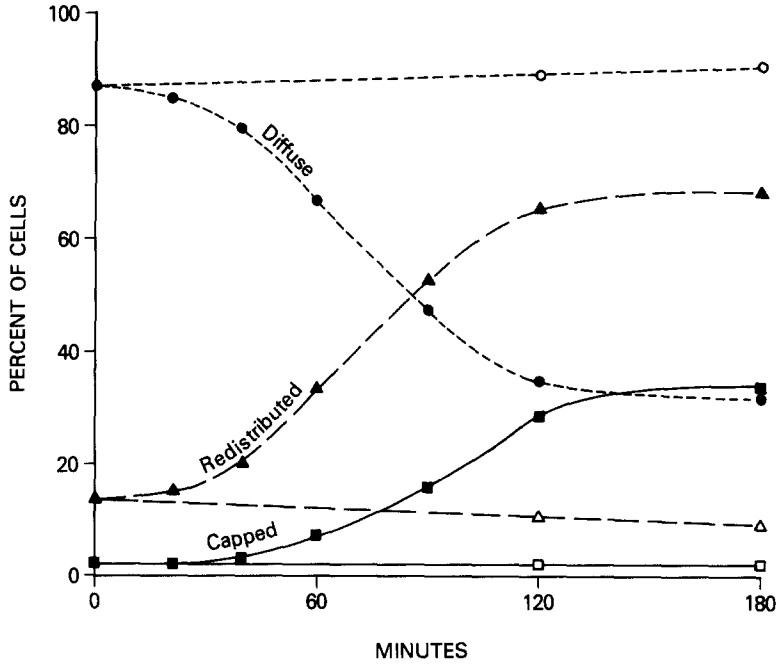

FIGURE 11 Effects of anti-fibronectin on the distribution of extracellular fibronectin. Cells in regular culture medium were treated with 50 μg/ml of affinity-purified anti-fibronectin or with control IgG, then fixed at regular intervals and stained with anti-antibody labeled with fluorescein. For each time-point, 250 cells were scored as described in the text, and the percent of cells in each category was calculated. Filled symbols indicate anti-fibronectin; open symbols indicate control IgG. (□) capped, (△) redistributed, and (○) diffuse (see text for criteria).

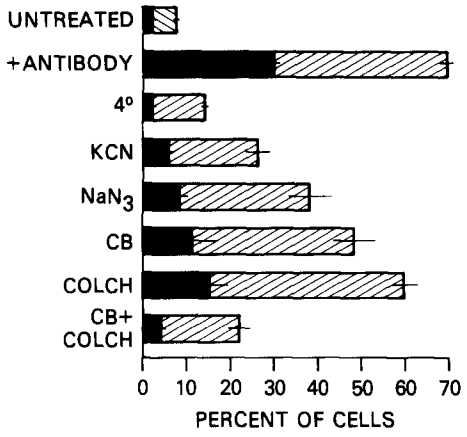

FIGURE 12 Effects of inhibitors on antibody-induced redistribution of fibronectin. Subconfluent chick fibroblasts were fixed without treatment ("untreated") or treated with 50 μg/ml affinity-purified anti-fibronectin for 3 h in the presence of the inhibitors indicated at the following concentrations: potassium cyanide, 2×10^{-3} M; sodium azide, 1×10^{-2} M; cytochalasin B, 10 μg/ml; colchicine, 1×10^{-4} M. Abscissa shows the percent of cells with caps (dark bars) or partial redistribution (cross-hatched bars).

tion of redistribution because of an apparently trivial mechanism.

The antibody-induced redistribution is inhibited only slightly by either 10 μg/ml of cytochalasin or 10^{-4} M colchicine alone. This concentration of cytochalasin resulted in cell rounding and the typical bizarre, arborized pattern of cell processes characteristic of many cytochalasin-treated cell types (51). The fibronectin patterns were consequently distorted, but 50% of the cells still possessed dorsally located fibronectin (Fig. 12). Cytochalasin-treated cells eventually lose substantial amounts of fibronectin (2, 25). Colchicine-treated cells had fewer elongated processes, and tended to be circular (46). After anti-fibronectin treatment, label was found in the characteristic dorsal locations in the majority of the cells (60%). Combining the cytochalasin and colchicine prevented redistribution (Fig. 12).

Transformation and Fibronectin

After transformation by the Schmidt-Ruppin strain of Rous sarcoma virus, antibody staining of

fibronectin is substantially reduced. Both the extracellular fibrillar and the intracellular, perinuclear granular forms of fibronectin appear to be decreased (Fig. 13*a*). The direct relationship of these alterations to transformation was examined using the temperature-sensitive ts68 strain of Rous sarcoma virus, which exhibits a transformed phenotype at 36°C and an apparently normal phenotype at 41°C (20). Fibronectin is also decreased in both extracellular and intracellular locations when cells infected by ts68 are maintained at 36°C, the temperature permissive for transformation (Fig. 13*c*).

In studies of dense cultures of chick fibroblasts infected by this virus, Wartiovaara et al. (48) reported that substantial amounts of the "SFA" (surface fibroblast antigen; 220,000- plus an apparent 150,000-dalton component) appeared to

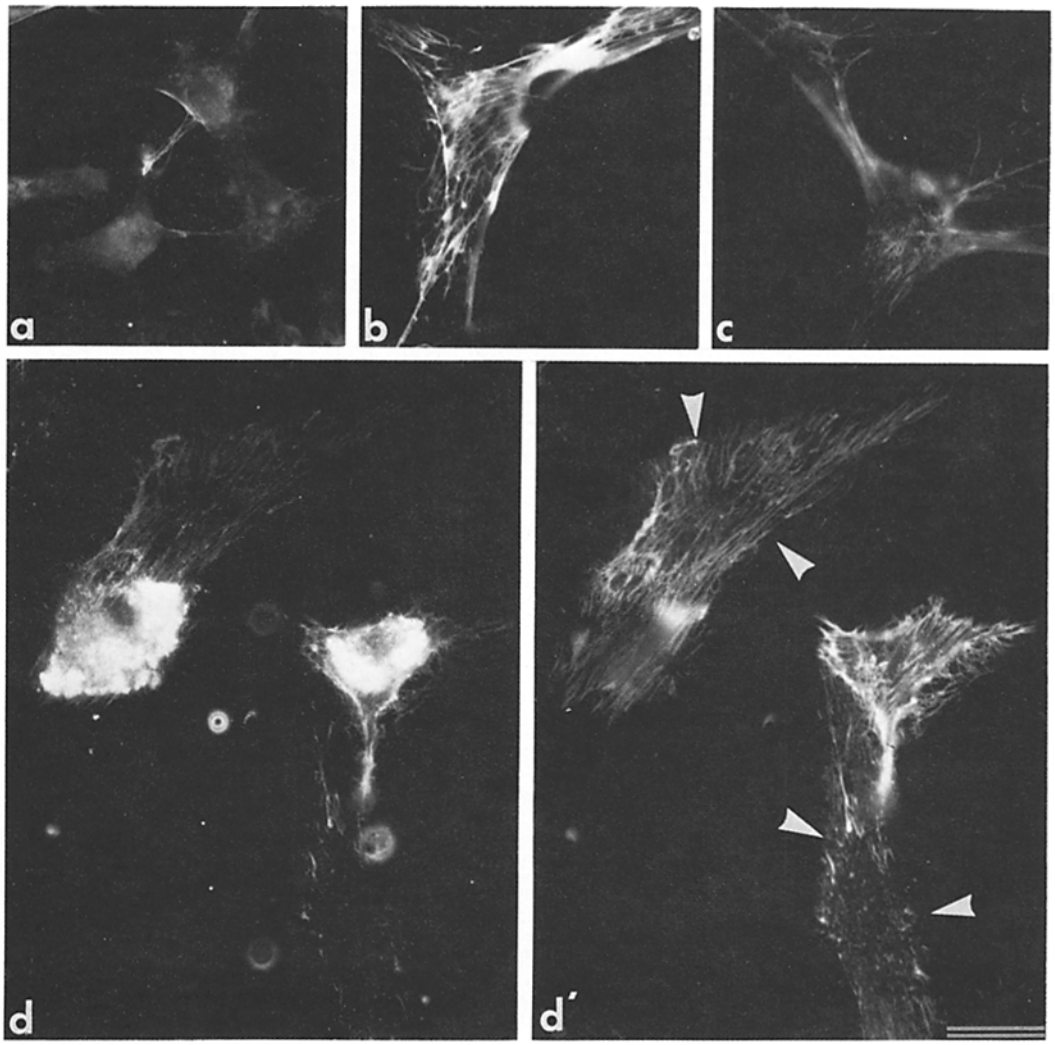

FIGURE 13 Alterations in fibronectin after transformation. Immunofluorescence microscopy. (*a*) Chick embryo fibroblasts transformed by Schmidt-Ruppin strain of Rous sarcoma virus; acetone-extracted. Note decreases in both intracellular and fibrillar extracellular fibronectin and smaller cell area compared to Fig. 5*a* (both 15-s exposures of parallel cultures). (*b*) Chick embryo fibroblasts transformed by the temperature-sensitive mutant ts68, 6 h after shift from 36° to 41°C. (*c*) Cells transformed by ts68, 48 h after shift from 41° to 36°C. (*d* and *d'*) Cells transformed by ts68, 6 h after shift from 41° to 36°C. Dark field (*d*) and immunofluorescence (*d'*). Note extensive loss of fibronectin onto substratum in trailing "tracks" (arrowheads). × 520. Bar, 25 μm.

be trapped under cells after such a temperature shift, and remained there until the cells were passaged with trypsin. Studies of sparser cultures in which it is possible to follow the fate of fibronectin from individual cells confirm and clarify these findings. Much of the fibronectin becomes dissociated from the cells during the rapid shape changes that occur upon temperature shift to the permissive temperature, and the fibronectin remains behind on the substratum while cells retract or move away (Fig. 13*d'*). This loss of fibronectin and the alterations in cell morphology are prominent 4–6 h after the temperature shift.

In contrast, cells shifted from 37° to 41°C (the temperature nonpermissive for transformation) rapidly regain fibronectin fibrils, which remain closely associated with the cells as on untransformed chick cells (Fig. 13*b'*).

Evaluation of Fibronectin "Reconstitution"

We previously showed that addition of exogenous cellular fibronectin CSP to a variety of transformed cells would restore a more normal morphology, surface architecture, and social behavior (53, 54, 60). All of the reconstituted fibronectin was on the exterior of the cell, inasmuch as gentle trypsinization removed all exogenous label (60). Similar experiments were recently reported by Ali et al. (3) using fibronectin added to a transformed hamster cell line.

The organization of this reconstituted fibronectin was examined using affinity-purified anti-fibronectin. The fibronectin is located in fibrils under and between cells, and organizes into a fibrillar extracellular matrix in confluent cells, as on normal cells (Fig. 15*c'*) (3). In sparse cultures, some amorphous aggregates are seen directly attached to the substratum in the absence of cells (Fig. 14*b*). In many instances, the fibronectin under cells is oriented towards the periphery of cells, as if holding the cell in a spread configuration (Fig. 14*b'*).

Incubation of "fibronectin-reconstituted" cells with affinity-purified anti-fibronectin results in a reversion back to a rounded cell morphology (this antibody also blocked the initial effects of fibronectin if added with the exogenous fibronectin [60]). Further incubation with the antibody resulted in redistribution or capping of the fibronectin, as on untransformed chick cells (Fig. 14*d–f*). The fibronectin is therefore appearing on the cell surface in a grossly normal pattern consistent with a role in adhesion to both cell and substratum (3, 54). Similar to normal fibronectin, reconstituted fibronectin is also subject to antibody-induced redistribution.

DISCUSSION

Specificity of the Antibody

Isolated cellular fibronectin is not soluble under conditions usually used to evaluate antibody specificity (56), which necessitated the use of modified double immunodiffusion and immunoprecipitation procedures in which fibronectin or cell homogenates are first solubilized at pH 11, then assayed at neutral pH. We have also used a new procedure for detecting antigens separated in SDS gels (31). Antisera produced against either electrophoretically or chromatographically purified fibronectin were specific for fibronectin by all of these procedures (see reference 31 and this paper). No differences in fluorescence staining patterns or in capacity to produce altered cell shape or fibronectin redistribution could be detected in comparisons of affinity-purified antibodies from either of these sources of antiserum.

There is no evidence that these antibodies can cross-react with a 150,000-dalton polypeptide (22) previously thought to associate with SF210 (fibronectin). To further ensure that the antibody was monospecific, it was affinity purified against the dimer fraction of isolated fibronectin. The antibodies used in this study did not cross-react with calf cold insoluble globulin in culture media.

Localization of Fibronectin

In cultures of chick fibroblasts at low cell density, fibronectin is localized to extracellular fibrillar structures under and between the cells, as well as to intracellular, diffusely granular structures. This intracellular localization is apparently due to recently synthesized fibronectin located in intracellular pools such as endoplasmic reticulum before export to the cell surface. By isotopic labeling procedures, such pools were found to contain ~10% of the fibronectin synthesized over 24 h (32).

Treatment of cells with the protein synthesis inhibitors cycloheximide or puromycin results in a progressive decrease in this intracellular immunofluorescent staining, which is complete in 2 h. The staining returns after removal of the protein synthesis inhibitors. These findings suggest that it will be possible to analyze the kinetics and exact pathways of secretion of this glycoprotein.

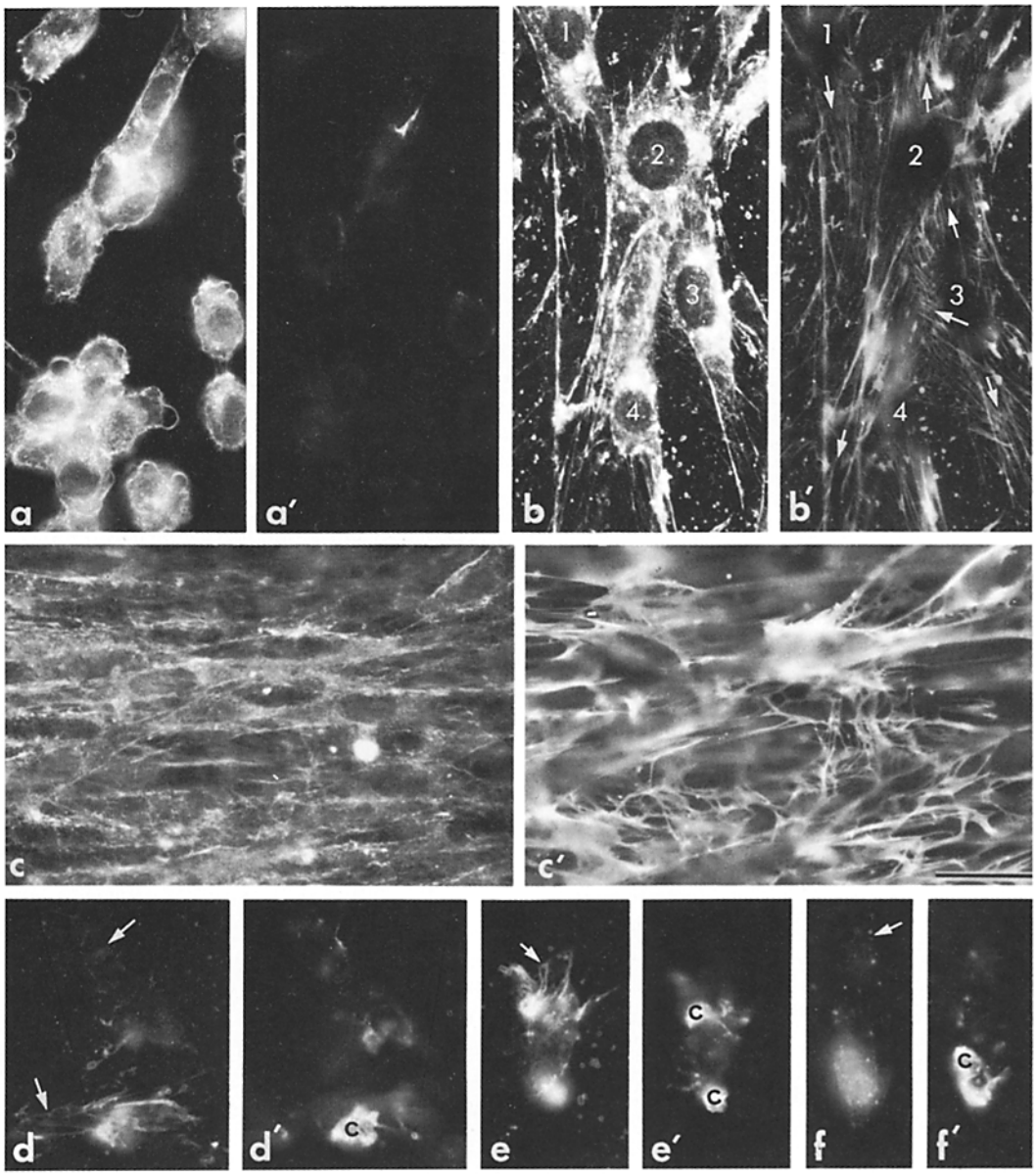

FIGURE 14 Localization and redistribution of cellular fibronectin reconstituted on transformed cells. SV1 cells were incubated with 25 μg/ml of purified fibronectin in regular culture medium for 48 h, then fixed (*b–c*) or incubated with anti-fibronectin before fixation (*d–f*). (*a–c*) Dark field. (*a'–c'*) Immunofluorescence of same fields. (*a* and *a'*) Control SV1 cells without fibronectin. (*b* and *b'*) Semiconfluent culture of cells plus fibronectin. Numbers indicate locations of nuclei. (*c* and *c'*) Confluent culture plus fibronectin. (*d*) Two cells with reconstituted fibronectin, incubated for 3 h with 50 μg/ml of affinity-purified anti-fibronectin, focused on the substratum. Arrows indicate fibrillar fibronectin remaining in cells. (*d'*) Same field as Fig. 14*d*, focused on caplike aggregates (*c*) on dorsal (upper) surface. (*e*) Cell plus 50 μg/ml of anti-fibronectin for 3 h, focused on substratum. (*e'*) Focused on dorsal surface. (*f*) Cell plus 50 μg/ml of anti-fibronectin for 3 h, focused on substratum; note minimal amounts of staining. (*f'*) Focused on dorsal surface. × 520. Bar, 25 μm.

As cells become confluent, the fibronectin bridging between cells becomes more prominent (6, 28, 44). Eventually cells are enmeshed in a complex fibrillar network. The intracellular staining in cells made permeable with acetone becomes much less prominent, and is detected only with difficulty in heavily confluent cells. These results suggest that the proportion of fibronectin on the cell surface increases at confluence, which would be consistent with the reported increases in the accessibility of LETS protein (galactoprotein a) of Nil cells to surface labeling procedures at confluence (11, 16) as well as the greater recoveries of fibronectin after treatment of confluent cells with 0.2 M urea (58).

Vaheri, Ruoslahti, and co-workers have extensively studied a cell surface antigen called "SFA" (22, 48). This antigen was initially identified using antibody prepared against papain digests of cell surface proteins. The SFA reportedly consisted of at least two cellular proteins of apparent mol wt 150,000 and 210,000 on SDS gels, which were thought to contain a common antigenic determinant. More recent work from that laboratory has utilized antibody against the human plasma protein CIg, which is reportedly immunologically indistinguishable from SFA. These antibodies also localize to complex extracellular fibrillar patterns and intracellular granular staining in cultured fibroblasts (39, 44). Mautner and Hynes (28) have recently described similar extracellular fibrillar patterns on hamster Nil cells.

The localization of fibronectin between cells and substratum as well as between cells is consistent with a role as an adhesive protein helping to maintain normal cell morphology (6, 28, 55). The similar localization of artificially reconstituted fibronectin on the surfaces of fibronectin-treated, morphologically "reverted" transformed cells also supports this hypothesis.

Antibody Effects on Cell Morphology

Another test of the role of fibronectin in cell shape is to treat living cells with anti-fibronectin and to determine its effects on cell morphology and adhesion. Treatment of chick embryo fibroblasts with affinity-purified anti-fibronectin results in rapid cell rounding and a blunting of cell processes that is nearly complete in 20 min. The effects are specific and reversible. The resultant cell morphology is similar to that of chick fibroblasts transformed by Rous sarcoma virus and to that of many other transformed cells that are deficient in cellular fibronectin.

These effects of anti-fibronectin are separable from the "capping" process (see below) by differences in kinetics (20 min vs. 2–3 h). In addition, monovalent antibody also produces the same rapid effects on cell morphology. The effects of anti-fibronectin on cell morphology are consistent with the notion that cell surface fibronectin is a major adhesive molecule of chick fibroblasts. However, the partial residual adhesiveness of chick cells treated with anti-fibronectin or transformed by Rous sarcoma virus (neither of which results in cell detachment) suggests that other cell-substratum adhesive mechanisms also exist in these cells.

Antibody-Induced Redistribution of Fibronectin

The redistribution of fibronectin in the presence of anti-fibronectin is similar to "capping" of concanavalin A and histocompatibility antigens on lymphocytes (29, 42) or on transformed fibroblastic cells (10, 41) in that it results in a well-circumscribed, supranuclear "cap" of antigen-antibody complexes. Fibronectin redistribution appears to be significantly slower than these other types of capping (2–3 h vs. 5 min on lymphocytes and 20–40 min on transformed cells). However, capping of a recently isolated glucose-regulated glycoprotein on untransformed fibroblastic cells also required antibody treatment for 2–3 h (36).

"Capping" of fibronectin occurred only on isolated cells and did not occur after fibronectin formed an extracellular matrix as cells grew to higher population densities, suggesting that formation of extensive cell-cell and cell-matrix attachments results in a stable meshwork of fibronectin that is resistant to reorganization. In contrast to lymphocytes, fibronectin redistribution on fibroblasts was not accompanied by detectable ingestion of antibody-antigen complexes. However, the redistribution of fibronectin was similar to lymphocyte capping in its sensitivity to metabolic inhibitors, and to the combination of colchicine and cytochalasin.

However, such antibody-induced redistribution was not found with hamster Nil cells (28). In contrast, fibronectin fibrils of human fibroblasts were also recently found to be disrupted by treatment with antibodies against human cold insoluble globulin, resulting in "patches" but not in capping (26). Whether that redistribution requires metabolic processes or is directly analogous to the

patching of lymphocytes, is not yet known.

The extensive antibody-induced redistribution of fibronectin on chick embryo cells indicates that fibronectin can be translocated laterally in the plane of the membrane. This redistribution involves a process requiring metabolic energy. Isolation of fibronectin "caps" might possibly provide information about plasma membrane binding sites for fibronectin, and the mechanisms of capping.

It is important to note that direct measurements of the lateral mobility of fibronectin showed that fibronectin is relatively immobile compared with other membrane-associated proteins that are rapidly mobile (38). However, the present results indicate that slow, directed movement of fibronectin will eventually occur in the presence of antibody. Whether the antibody-induced redistribution is due to bulk membrane flow (1) or to submembranous, cytochalasin- and colchicine-sensitive microfilament and microtubule complexes (5, 9, 29, 51) remains to be determined.

It is also important to note that a converse type of relationship between adhesive fibronectin molecules and the organization and distribution of microfilament bundles of cells may also exist. Treatment of untransformed cells with proteases that hydrolyze fibronectin results in a loss of microfilament bundles, and the reconstitution of fibronectin on transformed fibroblasts missing this protein results in a restoration of microfilament bundles (3, 52).

Transformation and Fibronectin

As reported previously by Wartiovaara et al. (48), who utilized antibodies against the "SFA" complex, antibody staining for extracellular fibronectin is decreased after viral transformation of chick fibroblasts. The decreases we find in both the intracellular and the extracellular pools of antigenic fibronectin staining are consistent with the decreases in both pools previously reported using radioactive labeling procedures (32). These decreases in isotopic labeling were due to (*a*) decreased biosynthesis (the major factor), (*b*) increased protein degradation, and (*c*) increased loss of intact fibronectin from the cell surface into the culture medium. The loss of fibronectin from the cell body to the substratum seen in this paper using fluorescent antibody procedures soon after shifting cells infected with ts68 to the temperature permissive for transformation may represent the last factor (*c*), which may play a significant role in the cell shape changes after temperature shift (18, 45). Such a decreased capacity of cells to bind cellular fibronectin may account for examples of incomplete restoration of normal cell morphology to certain transformed cell lines in previous reconstitution experiments (54, 60). These results suggest that it may be useful to search for cellular receptors for fibronectin that are altered after transformation.

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REFERENCES

1. ABERCROMBIE, M., J. E. M. HEAYSMAN, and S. M. PEGRUM. 1970. The locomotion of fibroblasts in culture. III. Movements of particles on the dorsal surface of the leading lamella. Exp. Cell Res. **62**:389–398.
2. ALI, I. U., and R. O. HYNES. 1977. Effects of cytochalasin B and colchicine on attachment of a major surface protein of fibroblasts. *Biochim. Biophys. Acta.* **471**:16–24.
3. ALI, I. U., V. MAUTNER, R. LANZA, and R. O. HYNES. 1977. Restoration of normal morphology, adhesion and cytoskeleton in transformed cells by addition of a transformation sensitive surface protein. *Cell.* **11**:115–126.
4. ANTOINE, J.-C., S. AVRAMEAS, N. K. GONATAS, A. STIEBER, and J. O. GONATAS. 1974. Plasma membrane and internalized immunoglobulins of lymph node cells studied with conjugates of antibody or its Fab fragments with horseradish peroxidase. *J. Cell Biol.* **63**:12–23.
5. BOURGUIGNON, L. Y. W., and S. J. SINGER. 1977. Transmembrane interactions and the mechanism of capping of surface receptors by their separate ligands. *Proc. Natl. Acad. Sci. U. S. A.* **74**:5031–5035.
6. CHEN, L. B., P. H. GALLIMORE, and J. K. McDOUGALL. 1976. Correlation between tumor induction and cell surface LETS protein. *Proc. Natl. Acad. Sci. U. S. A.* **73**:3570–3574.
7. CHEN, L. B., N. MAITLAND, P. H. GALLIMORE, and J. K. McDOUGALL. 1977. Detection of the large external transformation-sensitive protein on some epithelial cells. *Exp. Cell Res.* **106**:39–46.
8. DAVIES, P., Y. SHIZUTA, K. OLDEN, M. GALLO, and I. PASTAN. 1977. Phosphorylation of filamin and other proteins in cultured fibroblasts. *Biochem. Biophys. Res. Commun.* **74**:300–307.

9. EDELMAN, G. M. 1976. Surface modulation in cell recognition and cell growth. *Science* (*Wash. D. C.*). **192:**218–226.
10. EDIDIN, M., and A. WEISS. 1972. Antigen cap formation in cultured fibroblasts: a reflection of membrane fluidity and cell motility. *Proc. Natl. Acad. Sci. U. S. A.* **69:**2456–2459.
11. GAHMBERG, C. G., D. KIEHN, and S.-I. HAKOMORI. 1974. Changes in a surface-labeled galactoprotein and in glycolipid concentrations in cells transformed by a temperature-sensitive polyoma virus mutant. *Nature* (*Lond.*). **248:**413–415.
12. GALLIMORE, P. H., J. K. MCDOUGALL, and L. B. CHEN. 1977. In vitro traits of adenovirus-transformed cell lines and their relevance to tumorigenicity in nude mice. *Cell.* **10:**669–678.
13. GRINNELL, F., D. G. HAYS, and D. MINTER. 1977. Cell adhesion and spreading factor: partial purification and properties. *Exp. Cell Res.* **110:**175–190.
14. HYNES, R. O. 1973. Alteration of cell-surface proteins by viral transformation and by proteolysis. *Proc. Natl. Acad. Sci. U. S. A.* **70:**3170–3174.
15. HYNES, R. O. 1976. Cell surface proteins and malignant transformation. *Biochim. Biophys. Acta.* **458:**73–107.
16. HYNES, R. O., and J. M. BYE. 1974. Density and cell cycle dependence of cell surface proteins in hamster fibroblasts. *Cell.* **3:**113–120.
17. HYNES, R. O., A. T. DESTREE, and V. MAUTNER. 1976. Spatial organization at the cell surface. *In* Membranes and Neoplasia: New Approaches and Strategies. V. T. Marchesi, editor. Alan R. Liss, Inc., New York. 189–202 pp.
18. HYNES, R. O., and J. A. WYKE. 1975. Alterations in surface proteins in chicken cells transformed by temperature-sensitive mutants for Rous sarcoma virus. *Virology.* **64:**492–504.
19. JOHNSON, G. D., and E. J. HOLBOROW. 1973. Immunofluorescence. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications, Oxford. 2nd edition. 18.1–18.20 pp.
20. KAWAI, S., and H. HANAFUSA. 1971. The effects of reciprocal changes in temperature on the transformed state of cells infected with a Rous sarcoma virus mutant. *Virology.* **46:**470–479.
21. KESKI-OJA, J., D. F. MOSHER, and A. VAHERI. 1976. Dimeric character of fibronectin, a major fibroblast surface-associated glycoprotein. *Biochem. Biophys. Res. Commun.* **74:**699–706.
22. KESKI-OJA, J., A. VAHERI, and E. RUOSLAHTI. 1976. Fibroblast surface antigen (SF): the external glycoprotein lost in proteolytic stimulation and malignant transformation. *Int. J. Cancer.* **17:**261–269.
23. KLEBE, R. J. 1974. Isolation of a collagen-dependent cell attachment factor. *Nature* (*Lond.*). **250:**248–251.
24. KLEINMAN, H. K., E. B. MCGOODWIN, and R. J. KLEBE. 1976. Localization of the cell attachment region in types I and II collagens. *Biochem. Biophys. Res. Commun.* **72:**426–432.
25. KURKINEN, M., and A. VAHERI. 1977. Fibronectin matrix: antibody-induced reorganization in human fibroblast cultures. *Cell Biology International Reports.* **1:**469–475.
26. KURKINEN, M., J. WARTIOVAARA, and A. VAHERI. 1978. Cytochalasin B releases a major surface-associated glycoprotein, fibronectin, from cultured fibroblasts. *Exp. Cell Res.* **111:**127–137.
27. LINDER, E., A. VAHERI, E. RUOSLAHTI, and J. WARTIOVAARA. 1975. Distribution of fibroblast surface antigen in the developing chick embryo. *J. Exp. Med.* **142:**41–49.
28. MAUTNER, V., and R. O. HYNES. 1977. Surface distribution of LETS protein in relation to the cytoskeleton of normal and transformed cells. *J. Cell Biol.* **75:**743–768.
29. NICOLSON, G. L. 1976. Transmembrane control of the receptors on normal and tumor cells. I. Cytoplasmic influence over cell surface components. *Biochim. Biophys. Acta.* **457:**57–108.
30. NOTKINS, A. L., M. MAGE, W. K. ASHE, and S. MAHAR. 1968. Neutralization of sensitized lactic dehydrogenase virus by anti-γ-globulin. *J. Immunol.* **100:**314–320.
31. OLDEN, K., and K. M. YAMADA. 1977. Direct detection of antigens in sodium dodecyl sulfate polyacrylamide gels. *Anal. Biochem.* **78:**483–490.
32. OLDEN, K., and K. M. YAMADA. 1977. Mechanism of the decrease in the major cell surface protein of chick embryo fibroblasts after transformation. *Cell.* **11:**957–969.
33. PASTAN, I., M. WILLINGHAM, K. M. YAMADA, J. POUYSSÉGUR, P. DAVIES, and I. KLEIN. 1977. Biochemical basis of the morphologic phenotype of transformed cells. *In* International Cell Biology 1976–1977. B. R. Brinkley and K. R. Porter, editors. The Rockefeller University Press, New York. 161–167 pp.
34. PEARLSTEIN, E. 1976. Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. *Nature* (*Lond.*). **262:**497–500.
35. PORTER, R. R. 1959. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. *Biochem. J.* **73:**119–127.
36. POUYSSÉGUR, J., and K. M. YAMADA. 1978. Isolation and immunologic characterization of a glucose-regulated fibroblast cell surface glycoprotein (GRP) and its non-glycosylated precursor. *Cell.* **13:**139–150.
37. RUOSLAHTI, E., and A. VAHERI. 1975. Interaction of soluble fibroblast surface antigen with fibrinogen and fibrin. Identity with cold insoluble globulin of human plasma. *J. Exp. Med.* **141:**497–501.
38. SCHLESSINGER, J., L. S. BARAK, G. G. HAMMES, K. M. YAMADA, I. PASTAN, W. W. WEBB, and E.

L. ELSON. 1977. Mobility and distribution of a cell surface glycoprotein and its interaction with other membrane components. *Proc. Natl. Acad. Sci. U. S. A.* **74:**2909–2913.

39. STENMAN, S., J. WARTIOVAARA, and A. VAHERI. 1977. Changes in the distribution of a major fibroblast protein, fibronectin, during mitosis and interphase. *J. Cell Biol.* **74:**453–467.
40. STUDIER, F. W. 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J. Mol. Biol.* **79:**237–248.
41. SUNDQVIST, K. G. 1972. Redistribution of surface antigens—a general property of animal cells? *Nat. New Biol.* **239:**147–149.
42. TAYLOR, R. B., W. P. H. DUFFUS, M. C. RAFF, and S. DE PETRIS. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin. *Nat. New Biol.* **233:**225–229.
43. TENG, N. N. H., and L. B. CHEN. 1975. The role of surface proteins in cell proliferation as studied with thrombin and other proteases. *Proc. Natl. Acad. Sci. U. S. A.* **72:**413–417.
44. VAHERI, A., and D. F. MOSHER. 1978. High molecular weight, cell surface-associated glycoprotein (fibronectin) lost in malignant transformation. *Biochim. Biophys. Acta.* In press.
45. VAHERI, A., and E. RUOSLAHTI. 1975. Fibroblast surface antigen produced but not retained by virus-transformed human cells. *J. Exp. Med.* **142:**530–535.
46. VASILIEV, JU. M., I. M. GELFAND, L. V. DOMNINA, O. Y. IVANOVA, S. G. KOMM, and L. V. OLSHEVSKAJA. 1970. Effect of Colcemid on the locomotory behavior of fibroblasts. *J. Embryol. Exp. Morphol.* **24:**625.
47. VOGT, P. K. 1969. Focus assay of Rous sarcoma virus. *In* Fundamental Techniques in Virology. K. Habel and N. Salzman, editors. Academic Press, Inc., New York. 198–211 pp.
48. WARTIOVAARA, J., E. LINDER, E. RUOSLAHTI, and A. VAHERI. 1974. Distribution of fibroblast surface antigen. Association with fibrillar structures of normal cells and loss upon viral transformation. *J. Exp. Med.* **140:**1522–1533.
49. WEBER, K., and D. J. KUTER. 1971. Reversible denaturation of enzymes by sodium dodecyl sulfate. *J. Biol. Chem.* **246:**4504–4509.
50. WEINER, A. M., T. PLATT, and K. WEBER. 1972. Amino-terminal sequence analysis of proteins purified on a nanomole scale by gel electrophoresis. *J. Biol. Chem.* **247:**3242–3251.
51. WESSELLS, N. K., B. S. SPOONER, J. F. ASH, M. O. BRADLEY, M. A. LUDUENA, E. L. TAYLOR, J. T. WREHN, and K. M. YAMADA. 1971. Microfilaments in cellular and developmental processes. *Science (Wash. D. C.).* **171:**135–143.
52. WILLINGHAM, M. C., K. M. YAMADA, S. S. YAMADA, J. POUYSSEGUR, and I. PASTAN. 1977. Microfilament bundles and cell shape are related to adhesiveness to substratum and are dissociable from growth control in cultivated fibroblasts. *Cell.* **10:**375–380.
53. YAMADA, K. M., S. H. OHANIAN, and I. PASTAN. 1976. Cell surface protein decreases microvilli and ruffles on transformed mouse and chick cells. *Cell.* **9:**241–245.
54. YAMADA, K. M., K. OLDEN, and I. PASTAN. 1978. Transformation-sensitive cell surface protein: isolation, characterization, and role in cell morphology and adhesion. *Ann. N.Y. Acad. Sci.* In press.
55. YAMADA, K. M., and I. PASTAN. 1976. Cell surface protein and neoplastic transformation. *Trends Biochem. Sci.* **1:**222–224.
56. YAMADA, K. M., D. H. SCHLESINGER, D. S. KENNEDY, and I. PASTAN. 1977. Characterization of a major fibroblast cell surface protein. *Biochemistry.* **16:**5552–5559.
57. YAMADA, K. M., B. S. SPOONER, and N. K. WESSELLS. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* **49:**614–635.
58. YAMADA, K. M., and J. A. WESTON. 1974. Isolation of a major cell surface glycoprotein from fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **71:**3492–3496.
59. YAMADA, K. M., S. S. YAMADA, and I. PASTAN. 1975. The major cell surface glycoprotein of chick embryo fibroblasts is an agglutinin. *Proc. Natl. Acad. Sci. U. S. A.* **72:**3158–3162.
60. YAMADA, K. M., S. S. YAMADA, and I. PASTAN. 1976. Cell surface protein partially restores morphology, adhesiveness and contact inhibition of movement to transformed fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **73:**1217–1221.
61. YAMADA, K. M., S. S. YAMADA, and I. PASTAN. 1977. Quantitation of a transformation-sensitive, adhesive cell surface glycoprotein: decrease on several untransformed permanent cell lines. *J. Cell Biol.* **74:**649–654.