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Codistribution of pericellular matrix proteins in cultured fibroblasts and loss in transformation: Fibronectin and procollagen

(connective tissue matrix/cell surface/immunofluorescence/malignant transformation/cell adhesion)

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ABSTRACT **Antibodies to fibronectin and to distinct types of procollagens and collagens were used in immunofluorescent staining to localize these proteins in cell cultures. Normal human skin or lung fibroblasts produced a fibrillar pericellular matrix in which fibronectin and procollagen (types I and III) showed extensive codistribution. Fibronectin and procollagen were synthesized by the same cells as judged by double-stain immunofluorescence. Pericellular procollagen was specifically digested with collagenase without an effect on the fibrillar distribution of matrix fibronectin. Brief treatment with trypsin removed both matrix proteins. The human tumor cell lines HT-1080 (fibrosarcoma) and RD (rhabdomyosarcoma) produced little or no matrix fibronectin or procollagen. At sites of cell contact, simian virus 40-transformed lung fibroblasts (VA13) produced small amounts of pericellular fibrillar matrix fibronectin that codistributed with procollagen type I. Intracellular fibronectin and procollagen were visualized in all of these human sarcoma cell lines. When chicken embryo fibroblasts infected with a T class mutant (NY68) of Rous sarcoma virus temperature-sensitive for transformation were maintained at the nonpermissive temperature (41°) the cells had normal phenotype and a fibrillar matrix containing fibronectin and procollagen was present. At the permissive temperature (35°), the cells showed transformed phenotype and the matrix was lost. The failure to produce a pericellular fibronectin/collagen matrix may account for several phenotypic characteristics of transformed cultured fibroblasts.**

Fibronectin[¶] is a major pericellular matrix protein in cultures of adherent fibroblastic cells and is lost in layers of transformed cells (for reviews, see refs. 1 and 2). A role for the pericellular fibronectin in cell-to-cell and cell-to-growth substrate interactions has been suggested by immunofluorescent and immunoelectron microscopic studies (3, 4). *In vivo*, a protein antigenically indistinguishable from and structurally identical to cellular fibronectin by several criteria is present in the circulation (cold-insoluble globulin) and also in loose connective tissue matrix and basal laminae (1). The connective tissue matrix *in vivo* is composed of several types of collagen, proteoglycans, elastin, and other glycoproteins (5).

Development of antibodies specific for the distinct types of procollagen and collagen (6) has made it possible to study the distribution of collagenous proteins in cell cultures. Individual human fibroblasts were shown to synthesize both type I and type III collagen simultaneously (7). Use of anticollagen antibodies in immunofluorescence studies also showed that procollagen is present in fibrillar pericellular form (7, 8) in a manner resembling the distribution of pericellular fibronectin (9). We now report that the two matrix proteins, fibronectin and procollagen, show extensive codistribution in fibroblast cultures and that both matrix components are lost in transformation.

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MATERIAL AND METHODS

Cells. The cell lines used were WI-38 human (diploid embryonic human lung fibroblasts) and VA13 (their simian virus 40-transformed derivatives) (10). The transformed VA13 cells are positive for the simian virus 40-specific nuclear T antigen (11). The ER and ES (diploid human adult skin fibroblasts) cell lines were established locally. The WI-38, RD (human rhabdomyosarcoma) and HT-1080 (human fibrosarcoma) lines were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were grown at 37° in a humidified 5% CO_2 atmosphere in Eagle's basal medium modified for diploid cells and supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin(50 μg/ml). In some experiments, as indicated, the culture medium contained sodium ascorbate (30 μg/ml). The cells were subcultured (1:2) twice a week by using 0.25% trypsin/0.02% EDTA in Hanks' buffered saline to disperse the cells. All human cell lines were shown to be free of mycoplasma (12).

Tertiary cultures of chicken embryo fibroblasts, uninfected or infected with wild-type Rous sarcoma virus (RSV; Schmidt–Ruppin strain, subgroup A) or its T-class mutant (NY68, ref. 13) were prepared as described (14). Cells infected with NY68 mutant virus, when maintained at 41°, had a phenotype almost identical with that of normal uninfected cells. At 35° they were morphologically indistinguishable from cells infected with wild-type RSV.

Antisera. Antiserum to human plasma fibronectin was raised in rabbits. The purity of the antigen used for immunization was confirmed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis in which a single polypeptide band was detected. Immunofluorescence obtained in indirect staining was completely blocked by preincubation of the antiserum with purified plasma fibronectin. The antifibronectin antiserum gave a single precipitation line against normal human plasma, against extract of human fibroblasts, and against the purified protein in immunodiffusion analysis (15) and gave a single polypeptide band in immunoprecipitation with radiolabeled fibroblast extracts (4).

Antifibronectin goat antiserum, a gift from D. Mosher, was prepared as before (16). Purified plasma fibronectin specifically blocked staining reactions by this antiserum.

Rabbit antibodies to bovine type I and type III collagen and procollagens were prepared as described by Nowack *et al.* (17). These antibodies were rendered specific for the immunizing antigen by immunoabsorption (6). No significant crossreaction was observed between antibodies against type I or type III collagen proteins (17). The purified antibodies showed similar

Abbreviations: FITC, fluorescein isothiocyanate; RSV, Rous sarcoma virus; TRITC, trimethylrhodamine isothiocyanate.

[¶] This protein is also known as cell surface protein (CSP), cold-insoluble globulin, fibroblast surface (SF) antigen, and large external transformation-sensitive (LETS) protein.

reactions with bovine and human tissue components when studied by immunofluorescence techniques (6). Antibodies to chicken type I procollagen were prepared according to Dehm *et al.* (18). Antibodies to type II collagen (6) were used as negative controls. Absorption of the (pro)collagen antibodies with purified fibronectin had no detectable effect.

Immunofluorescence Staining and Microscopy. For study of pericellular proteins, the cell cultures were fixed with paraformaldehyde; for visualization of both pericellular and intracellular proteins, they also were treated with acetone at −20° (15). Indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG sheep immunoglobulin has been described (15). To visualize fibronectin and procollagen simultaneously, IgG was isolated from the antifibronectin goat antiserum, conjugated with tetramethylrhodamine isothiocyanate (TRITC) according to Bergquist and Nilsson (19), and used to stain the cell layers after indirect antiprocollagen FITC-staining. All results obtained for fibronectin with the direct conjugate could be duplicated by indirect staining using the rabbit antiserum. In the double-stain experiments, when the TRITC-conjugated antifibronectin goat IgG was applied first, (pro)collagen rabbit antibodies were omitted, and anti-rabbit IgG-FITC was used last, no FITC-fluorescence was detected. The rabbit antibodies were used in the following concentrations: antifibronectin antiserum, 1:40; anticollagens, 20 μg/ml; antiprocollagens type I, 30 μg/ml; and antiprocollagen type III, 15 μg/ml. The goat antifibronectin IgG fraction was used at 240 μg/ml.

For microscopy, a Zeiss Universal microscope with epi-illuminator III RS and an HBO/200-W lamp for specific fluorescence excitation together with filters for FITC (490 nm excitation light) or TRITC (545 nm excitation light) was used. There was no spillover of FITC-fluorescence into the TRITC-channel, and only a minimal amount of pale yellow TRITC-derived fluorescence, distinct from the "apple-green" FITC-fluorescence, was detected in the FITC-channel. To study codistribution of fibronectin and procollagen in the double-stained specimens, FITC fluorescence was photographed first and then the filters and dichroic mirrors were changed and the same field was photographed for TRITC fluorescence. Fluorescence micrographs were made on Kodak high-speed Ektachrome film with standard exposure times (30 sec for FITC and 45 sec for TRITC). The location of fibronectin fluorescence relative to the growth substratum and cell surfaces was determined by sequential use of phase-contrast and immunofluorescence optics without changing the objective or level of focus.

Enzyme Digestions. Live cultures were washed twice with serum-free medium, exposed to either 10 μg of bovine pancreatic trypsin (Trypsin-TPCK, Worthington, Freehold, NJ) per ml for 10 min or to 50 μg of bacterial collagenase (form III, 500 units/mg, Advance Biofacturers Corp., Lynbrook, NY) per ml for 60 min at 37° in serum-free medium, and then fixed for immunofluorescence.

RESULTS

Characterization of Antibodies. Possible antigenic crossreactions between fibronectin and (pro)collagen were tested by using passive hemagglutination and radioimmunoassay tests by procedures similar to those published previously (17). No significant crossreactivity was detected by either assay (Table 1).

Codistribution of Fibronectin and Procollagen. To study the distribution of fibronectin and collagenous proteins, double-stain immunofluorescence was used. The fixed cell layers were first stained indirectly for procollagen by using rabbit antibodies and FITC-conjugated anti-rabbit IgG and then directly for fibronectin by using TRITC-conjugated goat IgG. In confluent cultures of human fibroblasts, fibronectin and collagenous proteins showed extensive codistribution in pericellular fibrillar matrix. This is shown in Fig. 1 *A* and *B* for fibronectin and procollagen type I in cultures of WI-38 fibroblasts. A similar result was found for fibronectin and procollagen type III and collagen type I or type III. Human enbryonic and adult skin fibroblasts showed a similar superimposition of fibronectin and procollagen. Study of cell layers fixed with both paraformaldehyde and acetone to visualize intracellular antigen showed cytoplasmic staining of fibronectin and procollagen in the same cells (data not shown; see also Fig. 1 *I* and *J*). This codistribution of both pericullular and cytoplasmic proteins was observed irrespective of whether the culture medium was supplemented with ascorbate or not. When ascorbate was added, the intensity of cytoplasmic procollagen immunofluorescent staining was less. This was the only effect of ascorbate observed in the present studies.

In fibroblast cultures examined early (up to about 1 hr) after seeding of trypsinized cells, pericellular fibronectin, located

Table 1. Immunological reactivity between fibronectin and procollagens in passive hemagglutination and radioimmunoassays

	Agglutination titer* ($-\log_2$) with antibodies to					
	Fibronectin	Collagen		Procollagen		
		Type I	Type III	Type I†	Type III†	Type I‡
Erythrocytes coated with:						
Fibronectin, human	19	3	<2	3	3	<2
Type I collagen, bovine	<3	12				
Type III collagen, bovine	3		14			
Type III procollagen, bovine	<3				16	
Type I procollagen, COOH-terminal peptide, chicken	<3					11
	% binding in radioimmunoassays§					
Antigens labeled with ^{125}I:						
Type I collagen, bovine	0.9	102.0				
Type III collagen, bovine	3.7		100.4			
Type I procollagen, sheep¶	0.3			90.4		
Type III procollagen, bovine¶	−2.1				84.1	

* Normal rabbit serum reacts with titers of 3–4.
† Antibodies to the NH_2-terminal precursor-specific segment.
‡ Antibodies to the COOH-terminal precursor-specific segment.
§ Amount of binding by antiserum (diluted 1:10) minus nonspecific binding by normal rabbit serum; the error of the method is 3–5%, at most.
¶ Collagenase-derived peptide from the NH_2-terminal precursor-specific region.

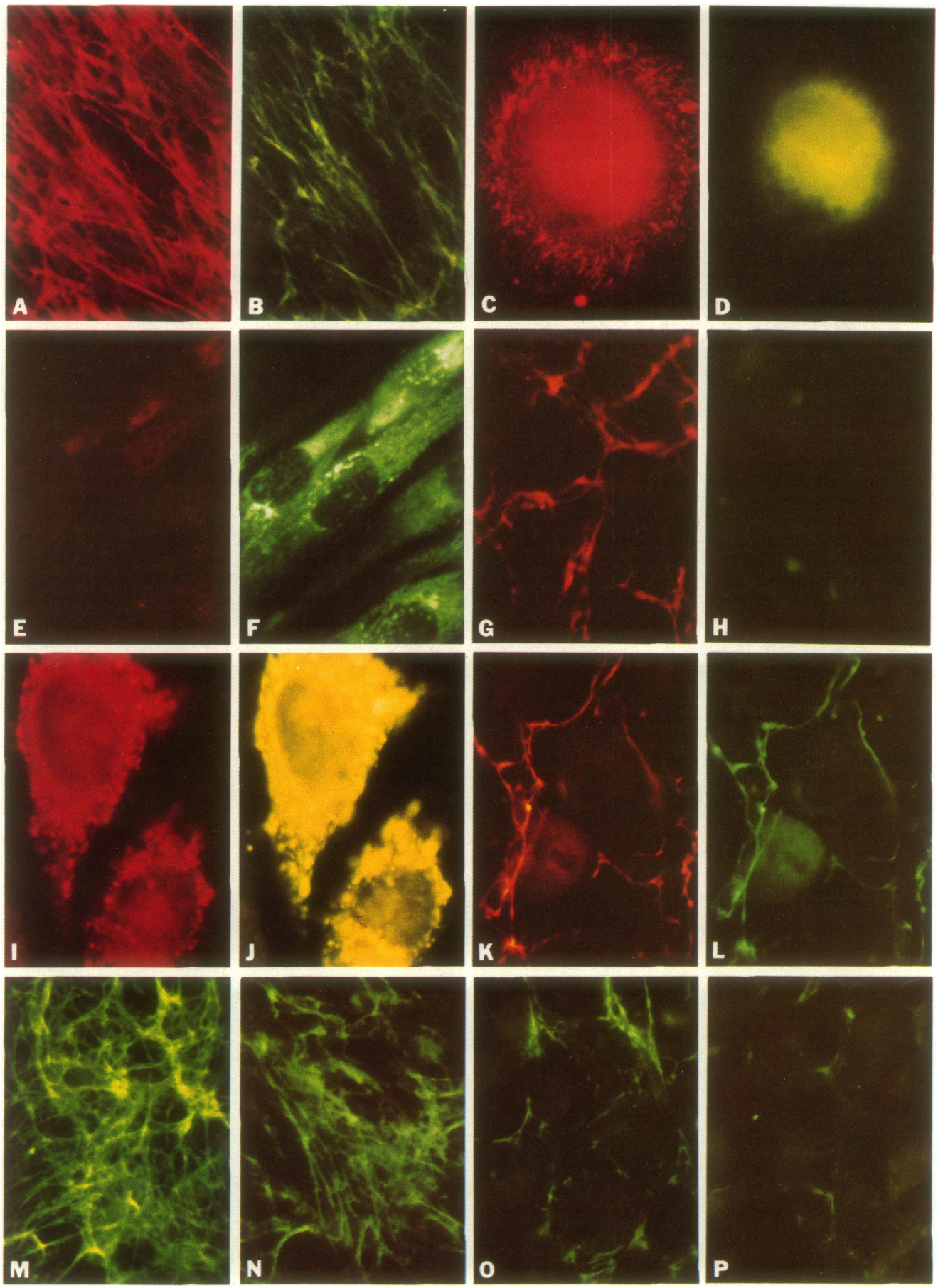

Fig. 1. (*Legend on following page.*)

by phase-contrast microscopy, was detected on the under surface of the cells (but procollagen was not) (Fig. 1 *C* and *D*) although in acetone-treated cells both fibronectin and procollagen were visualized in the cytoplasm. Examination of cells after longer subculture showed that, when present pericellularly, procollagen was detected exclusively at sites where matrix fibronectin was also found.

Effect of Trypsin and Collagenase. Brief treatment of live fibroblast cultures with trypsin removed all detectable pericellular fibronectin and procollagen type I without grossly affecting cell morphology. In such trypsin-treated fibroblasts, only cytoplasmic fibronectin and procollagen could be stained (Fig. 1 *E* and *F*). In contrast, digestion of cell layers with collagenase removed only pericellular procollagen type I but had no detectable effect on pericellular fibrillar fibronectin (Fig. 1 *G* and *H*) or on cytoplasmic staining of fibronectin and procollagen type I. The results of enzyme digestions were similar when collagen type I and procollagen type III antibodies were used.

Transformed Cells. The human tumor cell lines HT-1080 (fibrosarcoma) and RD (rhabdomyosarcoma) and the simian virus 40-transformed fibroblasts (VA13) are known (20) to produce large amounts of fibronectin in culture. Layers of HT-1080 or RD cells, even in dense culture, had little or no detectable pericellular fibronectin or procollagen, although cytoplasmic fibronectin and procollagen were detected (Fig. 1 *I* and *J*) in amounts varying with the type of procollagen and the cell line. The RD cells stained exceptionally strongly for procollagen type III (Fig. 1 *J*). In all experiments, VA13 cells at high cell density showed detectable amounts of pericellular fibronectin that codistributed with procollagen type I and was prominent at sites of cell-to-cell contact (Fig. 1 *K* and *L*). No matrix procollagen type III was detected.

To study the relationship of pericellular matrix protein and transformation more closely, the RSV/chicken embryo fibroblast system was used. Normal layers of normal chicken embryo fibroblasts had a dense meshwork of fibrillar matrix in which both fibronectin and procollagen type I were present. In cultures of fibroblasts transformed by RSV, little or no pericellular fibronectin or procollagen was detected. Chicken fibroblasts infected with the NY68 mutant of RSV had both fibronectin and procollagen in the pericellular matrix (Fig. 1 *M* and *N*) at the temperature non-permissive for transformation (41°) whereas at the temperature permissive for transformation (35°) they had lost most of the matrix (Fig. 1 *O* and *P*). Intracellular fibronectin and procollagen were detected both in cells of normal (uninfected or NY68-infected cells at 41°) or transformed phenotype (RSV-infected or NY68-infected cells) at 35°).

DISCUSSION

These studies on cultured fibroblasts show extensive codistribution of fibronectin and procollagen in the pericellular fibrillar matrix by immunofluorescence. The experiments with proteases as well as different specific ways of visualizing intracellular antigen (15) show the same distribution in the cytoplasmic space, indicating that the same cells synthesize both proteins. This was shown for embryonic and adult fibroblasts and for procollagen types I and III. The antibodies against procollagen types I and III are directed toward sites in the NH_2-terminal precursor-specific regions of the pro-α_1 chains (21). The strong staining of the pericellular matrix with anti-procollagen antibodies we observed is in line with previous reports on defective processing of procollagen under cell culture conditions (22, 23). The same structures could also be stained with antibodies specific for the collagenous portion of the procollagens (21). This agrees with a polypeptide analysis of the isolated pericellular fibrillar matrix (unpublished data) that shows procollagen chains with apparent molecular weights of 170,000 and 145,000 together with fibronectin (220,000) and glycosaminoglycans to be present in the matrix.

These immunofluorescence studies should not be taken to indicate molecular interaction between fibronectin and procollagen because the resolution of the technique is only of the order of 200–500 nm. However, an interaction may exist because soluble fibronectin is known to bind to collagenous proteins: collagen types I, III, and IV and, to a lesser extent, type II bind to plasma fibronectin. The binding site in collagen type I has been localized to a large cyanogen bromide fragment in the α_1 chain (residues 568–835). The binding does not require divalent cations and is enhanced by denaturation of the collagens to gelatin (24–26).

The pericellular fibronectin/procollagen matrix can be considered the *in vitro* equivalent of the connective tissue matrix, where fibronectin is found *in vivo* (27, 28). The digestion experiments suggest that procollagen is not essential for the stability of the pericellular matrix and support the possibility that fibronectin may have an organizing role in matrix formation. α,α'-Dipyridyl, which inhibits procollagen secretion (29), appears to prevent appearance of the procollagen matrix without grossly affecting fibronectin deposition (unpublished data). This also suggests that fibronectin may be associated with fibrillar matrix structures independently of the presence of procollagen. The way in which proteoglycans or other structural proteins (5) contribute to matrix formation and to the structure of the matrix is not known. Because fibronectin is also a distinct component of basal liminae, particularly during embryogenic development (27, 28), it seems likely that the matrix contains information important for the orderly growth and positioning of cells (30). Fibronectin may form a structural link between the cells and the matrix (31).

Loss of pericellular fibronectin in transformed cells has been

FIG. 1 (*on preceding page*). (*A* and *B*) Human embryonic lung fibroblasts (WI-38) cultured for 4 days, fixed with paraformaldehyde, and stained simultaneously for immunofluorescence by using TRITC-conjugated antibodies for pericellular fibronectin (*A*) or FITC-conjugated antibodies for procollagen type I (*B*). (×630.) The same field was first photographed for TRITC-fluorescence (*A*) and then for FITC-fluorescence (*B*). (*C* and *D*) Human adult skin fibroblasts (cell line ES) were trypsinized and, after 1 hr of subculture, were fixed with paraformaldehyde and acetone and stained simultaneously to visualize cellular fibronectin (*C*) and procollagen type I (*D*). (×1000.) (*E* and *F*) Human adult skin fibroblasts (cell line ES) were cultured for 2 days, treated briefly with trypsin, fixed with paraformaldehyde and acetone, and simultaneously stained for fibronectin (*E*) and procollagen type I (*F*). (×660.) (*G* and *H*) Human adult skin fibroblasts (cell line ES) were cultured for 2 days, treated with collagenase, fixed with paraformaldehyde, and stained simultaneously for pericellular fibronectin (*G*) and procollagen type I (*H*). (×630.) (*I* and *J*) Human rhabdomyosarcoma cells (cell line RD) were cultured for 2 days, fixed with paraformaldehyde and acetone, and stained simultaneously for fibronectin (*I*) and procollagen type III (*J*). The yellow color in *J* is due to high-intensity fluorescence that is reproduced in the photographic prints as yellow instead of green. (×1000.) (*K* and *L*) Simian virus 40-transformed embryonic lung fibroblasts (VA13) were cultured for 2 days, fixed with paraformaldehyde, and stained simultaneously for pericellular fibronectin (*K*) and procollagen type I (*L*). (×630.) (*M–P*) Tertiary chicken embryo fibroblasts infected with NY68 mutant of RSV were cultured for 2 days at either 41° or 35°, fixed with paraformaldehyde, and stained separately for either fibronectin or procollagen type I: (*M*) fibronectin, 41°; (*N*) procollagen type I, 41°; (*O*) fibronectin, 35°; (*P*) procollagen type I, 35°. (×630.)

consistently observed in cultures of transformed fibroblastic cells (1, 2). It was of interest to note that such a change was found also for matrix procollagen. Decreased synthesis and secretion has been observed in certain types of transformed cells (32–35). The distinct intracellular staining we observed for procollagen in the RD and VA13 sarcoma cells shows that these cells have not lost the ability to synthesize procollagen. Loss of the pericellular fibronectin/procollagen matrix is closely associated with the transforming process, as could be clearly shown in the experiments with the NY68 mutant virus-infected chicken embryo fibroblasts. This is also supported by results of Arbogast *et al.* (36), who measured the amount of collagen in layers of normal and RSV-transformed chicken fibroblasts and found none in the latter. Loss of matrix fibronectin in transformed cells, due to decreased synthesis (37) or decreased binding of the fibronectin molecules (11, 20), may interfere with the deposition of pericellular procollagens in transformed cells. Loss of the pericellular fibronectin/procollagen matrix represents a major structural change and may well relate to several of the *in vitro* characteristics of transformed cells (38).

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