Codistribution of Heparan Sulfate Proteoglycan, Laminin, and Fibronectin in the Extracellular Matrix of Normal Rat Kidney Cells and Their Coordinate Absence in Transformed Cells

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ABSTRACT   We used antibodies raised against both a heparan sulfate proteoglycan purified from a mouse sarcoma and a chondroitin sulfate proteoglycan purified from a rat yolk sac carcinoma to study the appearance and distribution of proteoglycans in cultured cells. Normal rat kidney cells displayed a fibrillar network of immunoreactive material at the cell surface when stained with antibodies to heparan sulfate proteoglycan, while virally transformed rat kidney cells lacked such a surface network. Antibodies to chondroitin sulfate proteoglycan revealed a punctate pattern on the surface of both cell types. The distribution of these two proteoglycans was compared to that of fibronectin by double-labeling immunofluorescent staining. The heparan sulfate proteoglycan was found to codistribute with fibronectin, and fibronectin and laminin gave coincidental stainings. The distribution of chondroitin sulfate proteoglycan was not coincidental with that of fibronectin. Distinct fibers containing fibronectin but lacking chondroitin sulfate proteoglycan were observed. When the transformed cells were cultured in the presence of sodium butyrate, their morphology changed, and fibronectin, laminin, and heparan sulfate proteoglycan appeared at the cell surface in a pattern resembling that of normal cells. These results suggest that fibronectin, laminin, and heparan sulfate proteoglycan may be complexed at the cell surface. The proteoglycan may play a central role in the assembly of such complexes since heparan sulfate has been shown to interact with both fibronectin and laminin.

Proteoglycans are implicated in a number of important cellular phenomena. They are present as components of various extracellular matrices including cartilage (1) and basement membranes (2–4). In addition to their structural role, proteoglycans are present on cell surfaces (5–8) and are thought to be involved in the adhesion of normal and malignant cells (9–12). They may also function as mediators of specific cell surface interactions directing cellular differentiation and movements during development (13).

Most studies on cell surface proteoglycans have focused on the glycosaminoglycan component of proteoglycans using [^{35}S]sulfate as marker or by staining with ruthenium red. Growing cells have been found to shed their cell surface-associated heparan sulfate during mitosis (14). Changes have

been observed in the quantity and properties of cell-associated glycosaminoglycans upon transformation (15–17). While there is no complete agreement on the nature of the changes, it appears that the amount of heparan sulfate in the cell layer decreases in cultures of transformed cells, while hyaluronic acid is present in increased concentrations. A transformation-associated increase in chondroitin sulfates has also been reported (18). The addition of sulfated polysaccharides to cell cultures decreases their saturation density and causes transformed cells to assume a more normal morphology (19–21).

Relatively little is known about the distribution and expression of intact proteoglycans. Recently, antibodies have been raised against both the protein core of a heparan sulfate proteoglycan (22) and a chondroitin sulfate/dermatan sulfate

proteoglycan (23). We used these antisera to examine the distribution of proteoglycans in cultured cells and to establish the relationship of the proteoglycans to fibronectin and laminin. We used a normal cell line which we have shown to contain fibronectin and laminin in the extracellular matrix, and its transformant lacking such a matrix (24). We now report that antibodies reveal marked differences in the distribution of proteoglycans at the surface of the normal and transformed cells and that heparan sulfate proteoglycan codistributes with fibronectin and laminin in the extracellular matrix.

MATERIALS AND METHODS

Cells

Normal rat kidney cells, subline 9 (NRK) (25, 26), and the transformed and tumorigenic (27) rat kidney cell line (TRK), 1255 B-7 (which was derived from NRK transformed by Kirsten sarcoma virus [26]), were grown in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Flow Laboratories, Inc., Inglewood, CA). Cultures were free of mycoplasma as tested by the method of Russell et al. (28). Sodium butyrate treatment of the TRK cells was performed by culturing the cells for 7–14 d in the presence of 2.5 mM butyrate (29).

Antisera to Proteoglycan

Preparation of antiserum to a heparan sulfate proteoglycan isolated from the basement membrane-producing EHS mouse tumor has been described (22). The antiserum was absorbed with laminin coupled to Sepharose.

Antiserum against a chondroitin sulfate/dermatan sulfate proteoglycan was prepared by immunizing a rabbit with proteoglycan purified from a rat yolk sac tumor. The preparation and properties of proteoglycan and the antiserum have been described (23). Antibodies were isolated from the antiserum by affinity chromatography on Sepharose to which the proteoglycan was coupled.

Antifibronectin and antilaminin sera have been described previously (30, 31).

Immunofluorescence

Cells to be examined by immunofluorescence were grown on glass coverslips and stained without fixation or after fixation with 3.0% paraformaldehyde (24). Fibronectin was detected using direct immunofluorescent staining with affinity-purified goat anti-rat fibronectin antibodies coupled to fluorescein. The conjugate was prepared according to the method of Johnson and Holborow (32) by adding dry fluorescein isothiocyanate to a solution of affinity-purified antibodies (30) followed by separation of the conjugated antibodies from the free dye by chromatography on Sephadex G-25.

Indirect immunofluorescence was used to detect laminin and the proteoglycans. Rabbit antibodies to laminin or the proteoglycans were incubated with the cells, and the binding of rabbit immunoglobulin was detected by incubation with rhodamine-conjugated goat anti-rabbit IgG (N. L. Cappel Laboratories, Cochranville, PA). For double fluorescence, the indirect staining for laminin or proteoglycan was followed by direct staining with the goat antifibronectin or vice versa. Negative controls included absorption of the antiheparan sulfate, antichondroitin sulfate, antifibronectin, and antilaminin with purified heparan sulfate proteoglycan, chondroitin sulfate proteoglycan, plasma fibronectin, and rat yolk sac tumor laminin, respectively, and substitution of the antibodies with normal rabbit IgG. The fluorescence was viewed using appropriate filters in a Zeiss microscope equipped with phase-contrast and epifluorescence optics.

RESULTS

Immunofluorescent Staining of Proteoglycans in NRK and TRK Cells

The normal cells exhibited in immunofluorescence a fibrillar staining pattern with the heparan sulfate proteoglycan antibodies (Fig. 1 a and b). The chondroitin sulfate proteoglycan antibodies revealed a punctate pattern distributed over the surface of the cell, clustering at random intervals into bright blebs (Fig. 1 c). The transformed cells, when stained with the heparan sulfate proteoglycan antibodies, lacked the fibrillar

network detected on the NRK (Fig. 1 d and e). They exhibited only marginally detectable diffuse fluorescence. The staining with the antichondroitin sulfate proteoglycan in TRK cells was in marked contrast to the staining observed with the other antibodies to extracellular matrix components. These cells exhibited intense cell surface staining similar to the punctate pattern seen on NRK cells. The TRK cells also showed the bright blebbing along the narrow processes of the cell body (Fig. 1 f). The staining patterns of fixed and unfixed cells were identical. The staining for the heparan sulfate and chondroitin sulfate proteoglycans could be inhibited in each case with the specific antigen.

Double-label Immunofluorescence

Double-label immunofluorescence was used to study the distribution of the proteoglycans in relation to the distribution of fibronectin. The same technique was also used to establish the relationship of fibronectin to laminin.

The staining of the NRK cells for heparan sulfate proteoglycan was coincidental with the staining pattern for fibronectin (Fig. 2). Similarly, the staining pattern for fibronectin was mostly coincidental with the staining for laminin (Fig. 3), although the laminin staining tended to have a more punctate appearance than fibronectin.

When the double-staining technique was used to detect simultaneously the chondroitin sulfate proteoglycan and fibronectin, it was found that this proteoglycan did not codistribute entirely with fibronectin. There were fibers containing fibronectin which lacked (within the limits of our detection) the chondroitin sulfate (Fig. 4). However, structures positive for chondroitin sulfate proteoglycan were also positive for fibronectin staining. The order of application of the various antibodies as described in Materials and Methods or focusing of the microscope to different planes did not affect the staining and codistribution patterns. The staining of the heparan sulfate proteoglycan, the chondroitin sulfate proteoglycan, and laminin could be inhibited by absorption of the appropriate antiserum with the specific antigens without affecting the staining of the fibronectin. Likewise, the staining of fibronectin could be inhibited with fibronectin without affecting the staining for the proteoglycans or laminin.

Reappearance of Fibronectin, Laminin, and Heparan Sulfate Proteoglycan Staining in Butyrate-treated TRK Cells

Culturing of the TRK cells in the presence of butyrate has previously been shown to change the morphology of these cells toward that of normal cells and to restore their cell surface matrix containing fibronectin (29). Butyrate treatment also led to the reappearance of the cell surface heparan sulfate proteoglycan and laminin, and double-labeled immmunofluorescent staining (Figs. 5 and 6) confirmed the codistribution of these macromolecules with fibronectin found in normal cells.

DISCUSSION

Antibodies to proteoglycans have made it possible to study their distribution in cell culture by immunofluorescence techniques. Much of the previous information about the distribution of proteoglycans has been obtained from chemical assays. This immunochemical method complements and extends the
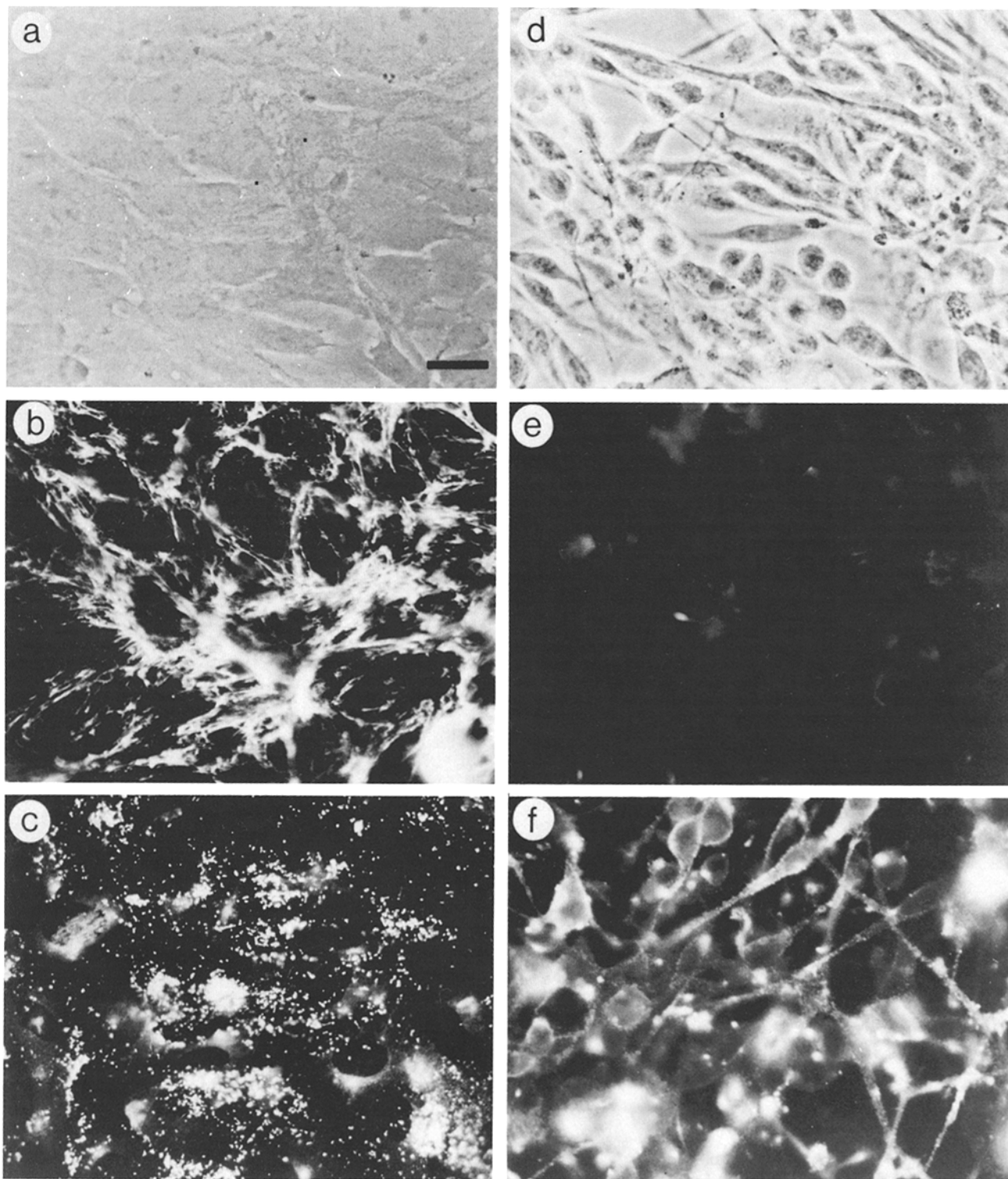
FIGURE 1   Immunofluorescent staining for proteoglycans in NRK and TRK cells. (a and d) Phase-contrast micrographs of the NRK and TRK cells, respectively. (b) NRK cells stained with antiheparan sulfate proteoglycan. (c) NRK cells stained with antichondroitin sulfate proteoglycan. (e) TRK cells stained with antiheparan sulfate proteoglycan. (f) TRK cells stained with antichondroitin sulfate proteoglycan. Bar, 25 μm. × 400.

previous information about their distribution. These antibodies are directed in the main against the protein portion of the proteoglycan and not against the attached glycosaminoglycans (22, 23). For this reason, they may recognize only certain of the proteoglycans containing either heparan sulfate or chondroitin sulfate.

Three findings emerge from our study: (a) heparan sulfate and chondroitin sulfate proteoglycans differ in their distribution in cultured normal rat kidney cells; (b) the heparan sulfate proteoglycan codistributes with fibronectin and laminin in the extracellular matrix of normal rat kidney cells; and (c) virally transformed rat kidney cells lack a detectable matrix containing
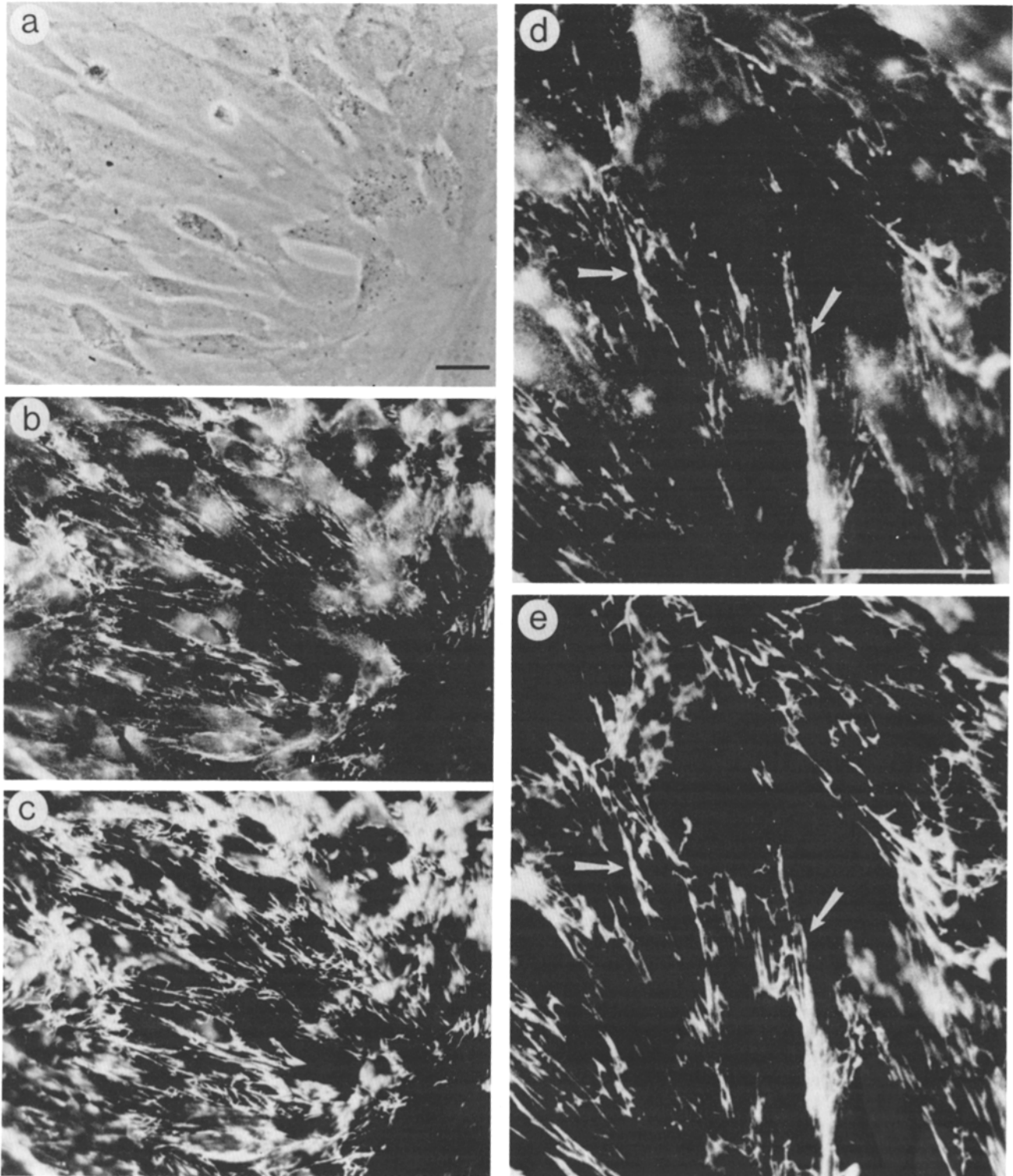
FIGURE 2 Double immunofluorescent staining of NRK cells with rabbit antiheparan sulfate proteoglycan followed by rhodamine-labeled goat anti-rabbit IgG (b) and fluorescein-labeled goat antifibronectin (c) as described in Materials and Methods. (a) The same field in phase contrast. (d and e) Higher magnification views of the staining patterns. Arrows indicate some of the fibers where staining with both the antiheparan sulfate proteoglycan (d) and the antifibronectin (e) is particularly obvious. Bar, 25 μm. (a, b, and c) × 400. (d and e) × 1,200.

these three components but retain the cell surface chondroitin sulfate proteoglycan.

The heparan sulfate proteoglycan was found localized in fibrils of extracellular matrix also containing fibronectin and laminin. In contrast, the chondroitin sulfate proteoglycan was present on the cell surface and, in a speckled distribution, in

the extracellular matrix. Presumably the functions for these two proteoglycans also differ. Previous studies suggest that cell surface heparan sulfate promotes cell adhesion (9, 12), whereas chondroitin sulfate proteoglycan is thought to retard the attachment of cells to the substratum (9, 33, 34). Our finding of the heparan sulfate proteoglycan in the same cell surface
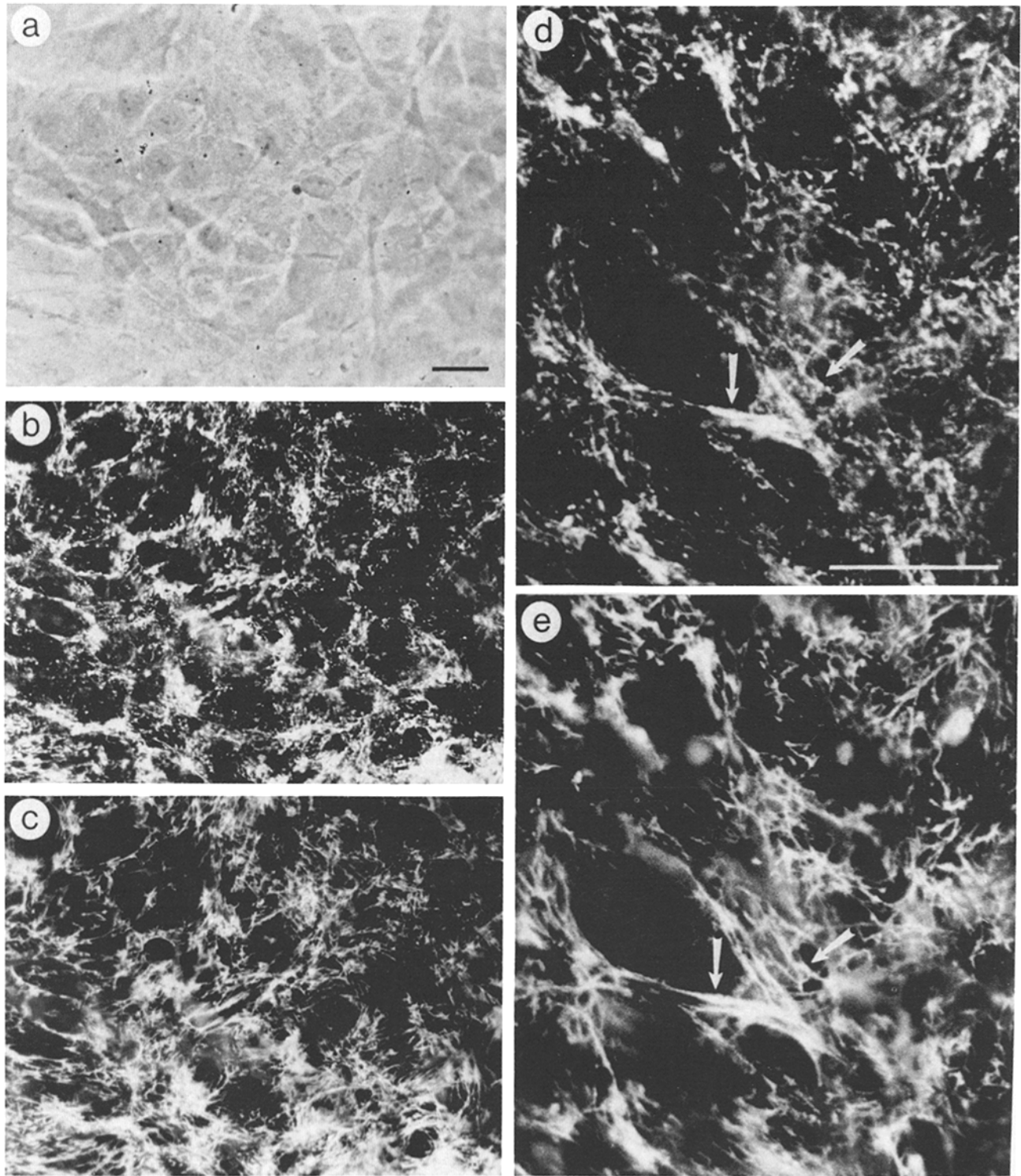
FIGURE 3 Double immunofluorescent staining of NRK cells with rabbit antilaminin (b) and antifibronectin (c). (a) The same field in phase contrast. (d and e) Higher magnification views of the staining patterns. Arrows indicate areas of coincidental staining with antilaminin (d) and antifibronectin (e). Bar, 25 µm. (a, b, and c) × 400. (d and e) × 1,200.

network as fibronectin and laminin, both of which are cell attachment-promoting proteins (24, 35, 36), supports these hypotheses.

The role of heparan sulfate proteoglycan may be to facilitate the deposition to the extracellular matrix of fibronectin and laminin. Both of these proteins interact with glycosaminoglycans (37, 38). Glycosaminoglycans (39–41) and a proteoglycan (42) have been found to enhance the binding of fibronectin to

collagen and cause precipitation of collagen-fibronectin complexes. Heparin and heparan sulfate are particularly active in this respect. Heparan sulfate proteoglycans have not been studied with regard to insolubilization of collagen-fibronectin complexes, but, on the basis of the results obtained with the glycosaminoglycans, one would expect them to have this activity.

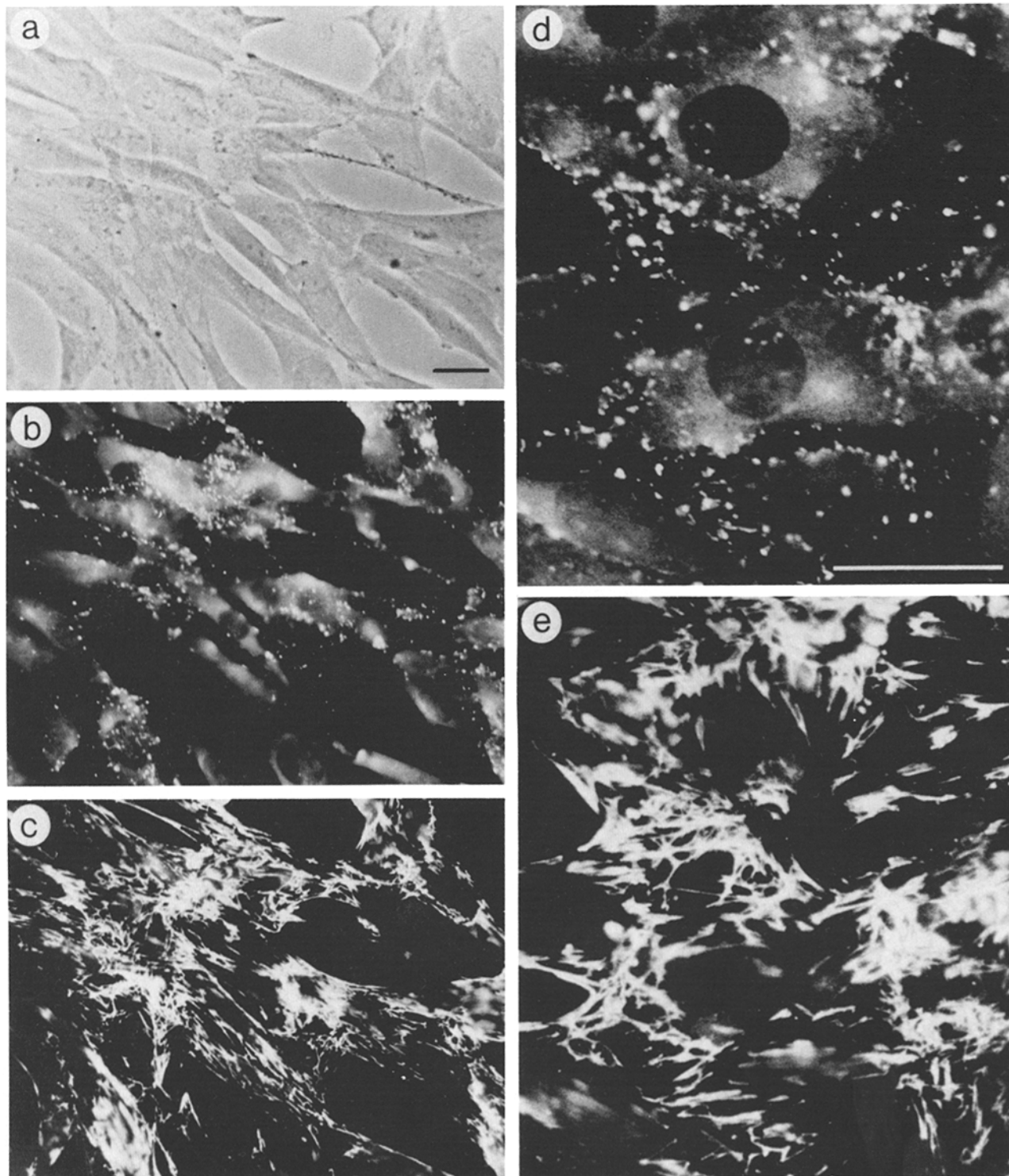The concordant absence of staining for heparan sulfate

FIGURE 4 Double immunofluorescent staining of NRK cells for chondroitin sulfate proteoglycan (b) and fibronectin (c). A sparse culture with few fibronectin fibrils was chosen to allow evaluation of the coincidence of the two stainings. (a) The same field in phase contrast. (d and e) Higher magnification views of the staining pattern, with antichondroitin sulfate proteoglycan (d) and antifibronectin (e). Bar, 25 μm. (a, b, and c) × 400. (d and e) × 1,200.

proteoglycan, fibronectin, and laminin from the extracellular matrix of transformed cells supports the idea that the heparan sulfate proteoglycan may play a role in the formation of extracellular matrix. Both fibronectin and laminin are synthesized by the transformed cells, but some as yet unknown cellular property prevents their incorporation into the extracellular matrix (24). The lack of staining for the heparan sulfate

proteoglycan at the surface of the transformed cells suggests that this component is also absent from the matrix. Our data do not exclude the possibility that the proteoglycan would be present but unavailable for binding of antibodies. It has been shown in cartilage (43) that the proteoglycan can be unavailable for antibody staining. However, we have not observed any staining for the heparan sulfate proteoglycan even in sparse
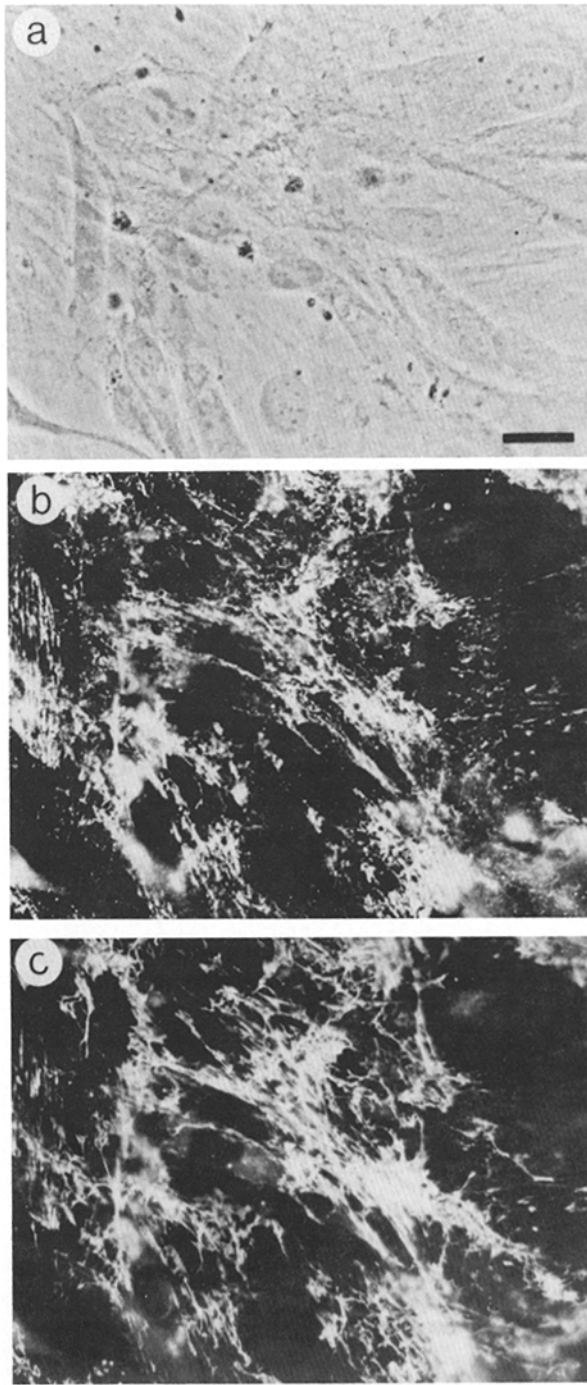
FIGURE 5 Double immunofluorescent staining of butyrate-treateu TRK cells for heparan sulfate proteoglycan (b) and fibronectin (c). (a) Same field in phase contrast. Bar, 25 μm. × 400.
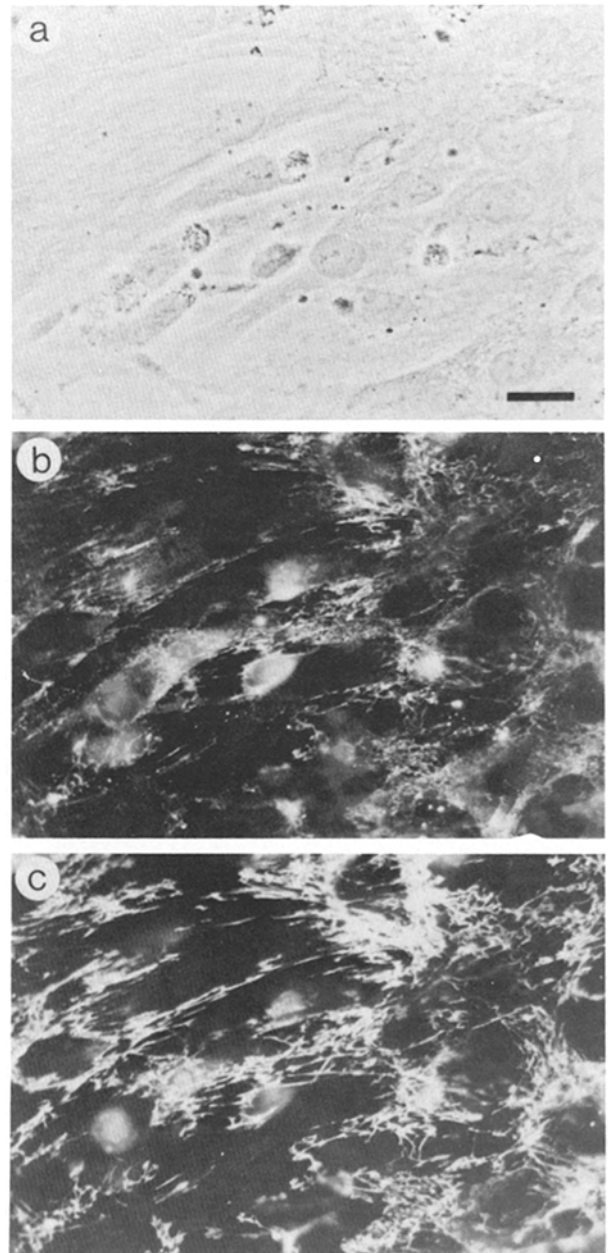

FIGURE 6 Double immunofluorescent staining of butyrate-treated TRK cells of laminin (b) and fibronectin (c). (a) The same field in phase contrast. Bar, 25 μm. × 400.

cultures of the TRK cells where the extracellular matrix is sparse and would be expected to offer much less of an impediment to the penetration of antibodies than the compact cartilage tissue. Moreover, since the proteoglycan is easily detectable on the normal cells, it appears likely that the reason for the lack of staining in the TRK cells is that it is absent.

Lack of normal heparan sulfate proteoglycan in the transformed cells could be critical to the assembly of the extracellular matrix. We have not yet been able to determine whether the transformed cells synthesize heparan sulfate proteoglycan with properties similar to that of NRK. Immunoprecipitation has revealed no heparan sulfate in spent culture media of either

cell type (E. G. Hayman, Å. Oldberg and E. Ruoslahti, unpublished results). This will be an area of further investigation with important implications to the understanding of extracellular properties such as adhesion.

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