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Focal adhesion formation by F9 embryonal carcinoma cells after vinculin gene disruption

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SUMMARY

The assembly of focal adhesions was investigated in F9 embryonal carcinoma cells in which the expression of vinculin was eliminated by a targeted disruption of the vinculin gene. Vinculin-deficient F9 cells were capable of adhering to fibronectin-coated surfaces, though they displayed a reduced spreading compared to the parental cells. Transmission electron microscopy as well as interference reflection microscopy of live cells showed that vinculin-null F9 cells formed focal adhesions that were indistinguishable from those of the control cells. Fluorescent labeling for actin, talin, α-actinin, paxillin and phosphotyrosinated components indicated that the organization of all these focal contact-associated components was essentially identical in the vinculin-containing and vinculin-null cells. However, quantitative, digitized microscopy indicated that the intensity of fluorescence labeling in focal adhesions for α-actinin, talin and paxillin was significantly higher in cells lacking vinculin. The results suggest that there are multiple molecular mechanisms for the formation of focal adhesions in the absence of vinculin.

Key words: adhesion plaques, vinculin knock out, focal contacts

INTRODUCTION

Adhesion of cultured cells to the extracellular matrix (ECM) is mediated by specialized regions of the plasma membrane known as focal contacts, or focal adhesion plaques (Burridge et al., 1988; Geiger and Ginsberg, 1991). At these sites, actin filaments are bound to transmembrane receptors of the integrin family (Hynes, 1987), through a complex of structural 'plaque' proteins including vinculin (Geiger, 1979), talin (Burridge and Connell, 1983) and α-actinin (Lazarides and Burridge, 1975). In addition to these and other structural components (Luna and Hitt, 1992), molecules involved in signal transduction, mainly tyrosine kinases (Volberg et al., 1992), are also localized at focal adhesion sites, suggesting a role in the transduction of contact-triggered signals (Burridge et al., 1992; Ben-Ze'ev, 1992; Juliano and Haskill, 1993). Such regulatory molecules include the pp60^{src} tyrosine kinase (Rohrshneider, 1980), the focal adhesion kinase p125^{FAK} (Schaller et al., 1992), the LIM domain-containing molecule zyxin (Sadler et al., 1992), the SH2 (Src homology 2)-containing actin binding molecule tensin (Lo et al., 1994), and a major phosphotyrosinated molecule paxillin (Turner et al., 1990).

The mechanisms underlying the association of actin filaments with integrin receptors in these plaques is largely unknown. Several in vitro binding studies suggested that F-actin may associate with integrin via talin, vinculin and α-actinin (Horwitz et al., 1986), or alternatively, through α-actinin, which may directly bind to the cytoplasmic tail of β_1 integrin (Otey et al., 1990, 1993). Other possible molecular interactions between actin and focal adhesion components such as talin, tensin or vinculin were also suggested (Muguruma et al., 1990; Lo et al., 1994; Menkel et al., 1994; Goldmann et al., 1994).

Vinculin is a major constitutive component of adhesion plaques and also of many adherens-type cell-cell junctions where it is involved, together with catenins and plakoglobin, in linking the microfilaments to the cadherin receptors of cell-cell contacts (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; Geiger and Ginsberg, 1991; Geiger et al., 1992; Kemler, 1993). In recent studies, the modulation of vinculin levels by overexpression or suppression by antisense cDNA transfection demonstrated dramatic effects on cell motility and spreading (Rodríguez et al., 1992a, 1993), stability of filopodia and lamellipodia (Varnum-Finney and Reichart, 1994), as well as anchorage-dependent growth and tumorigenicity (Rodríguez et al., 1992b, 1993).

To directly study the function of vinculin in focal adhesion assembly, we employed an F9 embryonal carcinoma cell line in which both alleles of the vinculin gene were inactivated by homologous recombination (Coll et al., unpublished data) and characterized the consequent effect on cell adhesion, mor-

phology and locomotion. In the present study we show that vinculin-deficient F9 cells, which have a reduced ability to spread on the substratum, are still capable of forming focal adhesions upon spreading on the ECM. These adhesion plaques were indistinguishable from those formed by the parental F9 cells, when observed by interference reflection or transmission electron microscopy. The focal adhesions of vinculin-deficient cells contained actin, α-actinin, paxillin and talin, as well as phosphotyrosinated proteins. Moreover, quantitative analysis indicated that the adhesion plaques in vinculin-deficient cells stain more intensely by anti-α-actinin, talin and paxillin antibodies, suggesting alternative linkage mechanisms between actin filaments and the membranes in adhesion plaques which may compensate for the lack of vinculin.

MATERIALS AND METHODS

Cell culture and differentiation

F9 embryonal carcinoma cells and the γ229 F9 mutant clone in which both alleles of the vinculin gene were inactivated by homologous recombination (Coll et al., unpublished data) were grown on gelatin-coated dishes in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum. Cells were induced to differentiate into parietal endoderm by treatment with retinoic acid and cAMP (Grover and Adamson, 1986).

Protein labeling and gel electrophoresis

Proteins were labeled for 20 hours with [^{35}S]methionine (100 μCi/ml, Amersham), and equal amounts of radioactive proteins were analyzed by two-dimensional (2-D) gel electrophoresis, using pH 4-8 range ampholytes in the first dimension and 8% polyacrylamide-SDS gels in the second dimension (Ben-Ze'ev, 1990).

Immunoblotting

Proteins, separated by SDS-PAGE or 2-D gel electrophoresis, were electroblotted onto nitrocellulose according to Towbin et al. (1979). To rapidly visualize the pattern of the transferred proteins, the blots were stained with Ponceau Red (Sigma). An area containing the proteins with a molecular mass higher than 46 kDa, and pI between 4.5 and 7, was excised and used for immunodetection with monoclonal anti-human vinculin antibody (clone hVIN-1, Sigma Immunochemicals, USA), followed by visualization using the ECL method (Amersham, UK), according to the manufacturer's instructions. To determine the profile of the radioactive proteins on the nitrocellulose blot, the blots were exposed to X-ray film.

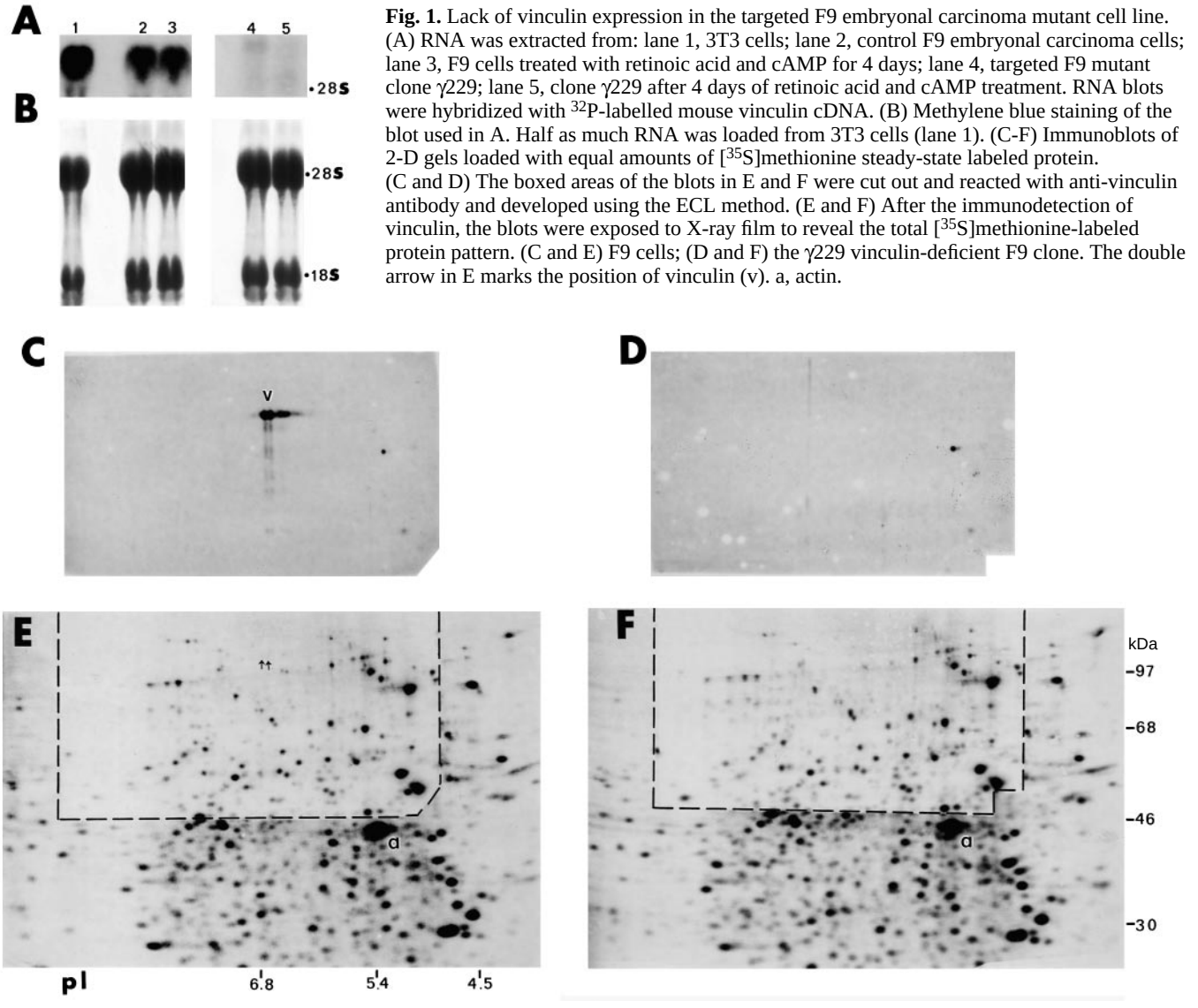

Fig. 1. Lack of vinculin expression in the targeted F9 embryonal carcinoma mutant cell line. (A) RNA was extracted from: lane 1, 3T3 cells; lane 2, control F9 embryonal carcinoma cells; lane 3, F9 cells treated with retinoic acid and cAMP for 4 days; lane 4, targeted F9 mutant clone γ229; lane 5, clone γ229 after 4 days of retinoic acid and cAMP treatment. RNA blots were hybridized with ^{32}P-labelled mouse vinculin cDNA. (B) Methylene blue staining of the blot used in A. Half as much RNA was loaded from 3T3 cells (lane 1). (C-F) Immunoblots of 2-D gels loaded with equal amounts of [^{35}S]methionine steady-state labeled protein. (C and D) The boxed areas of the blots in E and F were cut out and reacted with anti-vinculin antibody and developed using the ECL method. (E and F) After the immunodetection of vinculin, the blots were exposed to X-ray film to reveal the total [^{35}S]methionine-labeled protein pattern. (C and E) F9 cells; (D and F) the γ229 vinculin-deficient F9 clone. The double arrow in E marks the position of vinculin (v). a, actin.

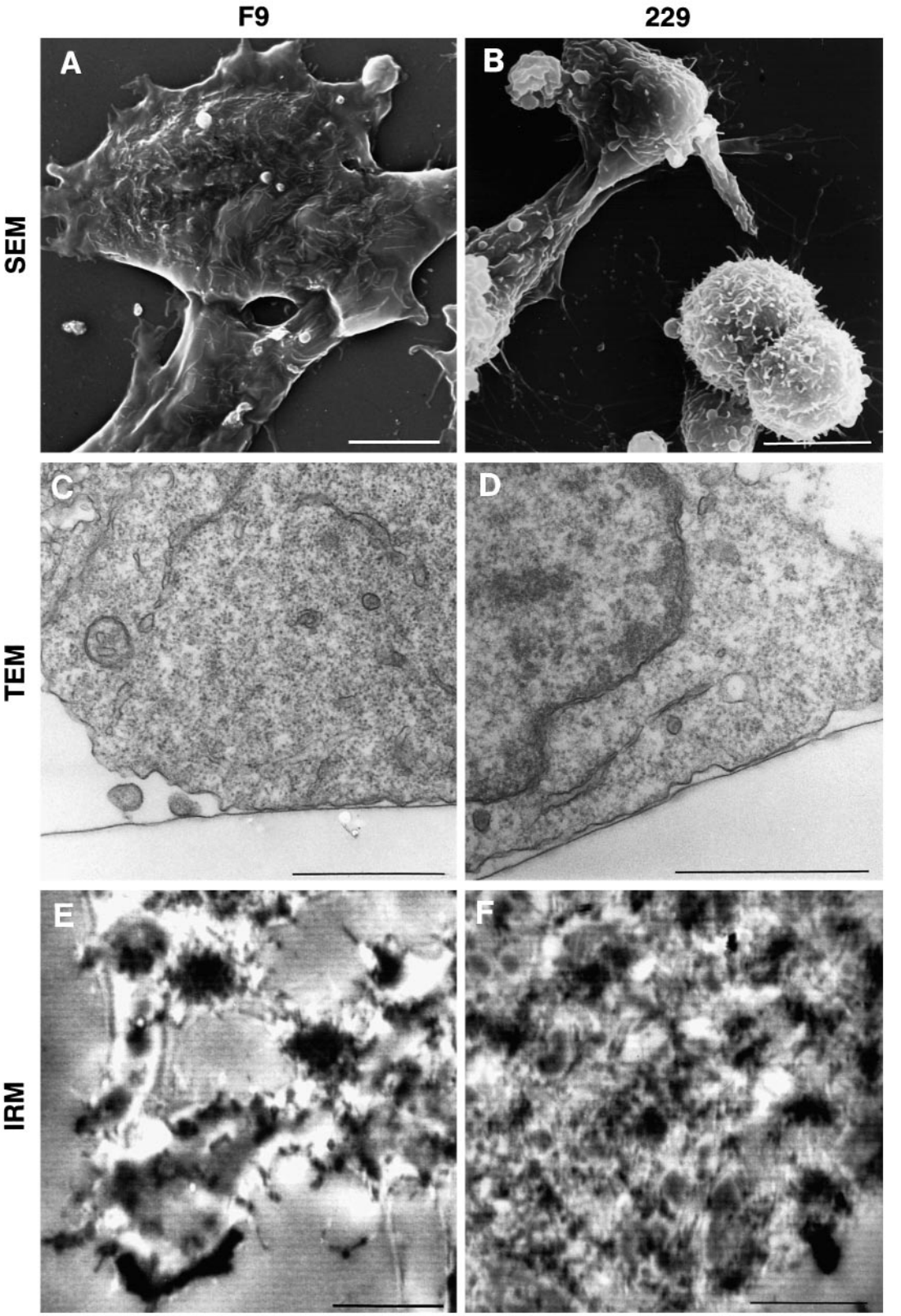

Fig. 2. Morphology and focal adhesion formation by F9 and vinculin-deficient γ229 cells. The morphology of F9 (A) and of the γ229 cells (B) on fibronectin was analyzed by scanning electron microscopy (SEM). The presence of focal adhesions in F9 (C) and γ229 (D) was examined by transmission electron microscopy (TEM) of sections cut perpendicular to the substratum. The formation of focal adhesions in live cells on fibronectin-coated substrata was visualized in F9 (E) and γ229 (F) cells by interference reflection microscopy (IRM). The bars in A, B, E and F are 10 μm; in C and D, 1 μm.

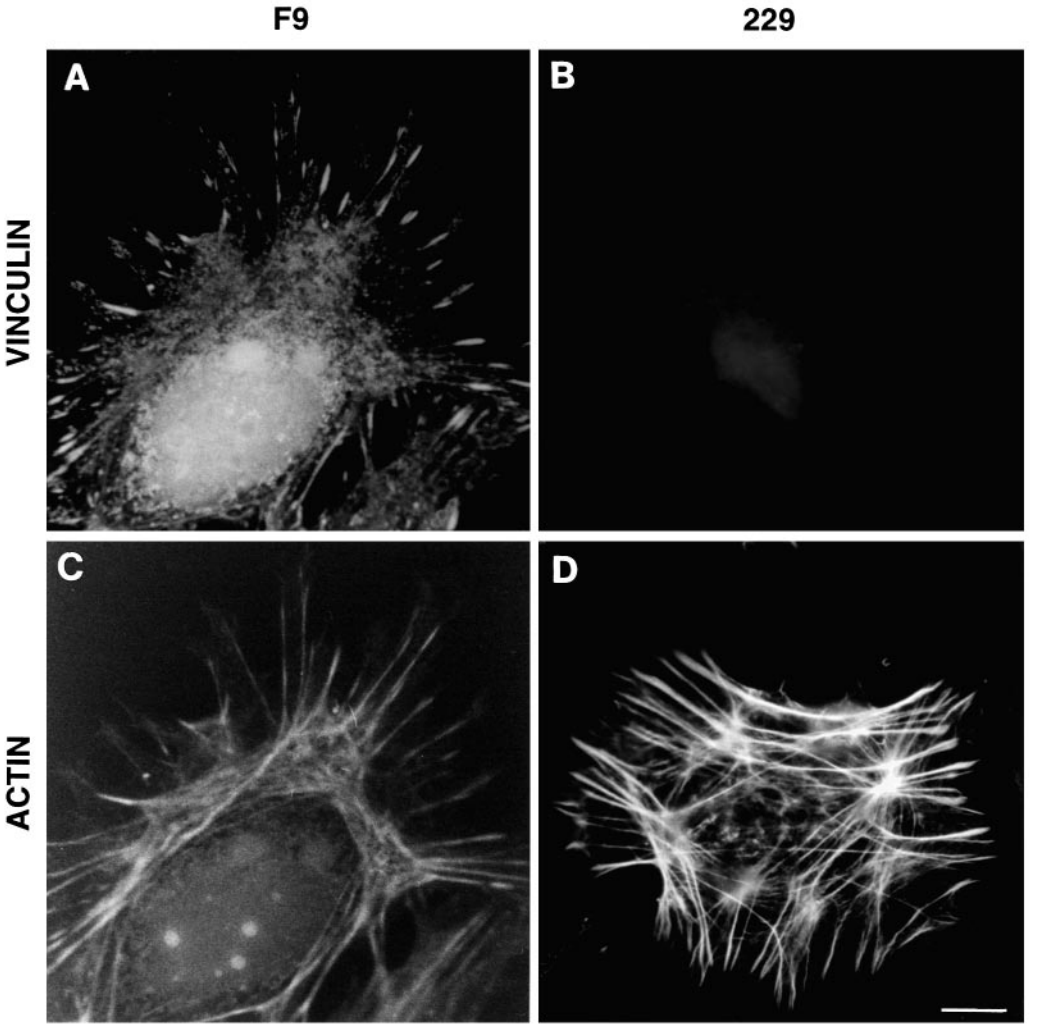

Fig. 3. Stress fiber assembly in the absence of vinculin. F9 and vinculin-deficient γ229 cells were doubly stained for vinculin (A, B) with monoclonal anti-vinculin antibody followed by rhodamine-labeled secondary antibody, and for polymerized actin (C, D) with FITC-phalloidin. (A and C) Control F9 cells; (B and D) γ229 cells. Note the presence of stress fibers in vinculin-null cells. Bar in D, 10 μm.

Northern blot hybridization

Total RNA was isolated by the acid-guanidinium method of Chomczynski and Sacchi (1987). A 20 μg sample of RNA was loaded per lane, except for RNA from 3T3 cells (10 μg), and the agarose gel electrophoresis-separated RNA was transferred to nitrocellulose paper. The RNA blots were stained with methylene blue to determine the efficiency of transfer, and then hybridized to a ^{32}P-labeled mouse vinculin cDNA probe, as described (Ben-Ze'ev et al., 1990).

Immunofluorescence

Cells were seeded on fibronectin (NY Blood Center) (50 μg/ml) - coated glass coverslips. The cells were simultaneously permeabilized and fixed with 0.5% Triton X-100 and 3% paraformaldehyde for 5 minutes, followed by 30 minutes post-fixation with 3% paraformaldehyde, as described (Volberg et al., 1992). Actin filaments were visualized with FITC-labeled phalloidin (Sigma, St Louis, MO). Monoclonal antibodies to α-actinin (BM-75.2) were from Sigma Immunochemicals. Anti-paxillin antibody was from Transduction Laboratories (Lexington, KY), while anti-talin antibody (clone 27.1) was prepared in our lab by injecting mice with purified chicken gizzard talin. An antiserum against talin obtained from Dr K. Burridge (University of North Carolina, Chappel Hill) was also used. Rhodamine labeled-goat anti-mouse IgG was from Jackson Laboratories (MI). The anti-phosphotyrosine antibody (clone 20.1) was described in a previous study (Volberg et al., 1992). The samples were viewed with an Axiophot microscope (Carl Zeiss, Germany). Fluorescent intensities of α-actinin, talin and paxillin in focal contacts and of α-actinin in stress fibers were determined using a cooled CCD camera (CC/CE 200, Photometrics, Tucson, AZ) coupled to a Zeiss Axioscop microscope. The recorded images were processed as previously described (Kam et al., 1993), and fluorescence intensity values per pixel were calculated. Since α-actinin was associated with stress fibers, in addition to its presence in focal contacts, the fluorescence intensity values in the adhesion site were normalized to the intensity of α-actinin along the attached stress fiber.

Microscopy

Interference reflection microscopy (IRM) setting on a Zeiss microscope was employed to visualize focal contacts in live cells. Scanning electron microscopy and transmission electron microscopy were performed as previously described (Hanein et al., 1993; Sabanay et al., 1991).

RESULTS

Morphology of focal contacts in vinculin-deficient cells

To study the role of vinculin in focal adhesion assembly, a mutant F9 embryonal carcinoma cell line (γ229) was employed in which both alleles of the vinculin gene were targeted by homologous recombination (Coll et al., unpublished data). These cells expressed no detectable vinculin RNA (Fig. 1A

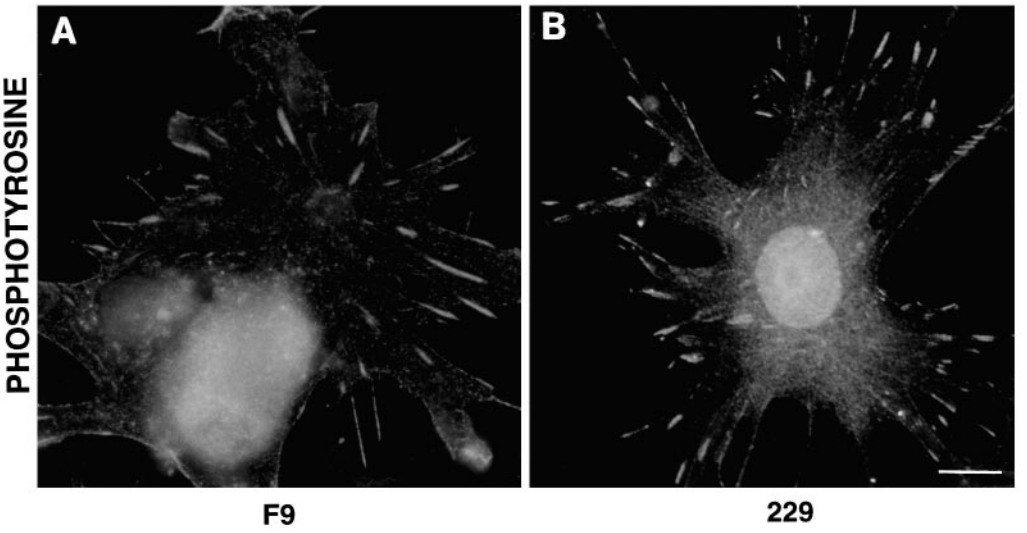

Fig. 4. Localization of phosphotyrosinated proteins in F9 and γ229 cells. F9 (A) and γ229 (B) cells were stained with mouse monoclonal antibody specific for phosphotyrosine followed by anti-mouse IgG labeled with rhodamine. Bar in B, 10 μm.

lanes 4 and 5). Immunoblot analysis of proteins separated by 2-D gel electrophoresis did not detect vinculin even in over-exposed Western blots with anti-vinculin antibody (Fig. 1D). Except for the lack of vinculin, the pattern of major cellular proteins was very similar in the parental (Fig. 1E) and the mutant (Fig. 1F) F9 cell lines.

The vinculin-deficient γ229 cells were capable of adhering to fibronectin- and gelatin-coated surfaces, but their spreading was apparently impaired (Fig. 2B) compared to the parental F9 cells (Fig. 2A), despite the fact that the expression of the major fibronectin receptor (integrin $\alpha_5\beta_1$) was similar in both cell types (results not shown). Transmission electron microscopic examination of sections cut perpendicular to the substratum revealed that the mutant cells contained electron-dense focal adhesions closely associated with the fibronectin substratum (Fig. 2D), which were indistinguishable from those formed by the control F9 cells (Fig. 2C). Interference reflection microscopy of the cells demonstrated that both were capable of forming dark regions characteristic of sites of close and focal cell-substratum contacts (Fig. 2E and F). These observations suggest that the vinculin negative cells did not loose the capacity to assemble focal adhesions with morphological characteristics similar to those of the parental cells.

Organization of focal adhesion components in vinculin-deficient cells

Immunofluorescence and fluorescent phalloidin staining revealed that despite the absence of vinculin (Fig. 3B), the mutant γ229 cells assembled stress fibers (Fig. 3D) that were similar to those of control F9 cells (Fig. 3C). Moreover, the focal adhesions of vinculin-deficient γ229 cells stained brightly with anti-phosphotyrosine antibody (Fig. 4B), similar to control F9 cells (Fig. 4A), suggesting that stress fibers and phosphotyrosine-rich adhesion plaques can assemble into focal adhesions in the absence of vinculin.

Recent in vitro studies suggested that actin filaments may associate with adhesion plaques via several alternative mechanisms including an interaction between the cytoplasmic tail of β_1 integrin with talin and vinculin or directly with α-actinin, an adhesion plaque component that is also a potent actin filament crosslinker (Otey et al., 1990, 1993). In addition, it was shown that talin can bind directly to actin in vitro (Muguruma et al., 1990; Goldmann et al., 1994), and that paxillin is a vinculin-binding molecule (Turner et al., 1990). We have therefore analyzed qualitatively and quantitatively the local intensities of immunolabeling for these proteins in control F9 compared to the vinculin-null γ229 cells, by digital fluorescence microscopy. As shown in Fig. 5 all these proteins were localized in characteristic focal adhesions, both in the vinculin-null γ229 cells and in the F9 parental line. Quantitative analysis of the local immunofluorescence intensities, however, pointed to significant differences between the vinculin-deficient and the control cells. The results, based on the analysis of a large number of focal contacts (300-400, in 3 independent experiments, for each cell type), indicated that the labeling intensities for α-actinin (Fig. 5A and B), talin (Fig. 5C and D) and paxillin (Fig. 5E and F) were higher by 40%, 60% and 80%, respectively, in the vinculin-null cells (Fig. 6). The differences were found to be highly significant (P<0.001-0.005). Western blot analysis with antibodies to paxillin, α-actinin and talin did not detect significant differences in the levels of these proteins between vinculin-deficient and the parental F9 cells (results not shown). These results imply that multiple modes of linking actin filaments to adhesion plaques exist, and that they are enhanced in the vinculin-deficient F9 cells.

DISCUSSION

The primary objective of this study was to investigate the role of vinculin in the assembly of focal contacts by structural and immunochemical analysis of the molecular structure of matrix adhesions after specific disruption of the vinculin gene. The results presented here, and those described (Coll et al., unpublished data), indicate that the elimination of vinculin expression leads to impaired adhesion to different substrata (Coll et al., unpublished data) and reduced ability to spread on fibronectin (Fig. 2B). Nevertheless, it does not inhibit the formation of focal adhesions, as judged by interference reflection and electron microscopic analyses. Moreover, the assembly of major components of focal adhesions such as actin, α-actinin, talin and paxillin, into adhesion plaques, was not impaired; it was even enhanced. This observation suggests

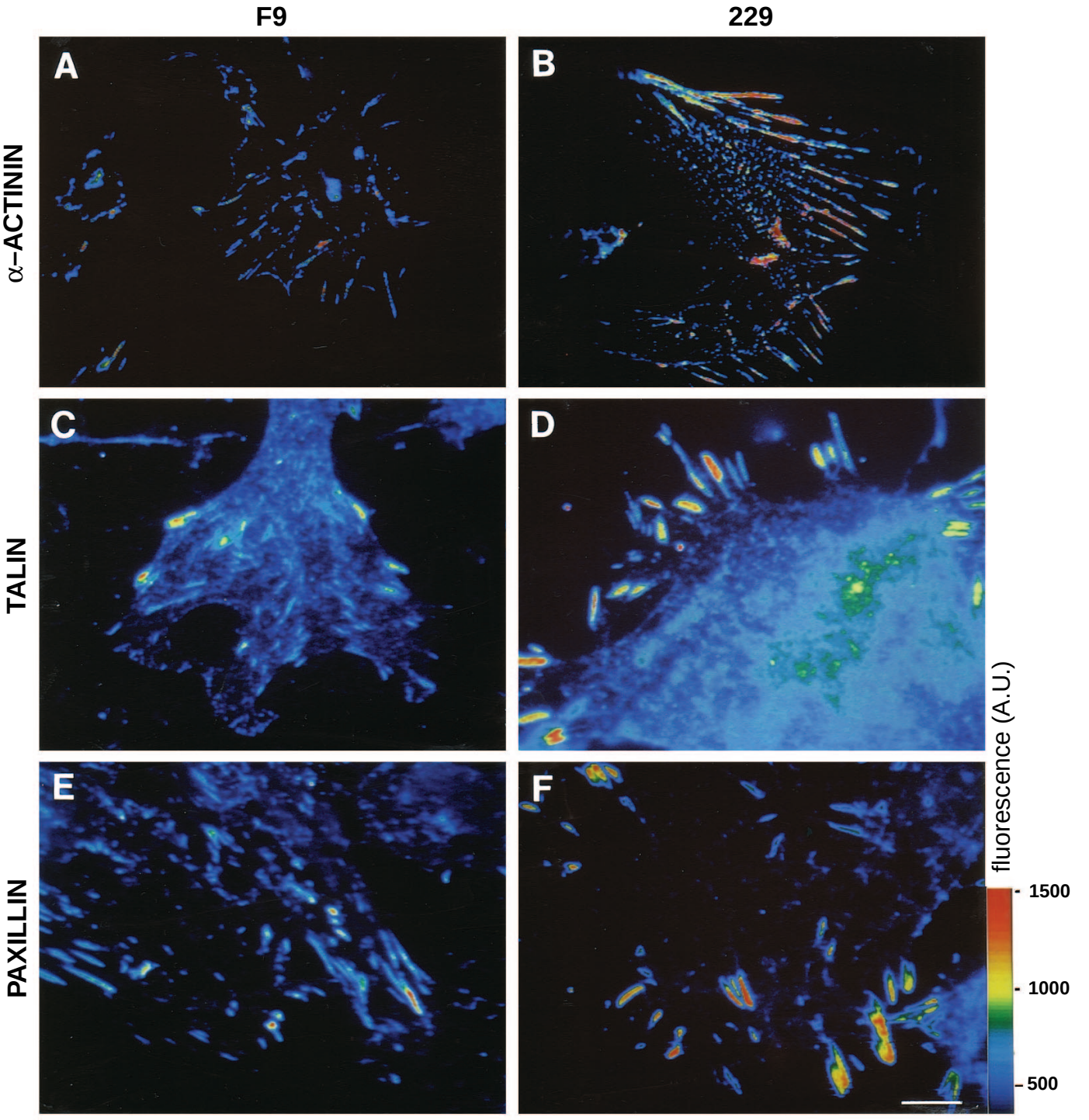

Fig. 5. Localization and quantitation of α-actinin, talin and paxillin in F9 and in vinculin-deficient γ229 cells. F9 (A, C, E) and γ229 cells (B, D, F) were immunofluorescently labeled with monoclonal antibodies to α-actinin (A, B), talin (C, D), and paxillin (E, F), followed by rhodamine-labeled anti-mouse IgG. The fluorescence was recorded using a CCD camera as described in Materials and Methods. and net fluorescence intensity per pixel was calculated and presented using a pseudo color scale. A.U., arbitrary units. Bar in F, 10 μm.

that the linkage of actin to the membrane in these sites includes a 'network' of multiple interactions between the various plaque proteins studied here, and that each of these proteins can bind to focal contacts, also in the absence of vinculin. Such interactions may include direct binding of α-actinin to the cytoplasmic tail of β_1 integrin (Otey et al., 1990, 1993). Similarly, it was shown that the association of talin with focal contacts might be driven not only by binding to vinculin (Horwitz et al., 1986), but also by interaction with other focal contact molecules such as $\alpha_5\beta_1$ integrin, which is expressed at normal levels in the vinculin-deficient F9 cells, as well as actin (Muguruma et al., 1990; Goldmann et al., 1994). On the other

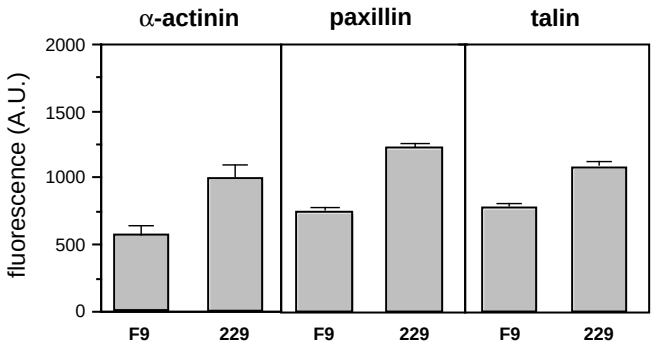

Fig. 6. Quantitative analysis of fluorescence intensity of adhesion plaque components in F9 and vinculin-deficient γ229 cells. The fluorescence intensity of α-actinin, paxillin and talin in individual adhesion plaques from 3 different coverslips for each type of protein was determined by digitized quantitative analysis of pseudo colors in 300-400 focal adhesions for each protein, as shown in Fig. 5. For α-actinin, the values were normalized against the intensity of staining of α-actinin in the corresponding stress fiber for each adhesion plaque. The average plus standard deviations are shown.

hand, the presence of paxillin in focal contacts in the vinculin-deficient cells was unexpected, since it was previously demonstrated that the major adhesion plaque component which binds paxillin is vinculin (Turner et al., 1990). Recent studies have shown, however, that paxillin can associate with SH3-binding domains of tyrosine kinases (Weng et al., 1993), and with the SH2-binding domain of v-Crk (Birge et al., 1993). The assembly of paxillin in focal adhesions may thus be attributed to such interactions in the vinculin-deficient γ229 cells (Fig. 4B).

The availability of the vinculin-null cell line provides further insight into the alternative linkage mechanisms of actin filaments to adhesion plaques. The F9 cell system and the vinculin-deficient mutant cells enabled us to investigate the involvement of vinculin not only in focal contacts, but also in cell-cell adherens-type junctions. It has been shown that upon retinoic acid stimulation both F9 and γ229 cells differentiate into parietal endoderm (Coll et al., unpublished data). Staining of the differentiated cells for actin, using fluorescent phalloidin, revealed an abundance of actin along the sites of cell-cell adhesion in both cell types (data not shown). Vinculin was apparently absent from these cell-cell contacts, both in F9 and in the vinculin-null cells. While the absence of vinculin from cell-cell adhesions in F9 may explain why the vinculin-deficient cells display normal intercellular adhesions, it raises an intriguing question concerning the role of vinculin in cell-cell adherens junctions in general: why is vinculin associated with some adhesions, for example, in cardiac muscle, various epithelial and endothelial cells (Geiger, 1979; Geiger et al., 1992), and not with others (such as those in F9 cells, as shown here)?

It should be pointed out that the absence of vinculin did not significantly affect the retinoic acid-mediated biochemical differentiation of F9-derived cells (Coll et al., unpublished data). Similar results were also reported for F9 cells in which the β_1 integrin genes were inactivated by targeted homologous recombination (Stephens et al., 1993). It is noteworthy that in both vinculin- and β_1 integrin-deficient F9 mutants, cell spreading on the ECM was partially inhibited, suggesting that while parallel structural linkages between the microfilament network and the ECM may exist, elimination of even a single molecule in this chain can affect the strength of cell adhesion and the ability of cells to spread on the substratum.

The vinculin-null F9 cells will be useful in structure-function analyses employing transfection studies with full-length and mutant forms of vinculin to define the role of various vinculin domains in its association with adhesion plaques.

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