Expression of Chicken Vinculin Complements the Adhesion-defective Phenotype of a Mutant Mouse F9 Embryonal Carcinoma Cell

Michael Samuels,* Robert M. Ezzell,‡ Timothy J. Cardozo,‡ David R. Critchley,§ Jean-Luc Coll,* and Eileen D. Adamson*

*La Jolla Cancer Research Foundation, La Jolla, California 92037; ‡Surgery Research Laboratory, Department of Anatomy and Cellular Biology, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129; and §Department of Biochemistry, University of Leicester, Leicester, LE1 7RH, United Kingdom

Abstract. A mutant cell line, derived from the mouse embryonal carcinoma cell line F9, is defective in cell-cell adhesion (compaction) and in cell-substrate adhesion. We have previously shown that neither uvomorulin (E-cadherin) nor integrins are responsible for the mutant phenotype (Calogero, A., M. Samuels, T. Darland, S. A. Edwards, R. Kemler, and E. D. Adamson. 1991. *Dev. Biol.* 146:499–508). Several cytoskeleton proteins were assayed and only vinculin was found to be absent in mutant (5.51) cells. A chicken vinculin expression vector was transfected into the 5.51 cells together with a neomycin-resistance vector. Clones that were adherent to the substrate were selected in medium containing G418. Two clones, 5.51Vin3 and Vin4, were analyzed by Nomarski differential interference contrast and laser confocal microscopy as well as by biochemical and molecular biological techniques. Both clones adhered well to substrates and both exhibited F-actin stress fibers with vinculin localized at stress fiber tips in focal contacts. This was in marked contrast to 5.51 parental cells, which had no stress fibers and no vinculin. The mutant and complemented F9 cell lines will be useful models for examining the complex interactions between cytoskeletal and cell adhesion proteins.

EARLIER studies have established that the cytoplasmic domains of two transmembrane glycoproteins, uvomorulin (Nagafuchi and Takeichi, 1988) and β1 integrins (Hayashi et al., 1990; Marcantonio et al., 1990; Reszka et al., 1992) are essential for transmembrane linkage to the cytoskeleton and for cell adhesion. The former glycoprotein links cells together in tight aggregates and epithelial sheets, the latter attaches cells to the extracellular matrix. Neither activity can be maintained without a set of interlinked cytoplasmic accessory proteins that are actively disassembled and reassembled onto the F-actin cytoskeleton. Uvomorulin, catenins α, β, γ (Kemler and Ozawa, 1989) and plakoglobin (Cowin et al., 1986) are components unique to the zonulae adherens junctions between cells. Others such as α-actinin, tenuin, vinculin, zyxin, are common to both zonulae adherens and cell-substrate plaques. Yet other proteins are unique to cell-substrate locations; certain integrins, fimbrin, paxillin, talin, and tensin for example (Geiger and Ginsberg, 1991).

We have previously described an F9 mutant cell line that is defective in both cell–cell and cell–matrix adhesion (Grover et al., 1987). The F9 att-5.51 line (called 5.51 hereafter) was produced by ethylmethanesulfonate mutagenesis, a process that can lead to a single base change in the genome (Ingle and Drinkwater, 1989) or to a wide range of mutational types (Wood and Moses, 1989). Unlike wild-type F9 cells, 5.51 cells cannot differentiate into an epithelial layer after treatment of cell aggregates with retinoic acid (5 nM RA), probably because they cannot aggregate into tight cell masses. Instead, they grow in suspension even in tissue culture dishes as floating, loose and uncompacted aggregates. RA treatment of 5.51 cells results in only parietal-type endoderm differentiation; epithelial layers of polarized visceral endoderm cells are not seen (Grover et al., 1987).

We have attempted to define the mechanism underlying the inability of the mutant cell line to undergo compaction by studying the expression of uvomorulin in 5.51 cells. Uvomorulin was expressed at 30–50% wild-type F9 levels in mutant 5.51 cells while the mRNA was barely detectable because it was highly unstable (Adamson et al., 1990). When uvomorulin was overexpressed in 5.51 cells, the cells aggregated better but did not form an epithelial layer of visceral endoderm cells and did not adhere to a substrate (Calogero et al., 1991). In short, the replacement of uvomorulin did not complement the cell–cell mutant phenotype.

We examine here the expression of several cytoskeletal

Address correspondence to Dr. Adamson at La Jolla Cancer Research Foundation, 10901 North Torrey Pines Road, La Jolla, CA 92037.

M. Samuels and Dr. Ezzell contributed equally to this work.

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proteins in 5.51 cells and find that, whereas some minor differences can be found in the expression of several proteins in mutant cells, vinculin is completely missing. We present evidence that this is the fundamental cause of the aberrant cell-cell and cell-matrix interactions displayed by 5.51 cells.

Vinculin (M_r 117 kD) localizes at focal contacts (Geiger, 1979; Burridge and Feramisco, 1980; Geiger et al., 1980) and intercellular adherens junctions (Geiger et al., 1981; Geiger and Ginsberg, 1991). Therefore, any cell that makes contact with other cells or with substratum, expresses vinculin. By inference, this includes all anchorage-dependent cultured cells (Otto, 1990). Vinculin is evolutionarily highly conserved among nematodes (Barstead and Waterston, 1989), birds (Price et al., 1989), and man (Weller et al., 1990). The structural and functional domains of vinculin have been summarized recently (Westmeyer et al., 1990; Critchley et al., 1991). There is a single gene, but a difference in splicing gives rise to the 150-kD metavinculin variant in muscle cells (Koteliansky et al., 1992). We describe here the results of the transfection of an expression vector coding for chicken vinculin into the mouse 5.51 cell line and the isolation of several clones that express different levels of vinculin protein. The conclusions are: (*a*) that the mutant phenotype is largely corrected, because both compaction and substrate adhesion are significantly improved; (*b*) actin stress fibers lacking in the mutant line are restored in the transfected lines; (*c*) cell surface topography and motility are restored in clones that express moderate amounts of exogenous vinculin protein but not in those that express very high levels. Our results suggest that subtle and complex regulatory interactions with other components in the cytoskeletal network are necessary for normal function.

Materials and Methods

Cell Culture

Wild-type F9 BIM cells were cultured as described earlier (Grover et al., 1983). The mutant F9 cell line was cultured in suspension in the same medium (Grover et al., 1987). Transfected cell lines are designated as 5.51UMI and 5.51Vin for clones expressing uvomorulin or vinculin, respectively. P19 embryonal carcinoma cells are cultured similarly to F9 cells but in noncoated dishes (McBurney et al., 1982).

Antibodies Used in Immunoblotting and Immunoprecipitation

Rabbit antibodies to uvomorulin, α-fodrin, and ankyrin were kindly provided by R. Kemler (Max Planck Institut, Freiburg, Germany), E. Repasky (Roswell Park Cancer Institute, Buffalo, NY) (Black et al., 1988), A. Dutton (University of California, San Diego, CA) and W. J. Nelson (Stanford University, Stanford, CA) (Nelson and Veshnock, 1986), respectively. Rabbit anti-talin antisera were from K. Burridge (University of North Carolina, Chapel Hill, NC) and M. C. Beckerle (University of Utah, Salt Lake City, UT). Anti-α-actinin was from A. Dutton and S. J. Singer (University of California, San Diego, CA). Three anti-vinculin antibodies were used: for immunoprecipitation, a polyclonal rabbit antibody to porcine smooth muscle vinculin from A. Dutton; for immunoblotting, mouse mAb V4505 (anti-chicken) and V9131 (anti-human) vinculin from Sigma Chemical Co. (St. Louis, MO). Anti-filamin has been described previously (Ezzell et al., 1988).

Immunofluorescence and Confocal Microscopy

F9 cells were cultured for 24 h (48 h for 5.51 mutant and 5.51Vin-clones 3 and 4) on polylysine-coated coverslips. Coverslips with attached cells were fixed for 8 min in 4% paraformaldehyde in PBS and permeabilized for 2 min in 0.2% Triton X-100 in PBS. After rinsing, the coverslips were incubated for 1 h in a blocking solution containing 3% BSA (Sigma Chemical Co.) and 1% normal donkey serum (Jackson Immunoresearch Labs, Inc., West Grove, PA). Wild-type F9 cells were stained with mouse anti-human smooth muscle vinculin (diluted 1:50, Chemicon International, Inc., Temecula, CA, catalog number MAB1624), and 5.51Vin-clones 3 and 4 were stained with mouse anti-chicken gizzard smooth muscle vinculin (diluted 1:10, Sigma Chemical Co., catalog number VIN-11-5) for 1 h. Both antibodies were diluted in blocking solution. The coverslips were washed (four changes, 15 min each), and incubated for 1 h in fluorescein (DTAF)-conjugated $F(ab')_2$ fragment of donkey anti-mouse IgG (diluted 1:200 in blocking solution, Jackson Immunoresearch Labs). The coverslips were washed overnight and then stained with rhodamine-phalloidin (diluted 1:100, Molecular Probes, Inc., Eugene, OR) to visualize F-actin. After washing for 15 min, the coverslips were mounted in a drop of 1 mg/ml p-phenylenediamine (Sigma Chemical Co.) to reduce photobleaching in 90% glycerol/PBS, pH 8.5. All specimens were examined in a confocal microscope (BioRad MRC 600 [BioRad Microsciences, Cambridge, MA]) attached to a Zeiss Axiovert 35 with a 100x Plan-Neofluar objective. Confocal images were collected and digitally stored using photon-counting Biorad COMOS software.

Time-lapse Video Microscopy

For time-lapse video microscopy, F9 cells were cultured on 25 mm circular coverslips coated with gelatin (for wild-type and 5.51 Vin clones 3 and 4) or polylysine (for 5.51 cells). The cells were maintained in a Leiden coverslip chamber (Medical Systems Corporation, Greenvale, NY) and examined in a Zeiss Axiovert 10 inverted microscope using a 100x Plan-Neofluar objective and Nomarski differential interference contrast (DIC)[1] optics. A Zeiss environmental microscope chamber was used to maintain the cell cultures at 37°C and 5% CO_2. Video images obtained with a Hamamatsu C2400 Newvicon camera were recorded onto a Sony LVR 5000 laserdisk recorder at 15-s intervals.

Plasmids

(*a*) For probing Northern blots, a 3-kb human vinculin cDNA, HV6, was used (Weller et al., 1990) after labeling by the random oligonucleotide method (Feinberg and Vogelstein, 1984). (*b*) An expression vector encoding full-length chicken cDNA was constructed from cVin1 and cVin5 (Bendori et al., 1989). The promoter was the Simian Virus-40 late promoter derived from pSVL. (*c*) A plasmid containing the 1.2-kb EcoRI fragment of the mouse vinculin cDNA (Ben-Ze'ev et al., 1990) in Bluescript KS (Stratagene Corp., San Diego, CA) was linearized with BamHI and cRNA of 220 bp corresponding to nts 1435–1649 of vinculin (Price et al., 1989) was synthesized from the T3 promoter according to the method of Melton et al. (1987). This was used in ribonuclease protection assays together with a probe that detects ribosomal protein mRNA, L32, used as a control for equal loading of RNAs, as described by Darland et al. (1991). The protected fragment of mouse vinculin RNA was 205 bp.

Transfections

Mutant F9 cells grow as loose aggregates in suspension. In contrast, we might expect to find adherent cells if vinculin expression rescued the inability to adhere. Dishes (10 cm) containing 1-2 × 10^6 suspended 5.51 cells were treated with 18 μg plasmid DNA as a mixture of 15 μg pSVL-cVIN (expressing chicken vinculin cDNA) and 3 μg pSV2neo (for G418 resistance). The method described by Graham et al. (1974) was followed and colonies resistant to 0.4 mg/ml G418 were selected in tissue culture dishes that were coated with gelatin. Clones that could adhere to the substratum were selected as well as less-adherent cell clones. Two clones, 5.51Vin3 and 4, are described in the most detail.

RNA Extraction and Analysis

RNA was extracted from cells as described by Chirgwin et al. (1974). Northern analysis was performed on formaldehyde-denaturing gels (Maniatis et al., 1982) with 20 μg of total RNA, after transfer to Nytran membranes (Schleicher and Schuell, Inc., Keene, NH). RNase protection assays were as described above with 50 μg total RNA.

1. *Abbreviations used in this paper*: DIC, differential interference contrast.

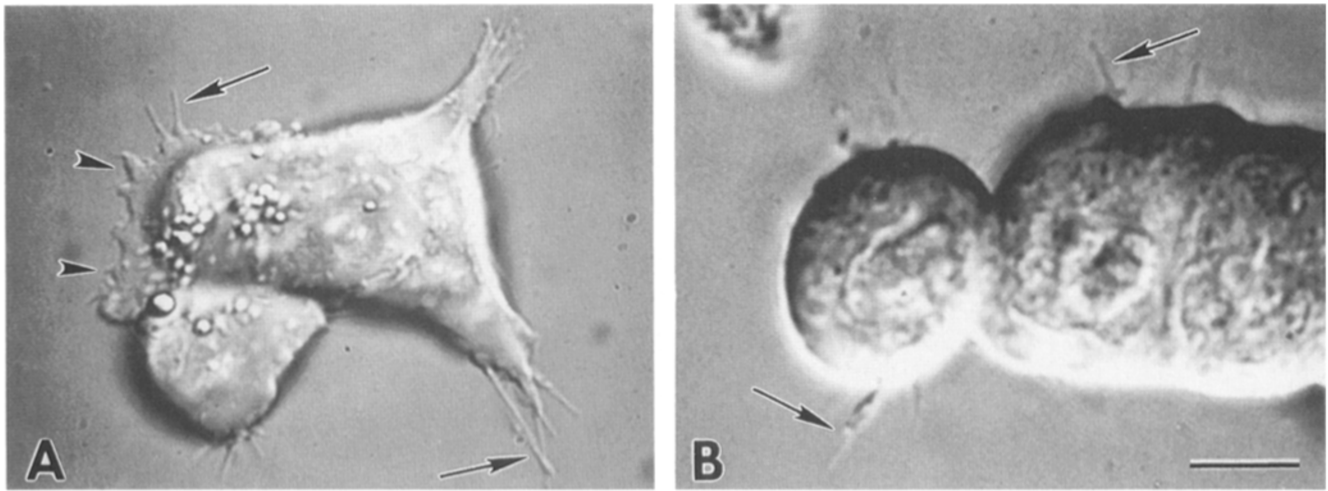

Figure 1. Morphology of wild-type F9 (*A*) and 5.51 (*B*) cells examined with Nomarski differential interference contrast optics. The wild-type cell is well-spread and has filopodia (*arrows*) and lamellipodia (*arrowheads*). In contrast. 5.51 cells are rounded and have only filopodia (*arrows*). 5.51 cells also grow as loose strings or clusters of non-adherent cells. Bar, 10 μm for *A*, and 6 μm for *B*.

Radiolabeling and Immunoprecipitation

Radiolabeling and immunoprecipitation were performed as in Darland et al. (1991). In brief, cells were labeled for 1 or 2 h in methionine-free medium containing 200 μCi/ml [^{35}S]methionine and cysteine (ICN ImmunoBiologicals, Lisle, IL, TranSlabel >1,000 Ci/mmol). Cells were lysed in RIPA buffer (1% deoxycholate, 1% Nonidet 40, 0.1% SDS, 25 mM Tris pH 7.5, 0.15 M NaCl, with protease inhibitors: 1 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin) and aliquots containing equal amounts of radioactive proteins were subjected to immunoprecipitation. Fixed *Staphylococcus aureus* (Boehringer-Mannheim Corp., Indianapolis, IN) precipitated complexes were analyzed by SDS-PAGE on 7 or 5% polyacrylamide gels. The radioactive proteins were detected by fluorography.

Immunoblotting

Known numbers of cells (10^5 to 10^6 cells per lane) were lysed and heated in sample buffer (Laemmli, 1970). SDS-PAGE analysis was followed by transfer to Immobilon membranes (Millipore Corporation, Bedford, MA). Rainbow Markers (Amersham Corporation, Arlington Heights, IL) were used to indicate the migration of proteins. Incubation of membranes in 1:200 to 1:500 dilutions of specific antibodies followed by peroxidase-labeled second antibodies at 1:500 was done according to the manufacturer's instructions (NOVEX, Encinitas, CA). Peroxidase was detected by staining using hydrogen peroxide and diaminobenzidine. Immunoblots in Fig. 9 were digitized by scanning with a Hewlett Packard ScanJet Plus attached to an Apple Macintosh IIci computer. A software program called Adobe Photoshop 2·0 (Adobe Systems, Mountain View, CA) was used to increase the contrast of the image. The contrast-enhanced digitized images were then photographed onto 35 mm Kodak TMAX 100 black and white negative film with a Lasergraphics LFR Plus film recorder.

Results

Mutant F9 (5.51) Cells Have Altered Morphology and F-Actin Organization

While wild-type F9 cells adhere and spread on gelatin-coated plastic, 5.51 cells float in the medium as loose aggregates (Grover et al., 1987). Wild-type F9 cells become flattened as they spread but remain in colonies on the substratum. Time-lapse video and laser confocal microscopy were used to examine the motile behavior and distribution of actin filaments (F-actin) and vinculin in wild-type and 5.51 cells. Wild-type F9 cells adhere to surfaces by first sending out long fingerlike filopodia. This is soon followed by protrusions of lamellipodia (Fig. 1 *A*). 5.51 cells have filopodia but no lamellipodia (Fig. 1 *B*).

In wild-type cells, F-actin is organized into stress fibers that terminate at focal contacts containing vinculin (Fig. 2, *C* and *D*). 5.51 cells do not have stress fibers and do not stain with vinculin antibodies (Fig. 2, *E* and *F*). Instead, F-actin is concentrated under the plasma membrane and in filopodia. Wild-type F9 cells observed 15 min after seeding have already begun to attach to a gelatin-coated substrate and while still rounded in shape are nevertheless able to organize vinculin into short nascent focal adhesion points (Fig. 2 *A*) even though actin stress fibers are not apparent (Fig. 2 *B*). These findings suggest that vinculin is required for attachment and spreading as well as for the formation of stress fibers.

Vinculin Levels in F9 Cells: Biochemical and Molecular Biological Evidence

Because vinculin staining is not evident in the mutant cells shown in Fig. 2 *E*, we extracted equal numbers of wild-type F9 and 5.51 cells to analyze their vinculin content by immunoblotting. The results show that wild-type F9 and P19 EC cells contain very similar steady-state levels of vinculin (Fig. 3, lanes *1*, *2*, and *5*) whereas 5.51 and its uvomorulin-expressing clone 1 (Calogero et al., 1991) have no visible vinculin protein.

Because vinculin could be unstable in 5.51 cells, it might be detectable with greater sensitivity if measured by metabolic labeling of newly synthesized protein. Equal amounts of radioactively-labeled lysate were analyzed with a polyclonal anti-vinculin to compare immunoprecipitates from wild-type and mutant cells (Fig. 4). Only wild-type F9 cells gave the expected 117-kD band. There was no such labeled polypeptide in 5.51 cells or with nonimmune serum. Fig. 4 *A* also shows the electrophoretic migration of uvomorulin in a similar aliquot of F9 lysate. In this case the F9 cells had been treated with tunicamycin and both glycosylated and underglycosylated versions of uvomorulin are seen as a doublet

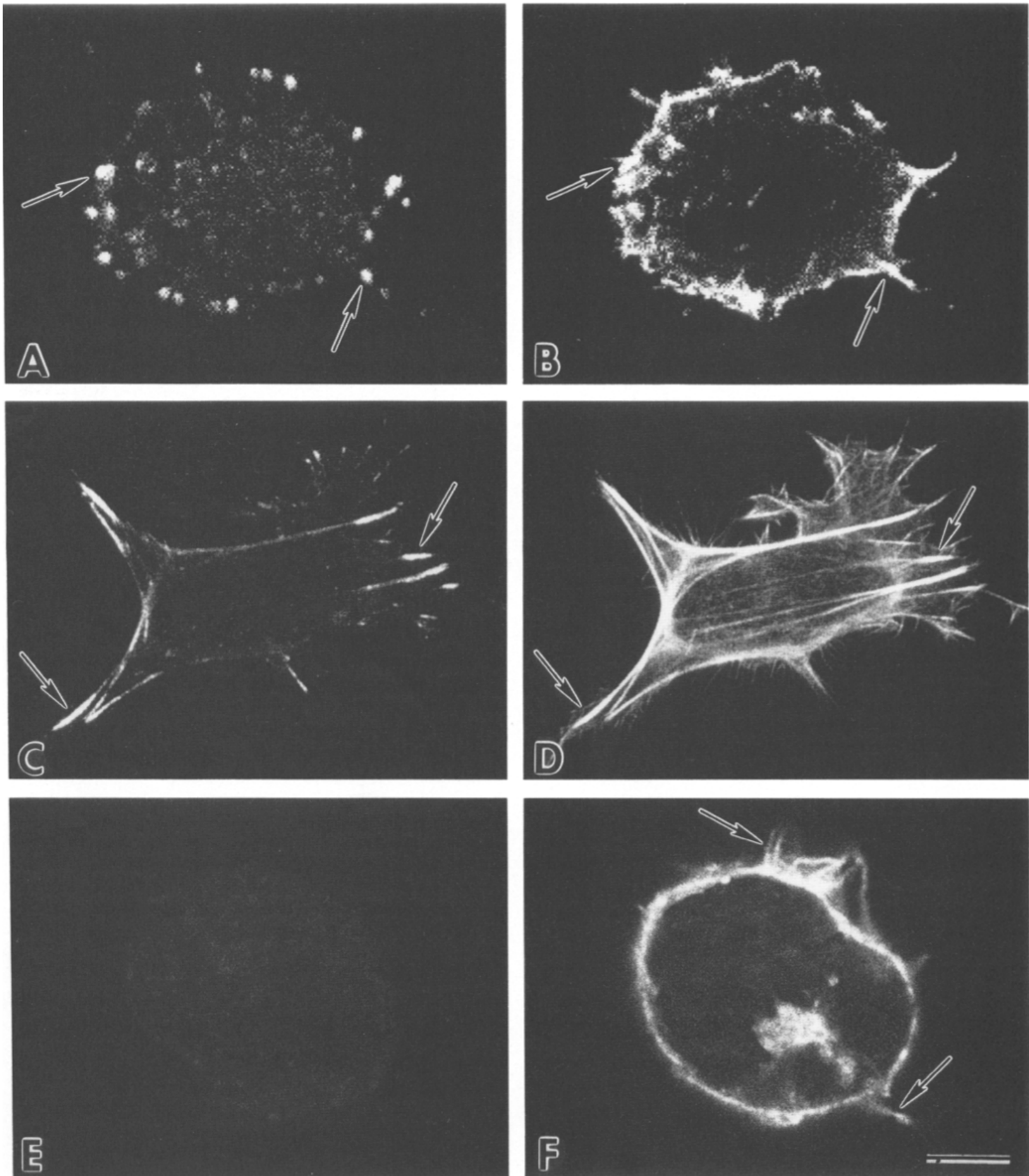

Figure 2. Colocalization of vinculin (*A*, *C*, *E*) and F-actin (*B*, *D*, *F*) in wild-type F9 (*A–D*) and 5.51 (*E* and *F*). Cells were strained with a mouse monoclonal antibody to human vinculin, and then stained with rhodamine-phalloidin (see Materials and Methods). Confocal images are of the ventral surface in contact with the substrate (for wild-type cells) and of the midsection of the cell (for 5.51). Wild-type F9 cells were observed 15 min (*A* and *B*) and 2 h (*C* and *D*) after seeding. After 2 h, F9 cells are fully spread and stress fibers are seen to terminate in vinculin-containing focal contacts (*arrows*). Even after 15 min, vinculin patches colocalize with F-actin at the cell periphery and in extending filopodia (*arrows*). *E* and *F* show a 5.51 cell fixed and stained 2 h after addition to the substrate. In this nonadherent cell type F-actin is concentrated at the cell periphery and in filopodia (*arrows*). Aggregates of F-actin inside 5.51 cells were frequently observed. Vinculin staining with antibodies to human (*E*) or chicken (not shown) vinculin was not detected in 5.51 cells. The round shape and F-actin organization in 5.51 cells resemble the adhering wild-type F9 cell (compare *B* with *F*). Bar, 5 μm for *A*, *B*, *E*, and *F*, and 10 μm for *C* and *D*.

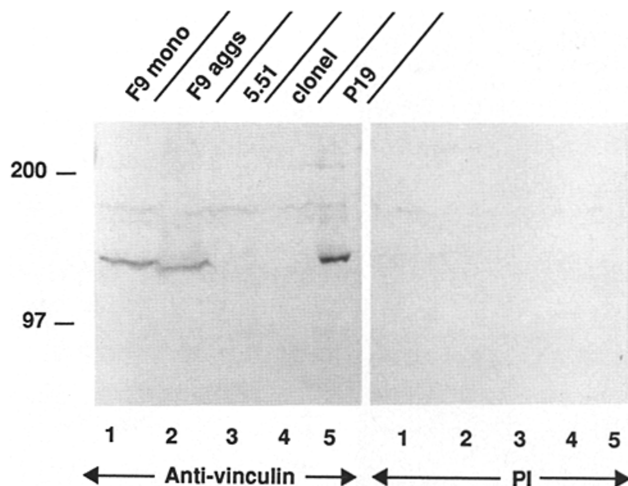

Figure 3. Immunoblotting to detect vinculin in EC cells. Equal numbers of cells were extracted as described in the Materials and Methods section. Vinculin was assayed by a two-layer method employing rabbit polyclonal anti-vinculin and peroxidase labeled second antibodies. The stained bands in the left-hand panel indicate that equal levels of vinculin were detected in monolayer (*mono*) and in aggregated (*aggs*) F9 EC cells and P19 cells but none were observed in mutant 5.51 or its uvomorulin transfected clone, 5.51 UM1 (denoted C1). PI was nonimmune serum negative control.

at about 120 kD. Vinculin moves at a slightly different migration rate between the uvomorulin doublet. To maximize the ability to detect vinculin in 5.51 cells, threefold more radiolabeled 5.51 cells compared to F9 wild-type were analyzed in Fig. 4 *B*. Even here, vinculin was not detected in 5.51 lysates although a number of other nonspecific proteins were seen in anti-vinculin as well as nonimmune lanes. We conclude that vinculin protein is absent from the mutant F9 cells.

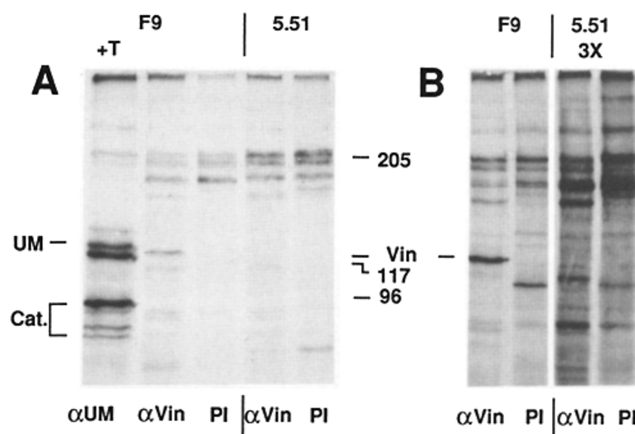

Figure 4. Immunoprecipitation of methionine-labeled F9 and 5.51 mutant cells. (*A*) Equal amounts of radioactivity were compared as described in the Materials and Methods section. To demonstrate the presence of uvomorulin and to distinguish between UM and vinculin, lane *1* shows uvomorulin protein synthesized in F9 cells treated with tunicamycin to inhibit the glycosylation of this protein. The triplet of proteins represents UM precursor (125 kD), mature UM (120 kD), and the under-glycosylated form. The catenins (*Cat*) coprecipitate with UM as indicated (Ozawa et al., 1989). Vinculin also migrates at ~120 kD and is seen only in lane 2, an extract of F9 cells. There is no detectable vinculin in 5.51 cells (lane *4*). (*B*) Here the amount of radioactive 5.51 lysate was threefold that of F9 cells but no immunoprecipitated vinculin could be seen. Lanes marked PI are controls with a nonimmune rabbit serum. The migration position of marker proteins is indicated in kD.

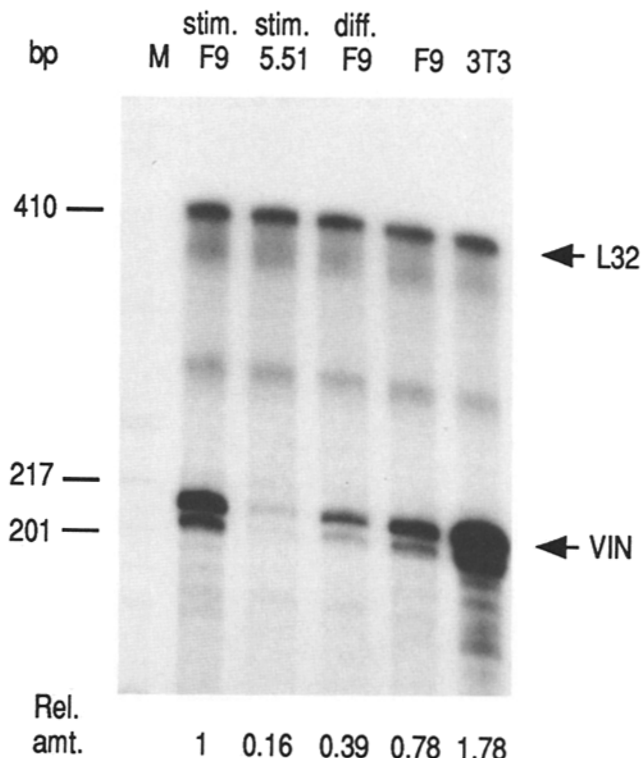

Figure 5. Ribonuclease protection analysis of the relative levels of vinculin transcripts in F9 cells stimulated with 20% serum and cycloheximide (10 μg/ml) for 1 h; 5.51 similarly stimulated; F9 cells after RA-stimulated differentiation for 4 d; unstimulated F9 monolayers, and 3T3 cells, respectively. L32 is the signal given by ribosomal protein transcripts and is used to control for RNA loading on the gel. The relative levels of vinculin mRNA in each sample are shown below. *M*, marker lane.

The expression of vinculin mRNA was detected by Northern blotting as a transcript of 6-7 kb in RNA extracts of F9 and 3T3 mouse fibroblasts, but no signal above background levels could be seen in 5.51 cells (data not shown). To detect and quantify vinculin mRNA levels with greater sensitivity, a ribonuclease protection assay was performed using a mouse cRNA that protects 205 bp of endogenous vinculin mRNA. Fig. 5 shows that the levels of vinculin mRNA in F9 cells are lower than in fibroblasts. Note that stimulating cells with 20% serum in the presence of cycloheximide (Ben-Ze'ev et al., 1990) for 60 min, or retinoic acid for several days, results in changes in the levels of vinculin mRNA. Unlike Northern blotting, a low level (approximately one-sixth of that found in F9) of vinculin mRNA was detected in mutant 5.51 cells using the ribonuclease protection assay. These levels were estimated by reference to the level of L32 ribosomal protein mRNA which was assumed to be invariant.

Transfection of Vinculin into 5.51 Cells Restores Adhesion, Morphology and Actin Organization

Co-transfection of an expression vector encoding full-length chicken vinculin, together with one that conveys neomycin

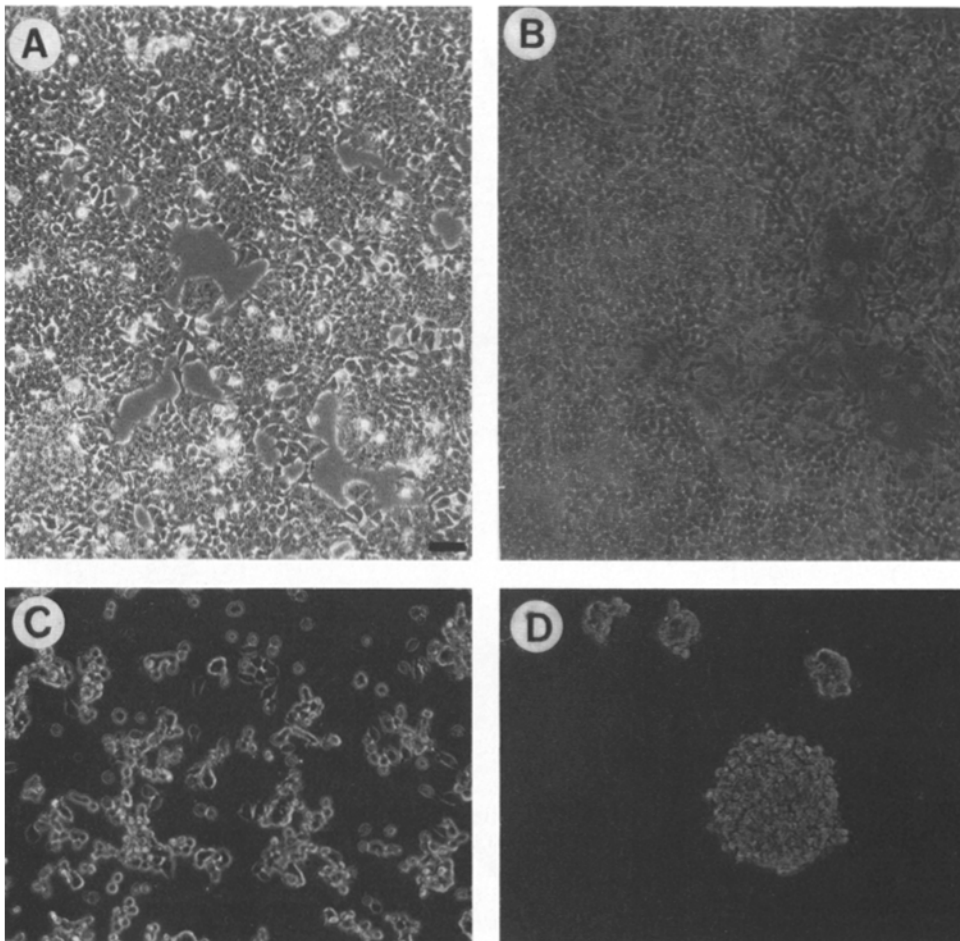

Figure 6. Phase contrast micrographs compare the general appearance of a population of F9 wild-type (*A*) with transfected cells, 5.51Vin4 (*B*) and 5.51Vin3 (*C*) grown as monolayers. 5.51Vin3 cells attach and spread comparatively poorly 2 d after seeding (*C*), but in suspension culture aggregate and compact well (*D*). Bar in *A*, 50 μm and applies to all panels.

resistance, allows the selection of stable cell lines carrying both genes. Selection in G418 gave a mixture of phenotypes, but we selected cells that either aggregated better or adhered better to the gelatin-coated substrate. The two properties appeared in parallel, thus supporting the idea that vinculin may be the major defect in the mutant cells. Two main clones, 5.51Vin3 and 5.51Vin4, were selected and subcloned. Clone 3 was less adhesive than clone 4 in both rate of adhesion and flattened appearance in a monolayer (Fig. 6 *C*) but formed moderately tight aggregates in suspension cultures (Fig. 6

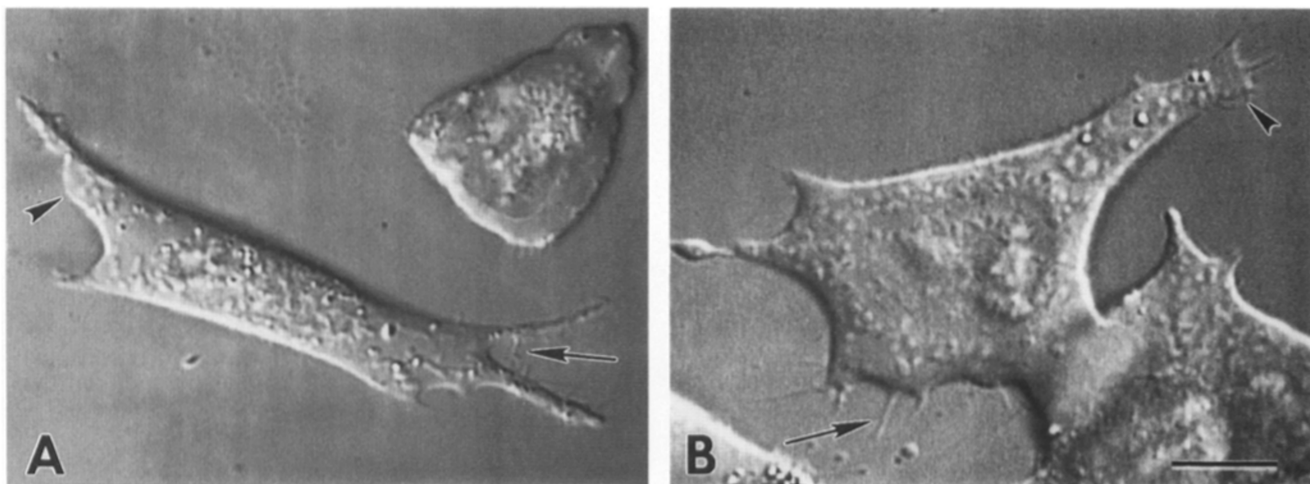

Figure 7. Morphology of 5.51Vin3 (*A*) and 5.51Vin4 (*B*) cells examined with Nomarski difference interference contrast optics. Both clones are attached to the substrate and have filopodia (*arrows*) and lamellipodia (*arrowheads*). Vin3 is not as well spread as Vin4 and wild-type cells (see Fig. 1). Bar, 10 μm for *A* and *B*.

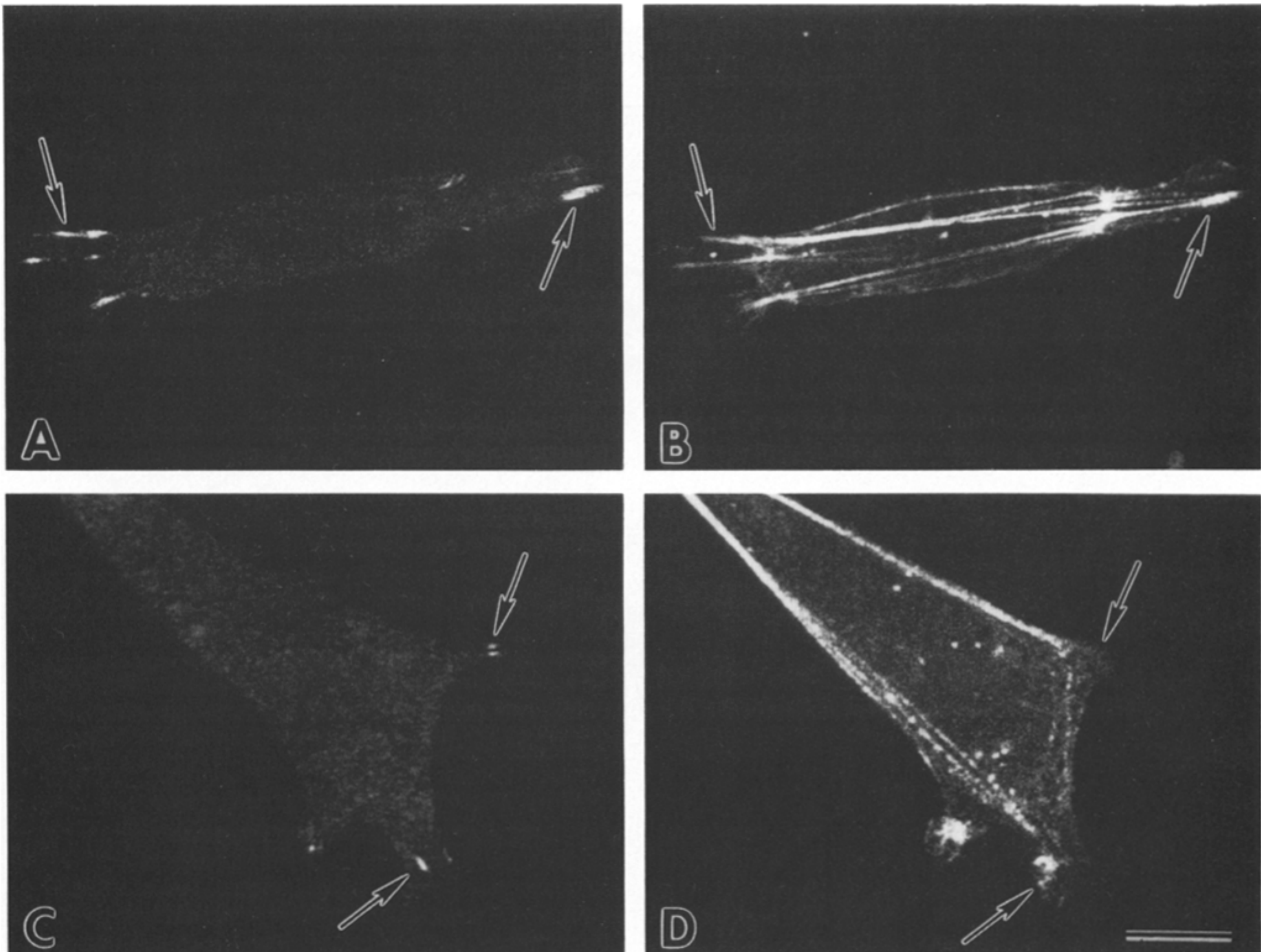

Figure 8. Colocalization of vinculin (*A, C*) and F-actin (*B, D*) in 5.51Vin3 (*A, B*) and 5.51Vin4 (*C, D*) cells. Cells were stained with a mouse mAb to chicken vinculin, and then stained with rhodamine-phalloidin. Confocal images are of the ventral plasma membrane in contact with the substrate. Both clone 3 and 4 cells have actin stress fibers that terminate in chicken vinculin-containing focal contacts (*arrows*). The abundance of stress fibers and vinculin staining in the 5.51Vin3 cell correlates with the increased expression of chicken vinculin (see Results). Bar, 10 μm for *A–D*.

D). Clone 4 monolayers adhered well to plastic and spread sufficiently to resemble wild-type F9 cells (Fig. 6, compare *A* with *B*).

5.51Vin clones 3 and 4 cells were observed with Nomarski DIC optics in order to observe spreading and membrane mobility (Fig. 7). 5.51Vin3 cells did not spread as well as 5.51Vin4 and wild-type cells (see Fig. 1 *A*), but contrasted clearly with the rounded shape and absence of lamellipodia in the 5.51 parental line (compare Fig. 1 *B* with Fig. 7).

We demonstrated next that the transfected cells not only expressed vinculin but also that they were able to place vinculin at the ends of actin stress fibers which were now restored. Fig. 8 shows that our two transfected 5.51 clones stained for chicken vinculin in a manner closely resembling that seen in wild-type F9 cells (see Fig. 2 *C* and *D*). Clone Vin3 had more actin stress fibers than clone 5.51Vin4, and more vinculin associated with the ends of the stress fibers (compare Fig. 8, *B* with *D*). The vinculin in 5.51Vin4 localized in focal contacts and clustered in small patches distributed over much of the cell surface. As shown in Fig. 8, a mouse mAb to chicken vinculin stained both 5.51Vin 3 and 4 cells. The anti-human vinculin antibody used to stain the wild-type mouse F9 cells (see Fig. 2) did not stain these cells (data not shown) proving that the vinculin associated with focal contacts in the transfected 5.51 cells is chicken vinculin and not endogenous mouse vinculin. These results demonstrate that chicken vinculin is being expressed in the transfected clones and suggest that this in turn is effecting the assembly of F-actin and the restoration of a normal F9 cytoarchitecture.

Biochemical Evidence for the Expression of Chicken Vinculin in Transfected 5.51 Clones

Immunoblotting with the polyclonal antibody to vinculin revealed that the two vinculin-transfected clones do indeed express higher levels of vinculin than even wild-type F9 cells (Fig. 9, *left*). We used a 10-fold range of cell lysates to compare the levels. At this point, we also tested a mixture of mouse mAb to human and chicken vinculin in order to try to increase the sensitivity of detection in cells that contained both antigens. It is clear that the differential recognition of

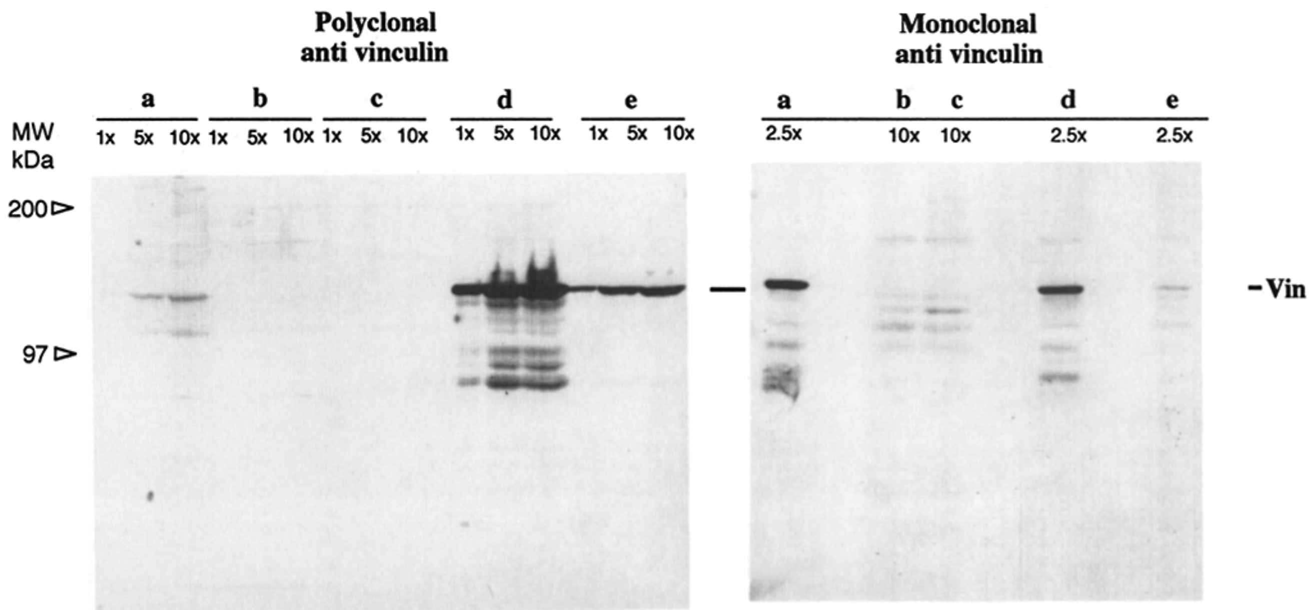

Figure 9. Immunoblotting to compare the levels of expression of vinculin in transfected clones. Lysates of wild-type (*a*), 5.51 (*b*), 5.51UM1 (*c*), 5.51Vin3 (*d*), 5.51Vin4 (*e*) cells were analyzed as described in the Materials and Methods section. Vinculin was detected on blots with the polyclonal antibody used in immunoprecipitation studies (*left*) and with a mixture of mouse mAb to human and chicken vinculin (*right*). The second layer peroxidase-labeled antibodies were detected with diaminobenzidine and hydrogen peroxide. The number of cells assayed is shown above each lane: 1x = 10^5, 5x = 5×10^5, 10x = 10^6 cells.

antigens by antibodies in different techniques can give rise to different signal strength (Fig. 9, *right*). We may be underestimating the expression of vinculin in wild-type cells (compare lanes *a* in Fig. 9), but even with this increased sensitivity, there is no vinculin protein in either the 5.51 cells or the uvomorulin-transfected clones (Fig. 9, *b* and *c*). We have not assayed the floating transfected cells for vinculin expression.

Because the rabbit polyclonal antibody recognizes both mouse and chicken vinculin albeit with different sensitivities, we used it to examine and compare vinculin biosynthesis in metabolically-labeled cells. Fig. 10 (upper left) confirmed that vinculin synthesis was not detectable in 5.51 cells in contrast to F9 cells. 5.51Vin3 synthesized chicken vinculin at extremely high rates while in 5.51Vin4 cells the rate was similar to F9 wild-type cells. Because all the vinculin-transfected clones gave more compact aggregates in suspension cultures, we were interested in seeing if uvomorulin synthesis might have been increased by the expression of extra vinculin. We routinely observed unchanged levels of uvomorulin synthesis in vinculin-transfected and parental 5.51 cells: 30–50% of the level in wild-type F9 cells (Fig. 10, *upper right*; see also Adamson et al., 1990). We have never observed the induction of vinculin synthesis by the expression of exogenous uvomorulin in 5.51 cells.

These and other clones were assayed for their steady-state levels of mouse vinculin mRNA by ribonuclease protection assay. Using the mouse cRNA probe (this probe could not detect chicken transcripts), we could detect little difference in the levels of transcripts between 5.51 and any of the transfected 5.51 clones (data not shown). Therefore, the expression of an exogenous chicken vinculin gene did not affect the low levels of endogenous mouse vinculin mRNA in 5.51 cells.

Other Cytoskeletal Protein Levels in F9 Cells

Because vinculin is known to interact with talin and with α-actinin, these and several other cytoskeletal proteins were analyzed in immunoprecipitation assays and in immunoblotting. We made the assumption that the solubility of cytoskeletal proteins of each cell type remained the same and hence that our extraction procedure was equally effective in F9 and mutant cells. Little difference was observed in the biosynthetic rates (Fig. 10, bottom) of the cytoskeletal proteins fodrin (α-spectrin), ankyrin (not shown) and filamin in 5.51, transfected 5.51 or wild-type cells. The rates of synthesis of filamin and fodrin were somewhat variable, but the differences between F9 mutant and transfected cell lines were not significant.

The steady-state levels of cytoskeletal proteins were also compared by immunoblotting. Fig. 11 shows that α-actinin was quite similar in all clones while filamin and fodrin steady-state levels were somewhat variable. Similar results were obtained for talin and ankyrin (not shown). However, there seemed to be a much lower level of filamin in 5.51 cells, while transfected clones derived from 5.51 all expressed higher levels of filamin. We think that it is unlikely that the excess expression of either uvomorulin or vinculin could have caused this increase and so again we conclude that clonal differences account for this result. In summary, there are no large consistent differences in the expression of cytoskeletal proteins among the five cell lines examined.

Discussion

The Mutant Phenotype Is Complemented by the Expression of Vinculin

The defect in adhesion and actin organization in 5.51 cells

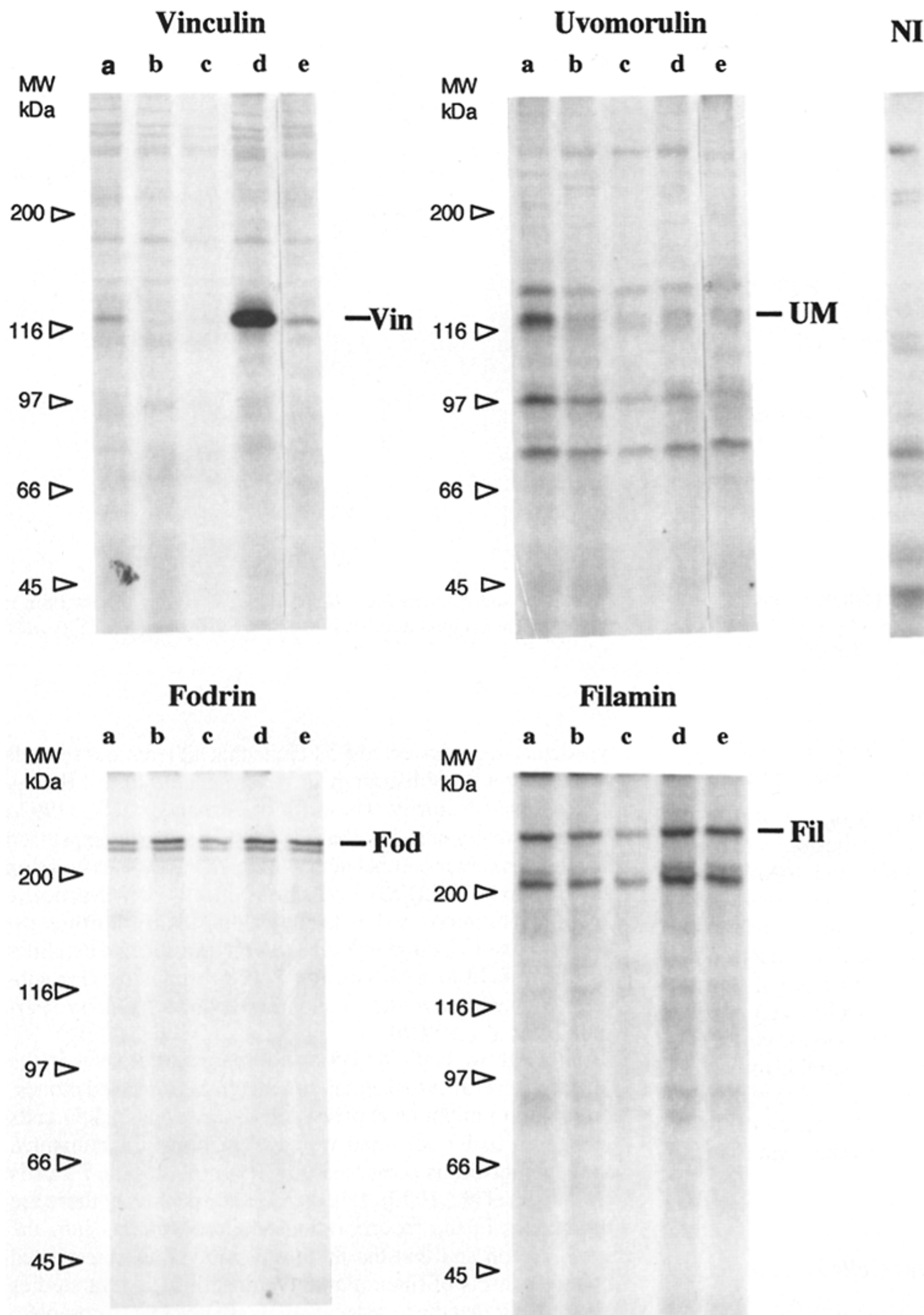

Figure 10. Biosynthesis of vinculin in transfected clones. Metabolically-labeled cells were examined by immunoprecipitation using the rabbit polyclonal antibody that recognizes both mouse and chicken vinculin. Analyses of the expression of vinculin (*upper left*) were made before the immunoprecipitation of uvomorulin from the same lysates (*upper right*). Fodrin (*lower left*) and filamin (*lower right*) antibodies were applied sequentially to an identical set of samples *a* through *e*, described in Fig. 9. All samples except *c* contained equal amounts of total radioactive protein; *c* contained half the amount. The position of the markers is shown as kD on the left. PI is nonimmune serum. See Materials and Methods for details of scanning and imaging.

is corrected by the expression of the transfected chicken vinculin gene. This is evidenced by the nearly normal rate and extent of substrate adhesion in two clones expressing chicken vinculin, especially in 5.51Vin4. In addition, cell–cell adhesion was improved. The introduction of moderate levels of vinculin into 5.51 cells (as in 5.51Vin4) allowed the mutant cells to adhere to each other and to the dish surface. We conclude that the major defect in the mutant cells is the loss of vinculin protein expression. A similar approach has been used by Barstead and Waterson (1989) to show that vinculin is essential for muscle development and function. They identified a population of nematodes that are paralyzed, fail to organize normal muscle structure, and have reduced levels of vinculin. This lethal mutation was corrected by injection of a cloned wild-type copy of the vinculin gene. On the other hand, microinjection of a mAb to chicken vinculin disrupted stress fibers and led to loss of focal contacts in chicken fibroblasts (Westmeyer et al., 1990). These observations are consistent with the hypothesis that vinculin is one of a number of interacting proteins which link F-actin to the cytoplasmic face of transmembrane glycoproteins involved in cell adhesion.

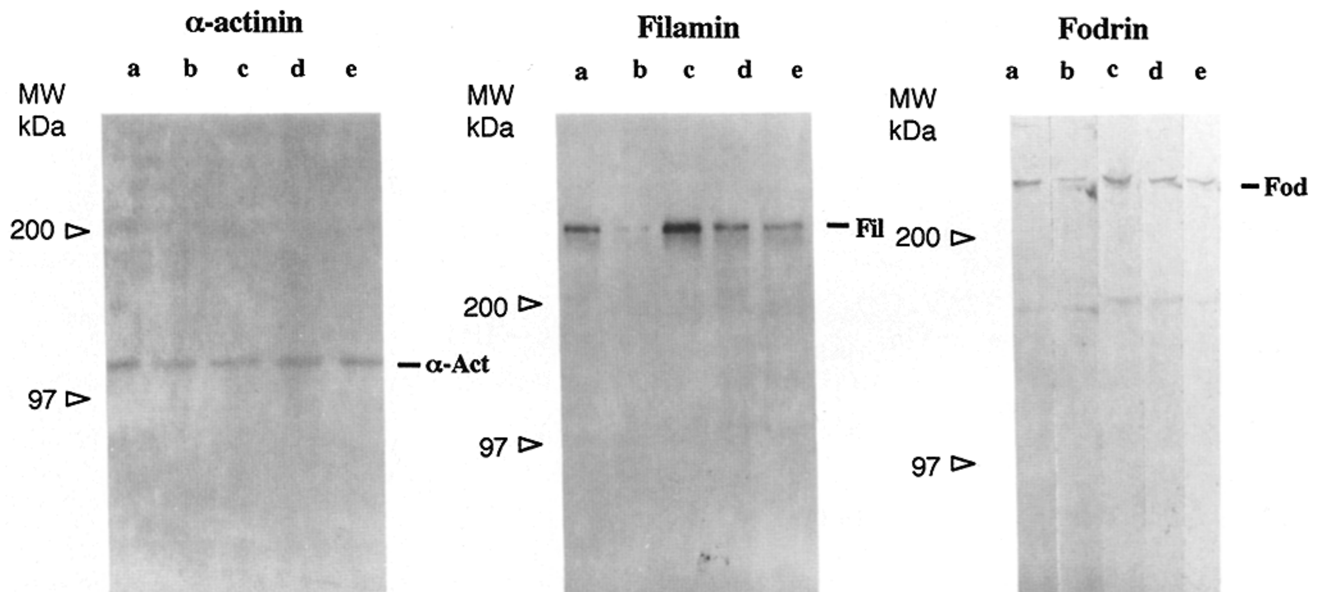

Figure 11. Steady-state levels of other cytoskeletal proteins in wild-type, mutant and rescued cells. (*Left*) Immunoblotting of α-actinin in the five samples (*a–e*) described in Fig. 9. (*Middle*) A similar analysis of filamin in an equal number of cells (5×10^5) in identical lysates; (*right*) analysis of the steady-state levels of fodrin.

Vinculin Expressed in Mutant Cells Is Correctly Localized and Restores the Actin Organization

We argue that the induced expression of vinculin in 5.51 Vin3 and 5.51Vin4 is causal in restoring the ability of the mutant cell line to adhere to substrates and to form actin-containing stress fibers (Fig. 8). Most of the over-expressed vinculin in 5.51Vin clones is located at the peripheral ends of actin stress fibers although some vinculin is also seen dispersed throughout the cytoplasm and this is not normally seen in wild-type F9 cells (Fig. 2). As observed by others (Pfeiffer and Vogl, 1991; Horvath et al., 1992), vinculin appears to be present in cells in a pattern similar to actin but at the peripheral ends of actin stress fibers. The presence of stress fibers is clearly correlated with the shape of the cell and to its ability to adhere to substrates via vinculin-containing cell matrix junctions.

The Complementation of Mutant Cells Is Imperfect

At least two explanations might account for the slightly incomplete complementation of the mutant phenotype by chicken vinculin to that exhibited by F9 parental cells. One is that chicken vinculin is sufficiently different to be unable to replace the mouse-type protein. This is unlikely, however, because there is 95% similarity between chick and human vinculin (Weller et al., 1990). This question can be studied when a mouse vinculin vector becomes available. The second possibility is that exactly the right amount of vinculin is needed. This hypothesis is supported by our observation that 5.51Vin3 which expresses very high levels of vinculin is less able to adhere than 5.51Vin4 which expresses moderately high levels (Fig. 9). The presence of excess vinculin as in 5.51Vin3 cells could inhibit cell spreading by over-stabilization of stress fibers and by affecting the disassembly and reorganization of actin filaments. However, we have not yet detected a transfected 5.51 clone that adheres and spreads perfectly on the substratum so we are unable to test the hypothesis satisfactorily. The work of Fernandez et al. (1992*a* and *b*) strongly suggests that the level of vinculin expressed in a cell can alter cell behavior, because the over-expression of vinculin in BALB/c3T3 cells by as little as 20% drastically reduces their motility (Fernandez et al., 1992*a*). Further, the restoration of vinculin levels in several transformed cell lines that express little or no vinculin results in an increase in substrate adhesiveness and in suppressed tumorigenicity (Fernandez et al., 1992*b*).

Although we could detect no consistent differences in the levels of several cytoskeletal proteins in 5.51-derived clones, only filamin might be expressed at lower levels in 5.51 cells compared to F9 cells and we have previously documented that uvomorulin is present at 30–50% of wild-type F9 cells (Adamson et al., 1990). It is therefore possible that there are differences in the incorporation of these proteins into the cytoskeleton and that the mutant phenotype is exacerbated by lower levels of filamin and uvomorulin. Current studies also suggest that the transfected cells are not able to complete the process of epithelium formation after the addition of retinoic acid to induce differentiation. The cells are not resistant to retinoic acid, however, because they do differentiate into parietal endoderm, a nonepithelial cell type. It should be noted that the ability of the mutant cells to proliferate is completely normal even in the absence of vinculin.

We believe that the major defect in 5.51 cells is in the vinculin gene or in a vinculin regulatory gene. We have never been able to select an F9 nonadhesive clone without mutagenesis. Therefore, this defect is not merely a characteristic of a clonal variation. Restriction enzyme analysis suggest that the structure of the vinculin gene in 5.51 cells is not detectably different from that in parental F9 cells and therefore, either a relatively subtle mutation has occurred or a regulatory gene is aberrant in the mutant cells.

The Role of Vinculin in Cell-Substrate Adhesion

The work of DePasquale and Izzard (1987, 1991) shows that talin is first located at the tips of the focal contact precursors and then accumulates in the adhesion plaque after the focal contact forms and before vinculin is seen there. The filopodia-like protrusions in 5.51 cells may be focal contact precursors because, based on the above findings, vinculin is not required for their assembly. In 5.51 cells, talin may be at the tips of the filopodia but focal contacts and actin connections cannot be made because vinculin is absent.

The difficulty of proper assembly of the cytoskeleton is reinforced by our growing knowledge of the level of complexity of interactions between the cytoskeletal proteins. Adhesion of cells to a substrate occurs through a chain of interactions from β-integrins to α-actinin and actin (Burridge et al., 1988; Geiger and Ginsberg, 1991; Critchley et al., 1991; Turner and Burridge, 1991; Hynes, 1992). However, although vinculin binds avidly to talin (Jones et al., 1989), vinculin lacking the talin binding site retains the ability to associate with focal contacts (Bendori et al., 1989). This interaction could occur through a binding site on vinculin, possibly the paxillin binding site, which is located toward the COOH-terminus of the molecule (Turner et al., 1990). Vinculin can also self-associate (Molony and Burridge, 1985). Furthermore, vinculin binds to α-actinin and through the latter protein to actin. Interestingly, α-actinin appears to bind directly to β1 integrin (Otey et al., 1990) even though it appears to be located some distance from the membrane (Chen and Singer, 1982).

We have shown here that vinculin is necessary for cell attachment, however, the spreading of cells after attachment is more complex and may not be a function of vinculin (although see Lehtonen et al., 1983). BALB/c-3T3cells replated from suspension cultures attach and spread extensively even in serum-free cultures when only basal levels of vinculin are present and vinculin is not detectable in the focal contacts. When serum is present however, vinculin expression is induced and large vinculin-positive plaques form (Ben-Ze'ev et al., 1990). Ben-Ze'ev et al. (1990) suggest that there may be two kinds of focal contacts, with vinculin present in only one type. It is possible that our complemented clones, 5.51Vin3 and 4, are unable to organize the nonvinculin focal adhesions. This may explain the incomplete appearance of adhesion. Vinculin expression is transcriptionally induced by serum growth factors and increases in vinculin synthesis requires both initial attachment to the substratum and serum factors. An autoregulatory mechanism linking the mode of vinculin organization to the growth factor-mediated control of vinculin gene expression has been suggested (Ben-Ze'ev et al., 1990). Such a control mechanism has been demonstrated for tubulin regulation (Ben-Ze'ev et al., 1979; Cleveland et al., 1981) and may operate for other cytoskeletal elements. Nevertheless, serum induction of cells in suspension culture is not effective in up-regulating vinculin expression; cell attachment to the substrate is necessary for induction. The mechanism of this requirement remains obscure. Our observations suggest that cell attachment is a catalyst for further adhesion and spreading. Although 5.51Vin clones attach at different speeds after trypsinization (perhaps depending on their level of expression of vinculin), all clones eventually appear similar in morphology, adhesion and strength of attachment.

The Role of Vinculin in Cell-Cell Compaction and Adherens Junctions

Wild-type F9 cells express uvomorulin at high levels (Peyrieras et al., 1985). Immunoprecipitation of uvomorulin coprecipitates α, β, and γ catenins (Vestweber and Kemler, 1984; Ozawa et al., 1989). Interestingly, α-catenin has ~30% similarity to vinculin (Nagafuchi et al., 1991; Herrenknecht et al., 1991). The cytoplasmic portion of uvomorulin is necessary to associate with α-catenin (Nagafuchi and Takeichi, 1989; Ozawa et al., 1990). The hypothesis is that uvomorulin forms a molecular complex with the actin-based cytoskeleton via the catenins and other proteins at the cytoplasmic domain. This association is essential for uvomorulin to act as a cell-cell adhesion receptor. The adherens junctions have tightly-associated actin filaments (Geiger et al., 1980; Tsukita et al., 1989; Tsukita et al., 1992) and although coprecipitation of vinculin does not occur in the presence of antibodies to actin, its distribution is markedly similar to actin patterns (Pfeiffer and Vogl, 1991; Horvath et al., 1992). It is possible that these associations are the trigger for the homotypic association between uvomorulin on adjacent cells that leads to cell-cell compaction and the formation of adherens junctions.

It has been observed that the shape of a cell, the nature of the substratum and the degree of contact with adjacent cells (Shirinsky et al., 1991) directly regulate the synthesis of vinculin in cultured fibroblasts (Ungar et al., 1986). In addition, vinculin is an immediate early gene, responding rapidly to the addition of serum growth factors by up-regulating its transcription (Ben-Ze'ev et al., 1990; Bellas et al., 1991). Fibronectin (Dean et al., 1990), β1-integrin, α-tropomyosin and γ and β-actin (Ryseck et al., 1989) are also early response genes. It is clear, therefore, that cell interaction, cell shape, molecular interactions at the cytoplasmic domains of adherens junctions and early signal transduction are all cross-regulated and correlated with long-term signals and cell behavior. The mutant F9 cell line will be an important model for studies of the molecular interactions between the components involved in adhesion and the consequences of perturbations made to single components.

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