

Overexpression of Vinculin Suppresses Cell Motility in BALB/c 3T3 Cells

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The content of vinculin, a cytoplasmic protein found in focal contacts and cell-cell junctions, was increased in BALB/c 3T3 cells by gene transfection. The vinculin expressed from the full length chicken cDNA, incorporated into focal contacts and its pattern was identical to that of the endogenous protein. Cells stably expressing vinculin by 20% over the endogenous level had altered locomotory properties. In these cells, the ability to migrate into a wound formed in a confluent monolayer and the locomotion of individual cells were drastically reduced. The results provide direct evidence that cell locomotion can be regulated by modulating vinculin expression. © 1992 Wiley-Liss, Inc.

Key words: vinculin overexpression, cell migration/locomotion, cell adhesion, cell motility-inhibition

INTRODUCTION

Vinculin, a widely distributed 117,000-dalton protein, is associated with the cytoplasmic aspects of adherens junctions (AJ) formed between neighboring cells, or at cell-ECM contacts [Burridge et al., 1988; Geiger and Ginsberg, 1991]. While vinculin belongs to the group of constitutive structural cell proteins, recent studies have demonstrated that its expression may be modulated in a variety of cell types in culture and in vivo. For example, cultured fibroblasts regulate vinculin mRNA and protein synthesis in response to changes in cell contacts and shape [Ungar et al., 1986; Bendori et al., 1987]. Moreover, vinculin organization and expression are both regulated during differentiation of granulosa cells in culture [Ben-Ze'ev and Amsterdam, 1987] and in the course of adipogenic conversion of 3T3 cells [Rodríguez Fernández and Ben-Ze'ev, 1989], in smooth muscle cells placed in culture [Belkin et al., 1988], and in migrating corneal epithelial cells in vivo [Zieske et al., 1989]. In recent studies, the changes in the organization of vinculin induced by serum stimulation of fibroblasts [Herman and Pledger, 1985], were shown to be accompanied by a transient elevation of vinculin gene transcription and protein synthesis [Ben-Ze'ev et al., 1990; Bellas et al., 1991]. These results suggested that vinculin regulation

may be involved in the response to growth activation and differentiation [Ben-Ze'ev, 1991], although the possible physiological significance of these changes in vinculin expression remained unknown.

To elucidate this aspect, we have isolated a series of 3T3 cell lines stably expressing different levels of a transfected, full length chicken vinculin cDNA. We show that the chicken vinculin in the transfected cells becomes incorporated into focal contacts and its pattern is similar to that of the endogenous protein. Moreover, 3T3 cells expressing the foreign vinculin, at a level about 20% of the endogenous vinculin content, have dramatically altered locomotory properties: they have a diminished ability to close a wound introduced in a confluent monolayer. Furthermore, the locomotion of individual transfected cells, manifested by their capacity to form phagokinetic tracks, was markedly suppressed. This is the first direct demonstration that a relatively modest increase in vinculin content may significantly affect cell motility.

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MATERIALS AND METHODS

Cell Culture and Transfection

BALB/c 3T3 clone A31 cells were grown in Dulbecco's modified Eagle medium plus 10% calf serum (Gibco, Grand Island, NY). The cells were transfected with a full length chicken cDNA coding for vinculin. The construct used for transfection was obtained by ligating the EcoRI-BamHI fragment of the *cvin1* clone [Bendori et al., 1989] containing the 5'-1440 nucleotides, with the EcoRI-BamHI of *cvin5* containing the 3'-sequences, including part of the noncoding 3'-end. Previous studies confirmed that the product obtained following expression in eukaryotic cells was indistinguishable from chicken fibroblast vinculin [Bendori et al., 1989]. This construct was cloned into the BamHI site of the polylinker of the pJ4 Ω expression vector provided by Dr. V. Rotter of the Weizmann Institute of Science [Shauly et al., 1991]. The pJ4 Ω vector consists of the Mo-MuLV LTR promoter-enhancer sequence, the SV40 small t antigen intron, and the SV40 early polyadenylation signal in the pBR322 plasmid. The neomycin resistance (*neo*^r) gene, which was co-transfected with the pJ4 Ω construct containing the full length chicken vinculin, was subcloned into the pSVL expression vector. Cells were transfected by the calcium phosphate precipitation method and colonies resistant to 600 μ g/ml of G418 (Geneticin, Gibco) were selected.

Immunofluorescence

For immunofluorescence microscopy, cells were grown on glass coverslips and fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) and permeabilized with 0.5% Triton X-100. The anti-chicken vinculin monoclonal antibody and the broad-range monoclonal anti-vinculin antibody were those used in a previous study [Bendori et al., 1989]. The second antibody was rhodamine-labeled goat anti mouse IgG. Actin filaments were stained with FITC-phalloidin (Sigma Chem. Co., St Louis, MO). The cells were examined by epifluorescence with a Zeiss Axiophot microscope.

Immunoprecipitation and Polyacrylamide Gel Electrophoresis

For immunoprecipitation, cells were labeled with 200 μ Ci/ml of ³⁵S-methionine for 4 hours. The cells were lysed in RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 25 mM TRIS, pH 8.0). The lysates were clarified by centrifugation at 15,000 g. Equal amounts of radioactive proteins (5×10^6 c.p.m) were incubated with the chicken vinculin specific antibody. The immune complexes were precipitated with *Staphylococcus aureus* (The Enzyme Center, Boston, MA). The proteins were separated on 8% poly-

acrylamide gels containing SDS. Two-dimensional (2-D) gel electrophoresis was performed as previously described [Ben-Ze'ev and Amsterdam, 1987]. Five $\times 10^7$ c.p.m. of ³⁵S-methionine labeled proteins were separated by 2-D gel electrophoresis and transferred to nitrocellulose by electroblotting. The radioactive proteins were visualized by autoradiography of the blot. The positions of the transfected and the endogenous vinculin on the 2-D gels were visualized by immunoblotting with the broad-range monoclonal anti-vinculin antibody, followed by alkaline phosphatase-linked anti-mouse antibody (Promega, Madison, WI). The levels of mouse and chicken vinculin, which were separated on the 2-D gels, were determined using a laser densitometer and computerized analysis of the images with the QUEST software system as described [Garrels, 1989].

Cell Motility and Phagokinetic Tracks

Cells were grown to confluence in 24-well dishes. A wound was introduced in the monolayers by a sharp 1-mm-thick plastic scribe. The medium was changed, and at various times after addition of the fresh medium, cells were fixed and stained with Giemsa. The number of cells migrating into an area of 1 mm² was determined randomly in 10 different areas along the wound for each cell type.

Coverslips dipped into 1% solution of bovine serum albumin were coated with gold chloride (Sigma Chem. Co.) according to Albrecht-Buehler [1977]. Five hundred cells were seeded per each 20 \times 20 mm coverslip, and after 24 hours of incubation the cells were fixed with paraformaldehyde (3%), and the tracks produced by the cells were viewed by darkfield microscopy with a $\times 5$ objective. The lengths of tracks were determined by projecting individual tracks on a screen and measuring randomly about 30 tracks for each cell type.

RESULTS

To study the properties of cells which stably express different levels of vinculin, BALB/c 3T3 cells were co-transfected with a full length cDNA coding for chicken vinculin [Bendori et al., 1989] and with the neomycin acetyl transferase gene (*neo*^r) conferring resistance to the aminoglycoside G418. Colonies resistant to G418 were selected and screened by immunofluorescence microscopy with a monoclonal antibody which selectively recognizes chicken but not mouse vinculin. In the transfected cells, regardless of the level of expression, the exogenous chicken vinculin (Fig. 1A) was localized in adhesion plaques at the termini of actin filaments (Fig. 1B), similarly to the endogenous mouse vinculin. In dense cultures, the transfected vinculin was

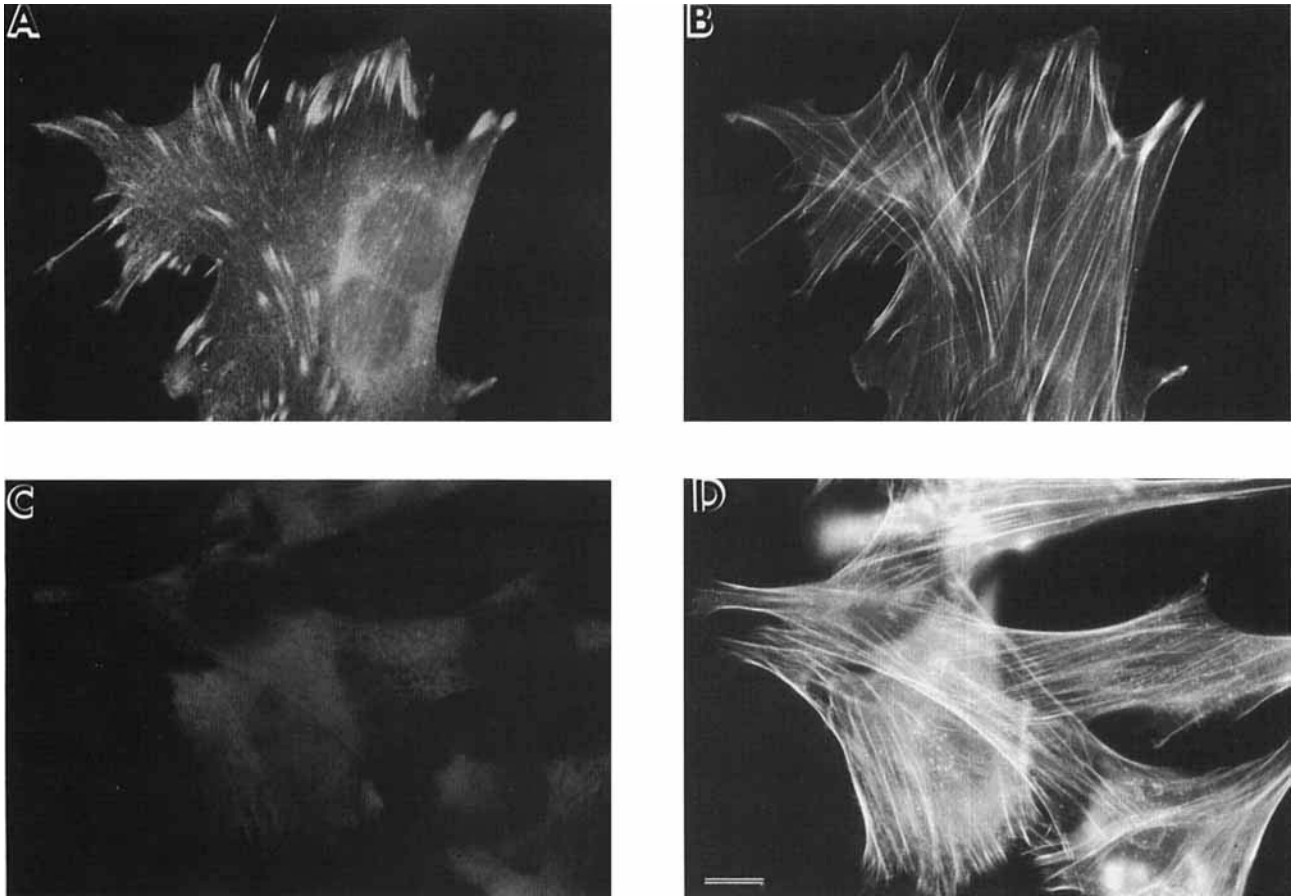


Fig. 1. Immunofluorescence localization of the transfected vinculin. BALB/c 3T3 A31 cells transfected with chicken vinculin (A, B) and cells transfected with the neo^r gene (C, D) were seeded on glass coverslips. Cells were immunostained with a monoclonal anti-vinculin

antibody specific for chicken vinculin, followed by rhodamine labeled goat anti-mouse IgG. (A, C). The labelling for actin was performed with FITC-phalloidin (B, D). Bar in D = 10 μ m, and applies to A–D.

detected also at cell-cell contact areas as in control 3T3 cells (results not shown). Cells expressing only the neo^r had, largely, a similar distribution of actin (Fig. 1D), but were not labeled with the chicken vinculin specific antibody (Fig. 1C).

The levels of chicken vinculin expressed in several transfected clones were highly variable, as determined by quantitative immunoprecipitation analysis (Fig. 2). Equal amounts of 35 S-methionine labeled total cell protein were immunoprecipitated with an antibody specific for chicken vinculin, and the immunoprecipitates separated by SDS-PAGE (Fig. 2). To evaluate the chicken vinculin content relative to the endogenous vinculin level in the transfected cells, total 35 S-methionine labeled cell lysates of selected transfected lines were examined by 2-D gel electrophoresis. The gels were electroblotted onto nitrocellulose paper and the total cellular protein pattern was visualized by autoradiography (Fig. 3A and C). The vinculin spots were identified with a broad-range

antibody against vinculin (Fig. 3B and D), and the levels of chicken and mouse vinculin were evaluated by quantitative, computerized densitometry of the autoradiograms. Chicken vinculin had the same M_r as mouse vinculin, but was slightly more basic than the latter (Fig. 3C), in agreement with previous 2-D gel analyses [Geiger, 1982; Ungar et al., 1986]. The levels of the transfected chicken vinculin in 10 different clones varied between 2 to 25% of the endogenous vinculin level. In clone V101, chicken vinculin represented 19% of the endogenous vinculin. The mouse vinculin levels in clones N87 (neo^r -transfected) and V101 were the same. No changes in the pattern of major cellular proteins, including microfilament associated proteins such as α -actinin, actin and the various nonmuscle tropomyosin isoforms, were detected between control and vinculin-transfected cells. There were small variations in the growth rate among different clones, but these were apparently not related to the amount of vinculin expressed.

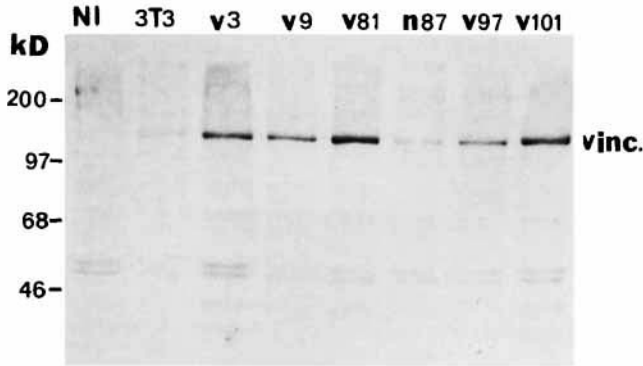


Fig. 2. Levels of chicken vinculin in stably transfected cell lines. Untransfected 3T3 cells, a *neo^r* clone (N87), and several chicken vinculin expressing clones (V3, V9, V81, and V101) were labeled for 6 hours with ^{35}S -methionine, and 5×10^6 c.p.m. of radioactive proteins were used for immunoprecipitation with an anti-vinculin antibody specific for chicken vinculin. The immunoprecipitates were analyzed by SDS-PAGE. An identical sample from clone V81 was immunoprecipitated with non-immune mouse serum (NI). vinc., vinculin.

Since vinculin is considered a major structural component of both cell-cell and cell-ECM contacts which are believed to affect, in a major way, cellular dynamics, we directly assessed the locomotory ability of the various transfected cells by two approaches: a "wound" was introduced in confluent monolayers of untransfected-3T3 cells, a vinculin-transfected clone (V101), and in a clone transfected with the *neo^r* gene alone (N87). Fresh medium was added and cultures were fixed and stained at various time points after wounding of the monolayer (Fig. 4). 3T3 cells and the N87 clone were able to close the wound within 20 to 24 hours, whereas in cells expressing the exogenous vinculin at 20% over the endogenous vinculin (clone V101), the ability to migrate into the wound was markedly reduced (about 3 to 4-fold) (Fig. 4). A similar result was also observed with 5 independent clones expressing chicken vinculin at 15–25% of the endogenous vinculin level. In cells expressing the transfected vinculin at less than 5% above the control, there were no apparent changes in the ability to migrate into the wound. Moreover, revertants were obtained from the clones originally expressing high levels of chicken vinculin after several months of culture in the absence of G418. The revertants which lost the expression of chicken vinculin closed the wound at a rate similar to that of control cells.

To determine whether the apparent immobilization of the transfected cells is due to increased intercellular interactions at the margins of the wound, or a genuine effect on cell locomotion, we examined the migratory capability of individual vinculin-transfected cells. The phagokinetic tracks produced by cells sparsely plated on

coverslips coated with colloidal gold were measured (Fig. 5). The ability to locomote randomly was reduced in single cells by about 4-fold in clones expressing the transfected vinculin at 20–25% over the endogenous level when compared to untransfected cells (Fig. 5).

DISCUSSION

The results obtained in this study demonstrated that overexpression of vinculin in 3T3 cells by about 20%, following transfection, brings about a major change in the dynamic properties of cells. This was manifested by an extensive decrease in the migration of the transfected cells into an artificial wound and in a significant reduction in locomotory activity of individual cells. These changes in cell motility were reversed in cell lines which have lost the expression of the transfected vinculin. It appears that the observations reported here are attributable to the elevated levels of vinculin in the transfected cells, rather than to subtle differences between the foreign chicken vinculin, and the endogenous protein. This suggestion is based on several observations implying that the transfected chicken vinculin is qualitatively equivalent to the intrinsic protein of 3T3 cells: (1) immunofluorescence studies have shown that the transfected vinculin is co-distributed in adhesion plaques with the endogenous vinculin; (2) chicken and mammalian vinculins, both human [Price et al., 1989; Weller et al., 1990] and partial sequences of mouse [Ben-Ze'ev et al., 1990], are over 90% homologous; (3) the partitioning of the transfected chicken vinculin between Triton X-100 soluble and insoluble fractions was identical to that of the endogenous protein; and (4) microinjection studies have shown that chicken vinculin is rapidly incorporated into, and becomes indistinguishable from, the endogenous vinculin pool of the injected mammalian cells [Kreis et al., 1985].

The precise mechanism whereby a specific increase in vinculin level suppresses cell motility is not clear. This reflects the limited current understanding of the molecular basis for cell adhesion and motility, and the interplay between the two. Nevertheless, the results of this study support the possible involvement of vinculin in the regulation of these processes.

Microscopic observations of focal contact formation in live cells indicated that these are highly dynamic structures which may grow, change their size and shape, and then disappear [Abercrombie et al., 1970; Abercrombie and Dunn, 1975]. Studies employing fluorescence photobleaching-recovery assays have shown that microinjected AJ components associate with either a soluble cytoplasmic pool, or with the cell-contact areas. These two pools are in a dynamic equilibrium which depends on the concentration of the various junctional

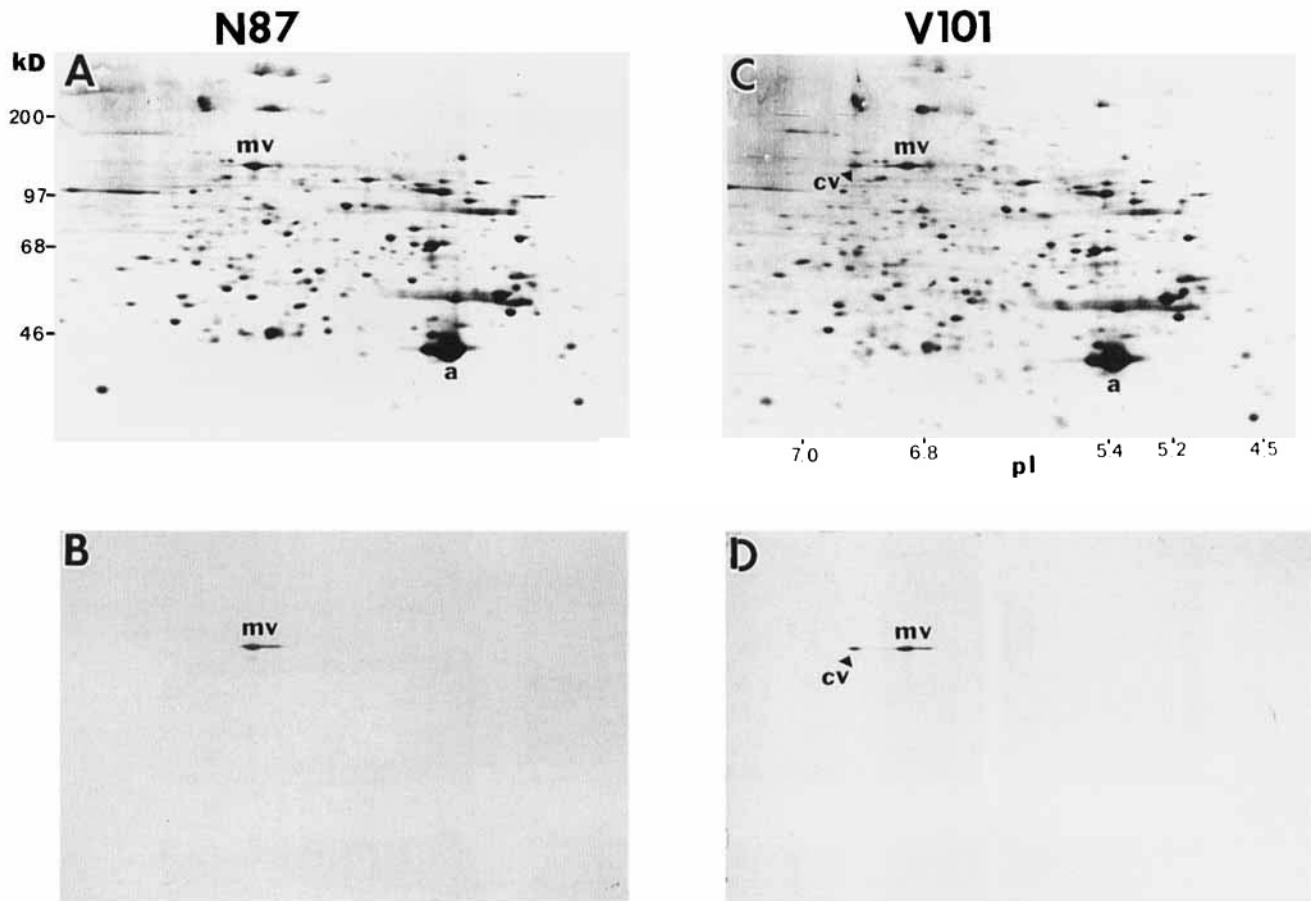


Fig. 3. Identification and levels of chicken vinculin determined by 2-D gel electrophoresis. Cells of a *neo^r* clone (A, B) and of a chicken vinculin-transfected clone (C, D) were labeled for 16 hours with ^{35}S -methionine. Equal amounts of radioactive total cell protein (5×10^7 c.p.m.) were analyzed by 2-D gel electrophoresis. The separated proteins were transferred to nitrocellulose by electroblotting and the pro-

tein pattern was obtained by autoradiography (A, C). The same blots were used to identify vinculin by incubation with a broad-species type anti-vinculin antibody, followed by alkaline phosphatase-coupled anti-mouse IgG (B, D). mv, mouse vinculin; cv, chicken vinculin; a, actin; pI, isoelectric point.

components [Geiger et al., 1984; Kreis et al., 1982]. The central role for vinculin in the control of this equilibrium was suggested by showing that vinculin contains at least two distinct binding sites for AJ components, and thus may contribute to the cooperativity in junctional assembly [Bendori et al., 1989; Geiger et al., 1990]. It is conceivable that changes in vinculin concentration alone could shift the equilibrium between diffusible and organized vinculin, leading to the formation of more stable AJ, which in turn could increase adhesion and reduce motility. It was previously demonstrated that stationary cells contain many large focal contacts in contrast to locomotory cells [Herman et al., 1981]. In spite of the heterogeneity in focal contact size of cultured cells, we consistently observed an increase in adhesion plaque size in 3T3 cells overexpressing vinculin.

The results of this study also lend support to the possible physiological relevance of the alterations in vin-

culin expression which occur in response to cell shape and contact changes [Ungar et al., 1986; Bendori et al., 1987], and following stimulation of cells with serum factors [Ben-Ze'ev et al., 1990; Bellas et al., 1991]. Regulation of vinculin expression may be required for the change in cell adhesion which accompanies cells progressing through the cell cycle, or when responding to a chemotactic signal in the serum.

Genetic studies employed to eliminate different microfilament components, especially in *Dictyostelium*, have shown both involvement of these proteins in various cellular functions, or no changes in cells where these genes were eliminated [Knecht and Loomis, 1987; De Lozanne and Spudich, 1987; Brink et al., 1990]. Studies with cultured mammalian cells have shown that it is possible to alter cell morphology and/or cell movement by transfecting into cells genes coding for individual microfilament associated proteins: the transfection of villin

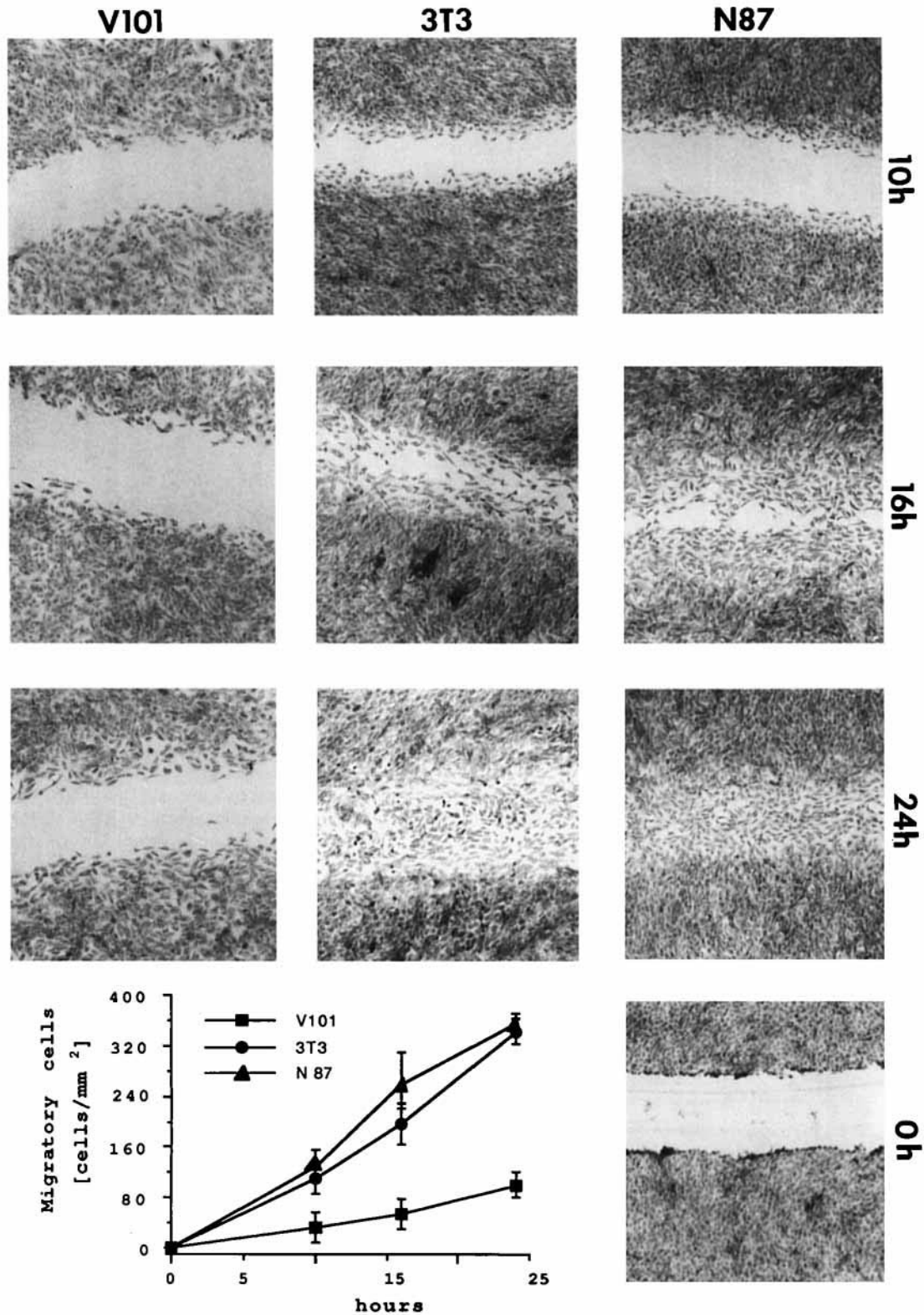


Fig. 4. Reduced ability to migrate into a “wound” of vinculin-transfected cells. 3T3 cells, transfected cells with the neo^r gene alone (N87), and cells transfected with chicken vinculin (V101), were cultured to confluence in 24-well dishes. A wound was introduced in the monolayers with a sharp plastic scribe and the cultures were incu-

bated in fresh medium. At various times after the “wounding”, the cultures were fixed and stained with Giemsa. The number of cells migrating into an area of 1 mm² was determined randomly in 10 different areas along the wound for each cell type at each time point.

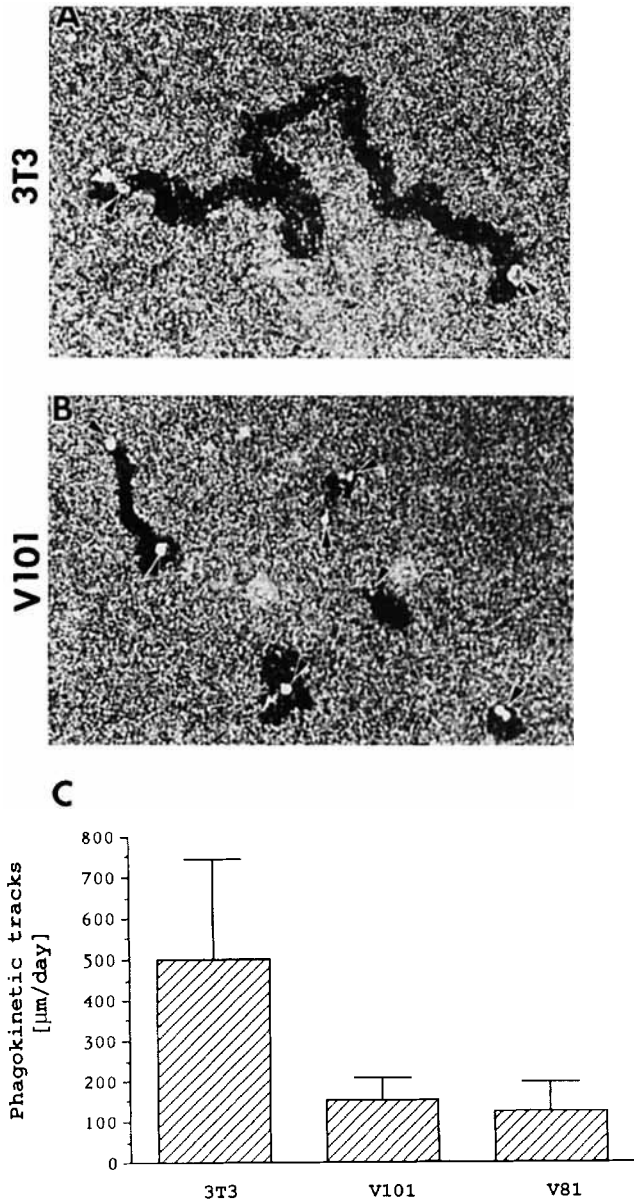


Fig. 5. The migratory ability of individual 3T3 cells and of vinculin transfected 3T3 cells. Cells were seeded sparsely, 500 cells per 20×20 mm coverslip precoated with colloidal gold. The phagokinetic tracks produced by 3T3 cells (A) were compared, 24 hours after seeding the cells, to those of vinculin-transfected cells (clones V81 and V101) by darkfield microscopy (B). The differences in the length of tracks produced by individual cells were determined by projecting the tracks on a screen and measuring randomly the length of about 30 tracks from each cell type (C). The arrowheads point to individual cells.

into cells not expressing this protein caused the formation of longer microvilli [Friedrich et al., 1989]. Most recently, the transfection of gelsolin into 3T3 cells caused an increase in chemotactic movement and in the migration of the cells into an artificially created wound

[Cunningham et al., 1991]. The changes induced in cellular properties in gelsolin-transfected cells were also obtained by elevating gelsolin expression by only 25% over the endogenous level, as was the case with vinculin in the present study. Overexpression of gelsolin was suggested to impair the assembly of the microfilament system and therefore enhance cell migration into the wound. Since many transformed 3T3 cells have an increased invasive ability and display a decrease in the expression of several microfilament components, including vinculin [Raz and Ben-Ze'ev, 1987], it will be interesting to transfect such cells with vinculin and observe their invasive and tumorigenic properties.

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