Linkage of Extracellular Plasminogen Activator to the Fibroblast Cystoskeleton: Colocalization of Cell Surface Urokinase with Vinculin

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Abstract. Several cell types display binding sites for [^{125}I]urokinase (Vassalli, J.-D., D. Baccino, D. Belin. 1985. *J. Cell Biol.* 100:86–92) which in certain cases are occupied with endogenous urokinase. These sites appear to focus urokinase at cell surfaces and hence may participate in tissue matrix destruction and cell invasion. Recently Pöllänen et al. (1987) demonstrated that the cell surface urokinase of human fibroblasts and fibrosarcoma cells is deposited underneath the cells in strands, apparently at sites of cell-to-substratum contact. Here, using immunofluorescence double labeling, we show that the urokinase strands present on human foreskin fibroblasts are colocalized with strands of vinculin, an intracellular actin-binding protein that is deposited at cell-to-substratum focal adhesion sites. Thus, this indicates linkage of the plasminogen/plasmin system both to sites of cell adhesion and to the cytoskeleton. The urokinase strands on HT 1080 fibrosarcoma cells are more numerous and have shapes that are more tortuous than those on normal fibroblasts. In intact HT 1080 cells, colocalized vinculin strands are obscured by an intense background of soluble vinculin but are apparent on isolated ventral plasma membranes. Certain properties of the urokinase strands suggest that they are related to the [^{125}I]urokinase-binding sites that have been described by several groups: (*a*) incubating fibroblasts with dexamethasone for 48 h or at pH 3 at 5°C for 10 min greatly decreases the number and intensity of the urokinase strands; (*b*) strands reappear when glucocorticoid-treated cells are incubated with exogenous 54-kD (but not 35-kD) urokinase, and this process is inhibited by a previously described 16–amino acid peptide that blocks [^{125}I]urokinase binding to the cells.

THE plasminogen activator urokinase has been implicated as a mediator of tissue remodeling and cell invasion in several normal processes (e.g., mammary gland involution [20] and angiogenesis [6]), and also in certain pathological states (12, 15).

A substantial body of evidence links tumor cell secretion of urokinase to tumor-mediated destruction of tissue matrices and tumor cell invasion. Although there are exceptions, generally tumor cells produce urokinase in much greater amounts than do normal cells (32). Antiurokinase antibody inhibits Hep3 human epidermoid carcinoma cells from metastasizing in chicken embryos (19) and penetrating human placental amnion membranes (18). Both antiurokinase antibody and the urokinase inhibitor protease nexin I inhibit HT 1080 human fibrosarcoma cell destruction of vascular smooth muscle extracellular matrix (5). A monoclonal antibody against the avian counterpart of urokinase inhibits the destruction of chick fibroblast extracellular matrix by Rous sarcoma virus–transformed chick fibroblasts, and causes these transformed cells to assume normal morphology (28).

It has been demonstrated that monocytes have urokinase "receptors" on their surfaces that do not mediate the internalization of urokinase but hold the enzyme at the cell surface (29). These binding sites bind the 54-kD but not the 35-kD form of the enzyme. Similar sites have been detected on normal fibroblasts and several types of tumor cells (3, 7). Blasi et al. (7) have identified a 16–amino acid sequence at the NH$_2$ terminus of the 54-kD urokinase light chain that recognizes the binding sites on A431 cells. The sites are partially or completely occupied with endogenous urokinase on normal fibroblasts and tumor cells, respectively (4, 27). In the case of monocytes, the sites become occupied with endogenous urokinase after activation of these cells (26). Immunofluorescence detection of urokinase on monocytes (29) and A431 carcinoma cells (27) suggests that this antigen is relatively diffusely distributed on the surfaces of these cells. Recently, Pöllänen et al. (22) reported that urokinase on fibroblasts and fibrosarcoma cells is concentrated in fibers that are located beneath the cells, apparently at sites of cell contact with the substratum. Here we show that the urokinase immobilized on human foreskin fibroblasts is concentrated in arrays of strands and that many of these are colocalized with strands of vinculin, a protein that links focal adhesion sites with the cytoplasmic actin-containing micro-

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filament system. In their urokinase-binding properties, the binding sites that give rise to these arrays in certain, but not all respects resemble the urokinase-binding sites previously detected in binding studies carried out with radioiodinated urokinase (3, 26, 29).

Materials and Methods

Materials

Human 35-kD urokinase was purchased from Collaborative Research, Inc., Waltham, MA. Human 54-kD urokinase and goat antiurokinase IgG were purchased from American Diagnostica Inc. (Greenwich, CT). Affinity purified rabbit antiurokinase IgG was obtained as previously described (13). Rabbit antivinculin serum was a gift from Dr. Gary C. Rosenfeld of the University of Texas Medical Center at Houston. Rabbit antithrombospondin IgG was a gift from Dr. Barry W. Festoff of the Kansas University Medical Center at Kansas City. Rabbit antifibronectin IgG and rhodamine-conjugated goat anti-rabbit IgG were purchased from ICN K&K Laboratories Inc., Plainview, NY. Fluorescein-conjugated swine anti-goat IgG and soybean trypsin inhibitor were obtained from Boehringer Mannheim Diagnostics, Inc., Houston, TX and fluorescein-conjugated goat anti-rabbit IgG was from Sigma Chemical Co., St. Louis, MO. We used 30-mm cell culture dishes (Falcon 3001; Becton Dickinson and Co., Mountain View, CA). DME was purchased from Hazleton Systems, Inc., Aberdeen, MD. Penicillin-streptomycin solution and FBS were obtained from Irvine Scientific, Santa Ana, CA. Trypsin and L-glutamine were purchased from KC Biological Inc., Lenexa, KS. Selenous acid was obtained from Aldrich Chemical Co., Milwaukee, WI, and paraformaldehyde and DMSO were from Fisher Scientific Co., Pittsburgh, PA. RIA-grade BSA, dexamethasone, human transferrin, saponin, and diisopropylphosphofluoridate were from Sigma Chemical Co. The 16-mer peptide corresponding to the urokinase-binding site sequence (G-T-C-V-S-N-K-Y-F-S-N-I-H-W-C-N) was synthesized by Biosearch, San Rafael, CA.

Cell Culture

Unless otherwise noted, human foreskin fibroblasts (HF)[1] cells and human HT 1080 fibrosarcoma cells were grown in DME supplemented with FBS (4% for HF cells and 10% for HT 1080 cells) and L-glutamine as previously described (25). Culture media contained 200 U/ml penicillin G and 200 µg/ml streptomycin sulfate. Cultures were maintained in a 5% CO_2 atmosphere at 37°C. Unless indicated otherwise, cells were seeded at 2 × 10⁴ (HF) or 1 × 10⁴ (HT 1080) cells/dish, grown for 24 h in serum-containing medium, and then incubated in serum-free medium for at least 2 h before immunofluorescence or [^{125}I]urokinase-binding experiments.

Dexamethasone Treatment

HF cells (at 2 × 10⁴/ml) were seeded in 30-mm dishes in serum-containing medium. After 12 h the cells were washed twice with and then cultured for 48 h in serum-free DME, 0.15 µg/ml dexamethasone, 0.1% BSA, 2 µg/ml human transferrin, and 4 µg/ml selenous acid (ATS-DX medium). In one experiment, confluent HF cells were grown in serum-free DME containing L-glutamine and dexamethasone for 12 h, passaged with 0.05% trypsin-containing dexamethasone, and plated in ATS-DX medium containing 10 µg/ml soybean trypsin inhibitor. After 5 h, the cells were washed twice with and incubated in ATS-DX medium for 48 h.

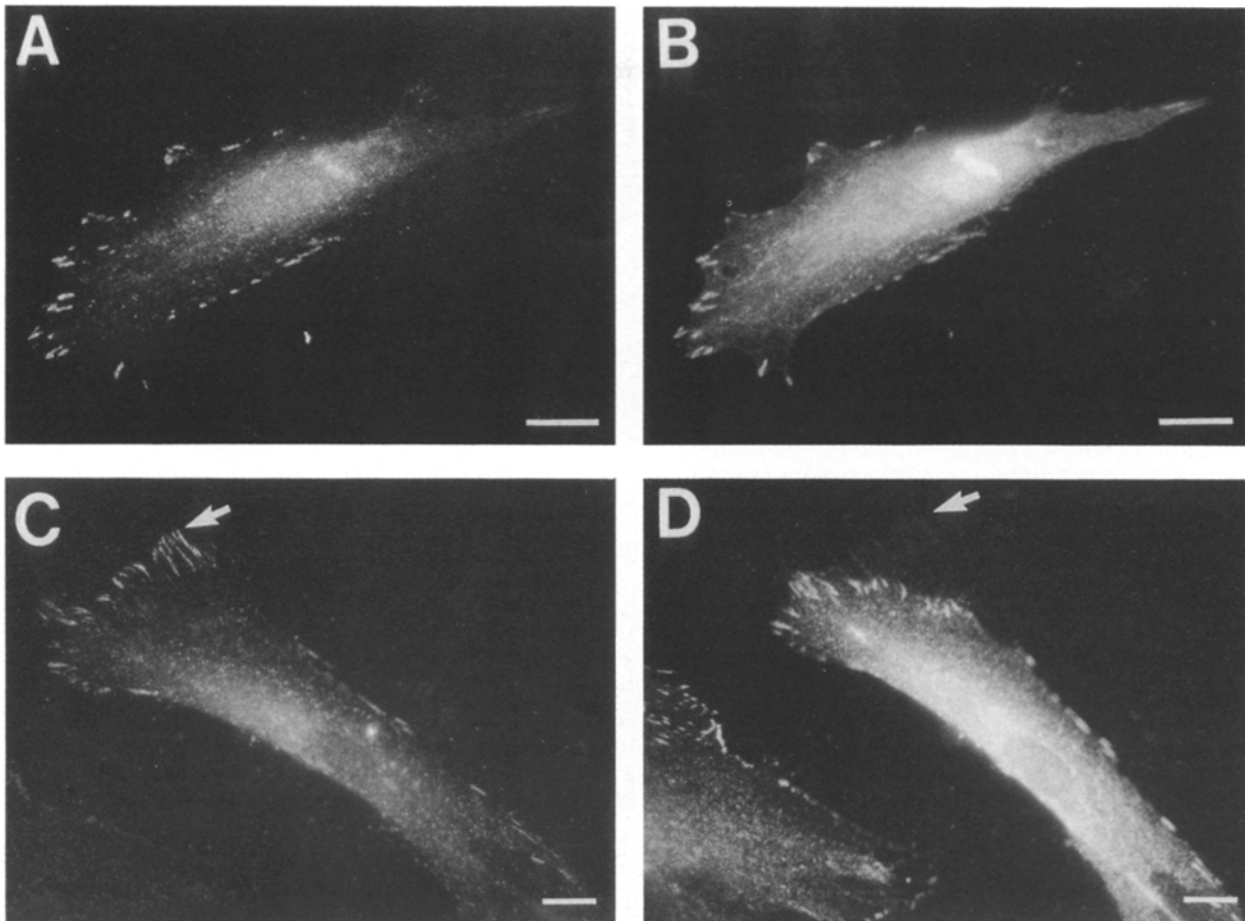### Immunofluorescence Labeling of Urokinase and Vinculin

Immunofluorescence labeling was done according to the procedure of M. C. Willingham and I. Pastan (31). The cells were fixed on ice with 3.7% paraformaldehyde in PBS. Treatment of the cells with acidic buffer (0.1 M glycine-HCl, pH 3, for 10 min), attempts to reload the urokinase-binding sites with 54- or 35-kD urokinase, and competition by the 16-mer peptide were all carried out on ice before cell fixation. Nonspecific antibody binding was blocked by incubating the cells at room temperature with 1% BSA.

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1. Abbreviations used in this paper: ATS-DX medium, serum-free DME, 0.15 µg/ml dexamethasone, 0.1% BSA, 2 µg/ml human transferrin, and 4 µg/ml selenous acid; HF, human foreskin fibroblasts.
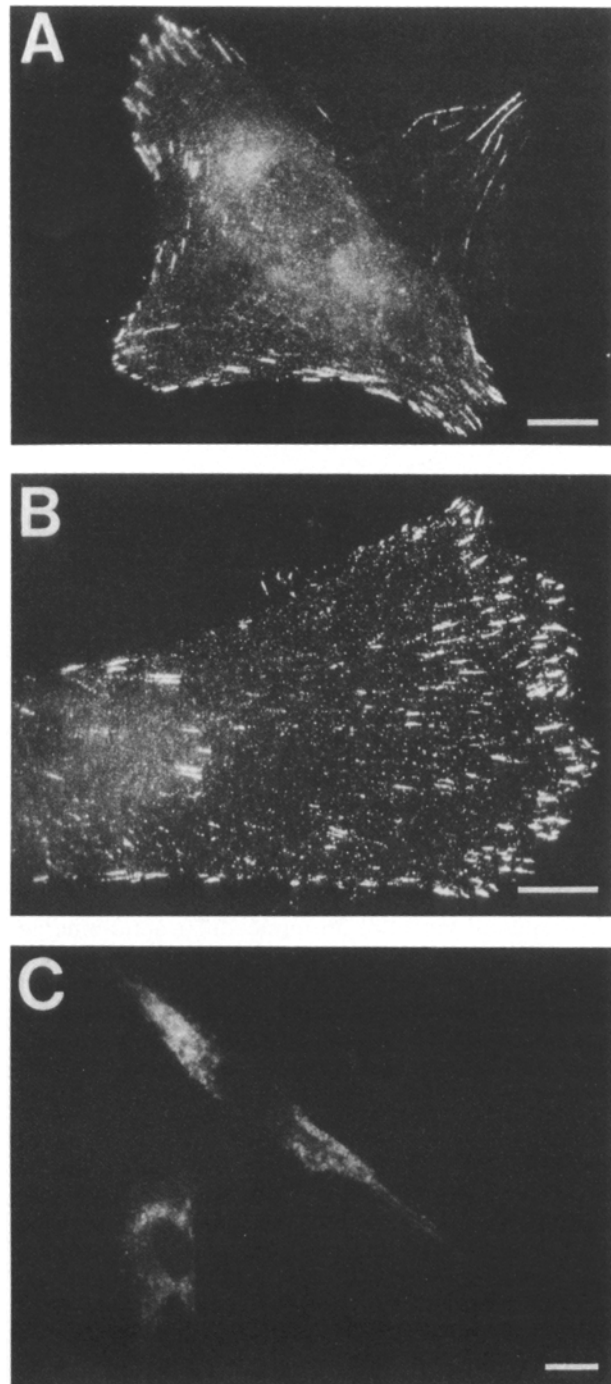
Figure 1. Immunofluorescence localization of urokinase on the surfaces of fixed human foreskin fibroblasts. Coverslips containing fixed fibroblasts were incubated with affinity-purified rabbit antiurokinase IgG (A and B) or nonimmune IgG (C) followed by fluorescein-labeled goat anti-rabbit IgG. Bars, 20 µm.

Fixed cells were incubated for 45 min at room temperature with immune or nonimmune IgG (30 µg/ml) and for 15 min at room temperature with fluorochrome antibody conjugate (50 µg/ml). In the case of vinculin localization, 0.1% saponin was added to all the reagents to permeabilize the cells. Immunofluorescence staining of vinculin was always performed after staining of urokinase to avoid permeation of antiurokinase IgG inside the cells. All coverslips were mounted in 0.05 M Tris-Cl, 50% glycerol, 0.02% sodium azide (pH 8.1) and observed with a microscope (E. Leitz Inc., Rockleigh, NJ) equipped with epifluorescence and a Fluoreszenz 40:1.3 oil immersion objective.

Figure 2. Urokinase and vinculin of fixed human foreskin fibroblasts detected by double immunofluorescence labeling. Goat antiurokinase IgG and fluorescein-labeled swine anti–goat IgG were applied before permeabilization of the cells with 0.1% saponin. The permeabilized cells were treated with rabbit antivinculin serum and rhodamine-conjugated goat anti–rabbit IgG. (*A* and *C*) Distribution of urokinase on the cell surface as indicated by fluorescein fluorescence. (*B* and *D*) Distribution of vinculin as indicated by rhodamine fluorescence. Control cells, stained for just vinculin or just urokinase, demonstrated no spillover between rhodamine and fluorescein filters. Arrows indicate where the relative intensities of the vinculin and urokinase strands have shifted. Bars, 20 μm.

Results

Foreskin fibroblasts were fixed without permeabilization, incubated with rabbit antiurokinase antibody, followed by fluorescein-labeled goat anti–rabbit IgG, and examined by epifluorescence microscopy. The antiurokinase antibody stained fiberlike strands, arrays of which tended to outline the cell borders (Fig. 1) and resembled the urokinase strands recently described by Pöllänen et al. (22). Occasionally the fluorescence formed almost continuous frames around the cells. When nonimmune IgG was substituted for the primary antibody, diffuse yellow fluorescence, also observed with the immune IgG, was evident, but no strands were present. The strands of urokinase antigen were also observed when affinity-purified rabbit antiurokinase IgG, another rabbit antiurokinase antiserum, or goat antiurokinase IgG were used as primary antibody.

We were struck that the patterns of urokinase immunofluorescence resembled published immunofluorescence patterns generated by staining of vinculin (14), a cytoplasmic actin-binding protein that is deposited in strands at cell-to-substratum focal adhesion sites (9). The spatial distribution of the urokinase and vinculin strands was investigated by double immunolabeling. The antivinculin antibody did not stain cells that had not been permeabilized (data not shown). The pattern of vinculin immunofluorescence in saponin-permeabilized cells is shown in Fig. 2, *B* and *D*. Much of the cell cytoplasm was covered by even and rather intense fluorescence, consistent with findings that a large fraction of the vinculin in cells is soluble (21). In addition, characteristic discrete strands of vinculin were visible at the edges. Intriguingly, Fig. 2 demonstrates that a large fraction of the vinculin strands (*B* and *D*) were colocalized with strands of urokinase (*A* and *C*), and vice versa. All of the vinculin and urokinase strands were in the same plane of focus, essentially at the substratum (coverslip) surface.

Examination of Fig. 2 also indicates that some urokinase strands lacked detectable colocalized vinculin strands, and some vinculin strands lacked detectable colocalized urokinase strands. At present there is no basis for deciding whether these actually represent instances of vinculin and urokinase strands not being colocalized or instances of the antigen strands being inaccessible to antibody. In some cases,
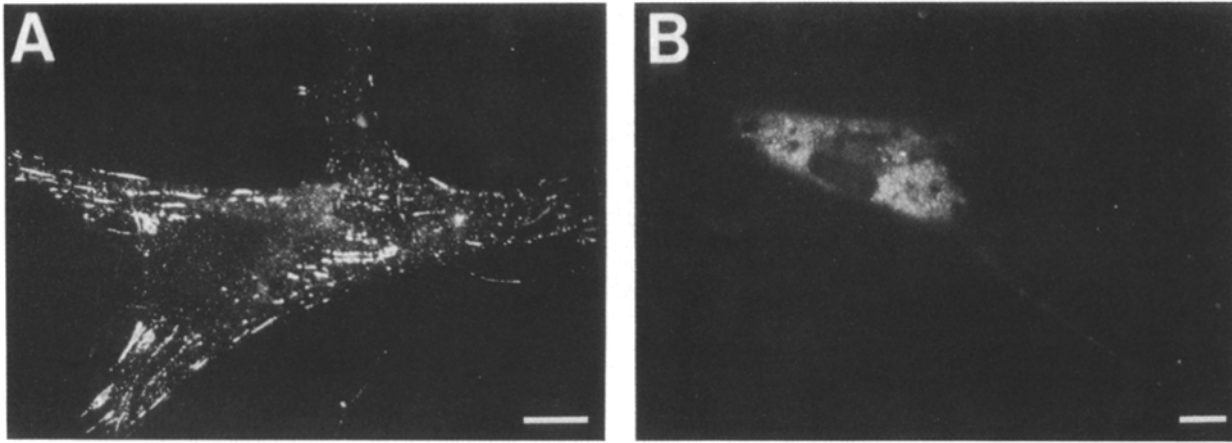
Figure 3. Distribution of urokinase on human foreskin fibroblasts before (*A*) and after (*B*) pH 3.0 treatment. The cells were incubated on ice for 10 min with 0.1 M glycine-HCl (pH 3.0), rinsed with PBS three times, and fixed. Immunofluorescence staining of urokinase was performed as mentioned in Fig. 1. Bars, 20 μm.

arrays of vinculin and urokinase strands were offset along the cells, as shown in Fig. 2, *C* and *D*. These offset patterns suggest that the focal contacts had loosened at one region of the cell, leaving urokinase strands, and had reformed at another region of the cell where they had not yet become loaded with urokinase.

Fragments of ventral cell surfaces that are enriched in focal adhesion plaques can be isolated, still attached to the culture dishes by rinsing cells after an incubation in a solution containing 1 mM $ZnCl_2$ (2). Use of this procedure on foreskin fibroblasts yielded culture dish surfaces that contained numerous superimposed strands of urokinase and vinculin that were similar to those present in intact cells (data not presented).

Brief treatment of foreskin fibroblasts at pH 3 on ice extracts urokinase from the cells and simultaneously exposes ~50,000 previously cryptic urokinase-binding sites (3, 4). This, and a similar finding made with A431 carcinoma cells (27), have been interpreted as evidence that pH 3 extracts endogenous urokinase from its cell surface-binding sites. Fig. 3 shows that incubation of the fibroblasts at pH 3 for 10 min on ice caused the disappearance of the urokinase strands. Addition of 54-kD urokinase to the cells after their incubation at pH 3, in general, did not lead to the reappearance strands of urokinase immunofluorescence, although occasionally strands did appear which were thinner, less intense, and less clearly confined to the cell borders than the urokinase strands present on cells before pH 3 treatment (data not presented). The incubation at reduced pH caused the edges of the cells to become jagged or crenated, but the vinculin fibers were not notably perturbed.

An alternative way to deplete the endogenous urokinase is to incubate the cells with cortisol or dexamethasone (4). These glucocorticoids inhibit the synthesis and secretion of urokinase by a wide variety of cells (e.g., see references 17, 30). Treatment of the foreskin cells with 0.15 μM dexamethasone for 48 h resulted in a pronounced reduction, but not virtual elimination, of the number of urokinase strands (Fig. 4). Incubation of these dexamethasone-treated cells with 54-kD urokinase resulted in the reappearance of numerous urokinase-containing strands that resembled those present on

cells that had not been incubated with dexamethasone. It is noteworthy that the urokinase immunofluorescence was not restored when 35-kD urokinase was used (Fig. 4). This result was probably not caused by failure of the antibody to recognize 35-kD urokinase, because preincubation of the antibody with this form of the enzyme eliminated its staining of cellular urokinase strands on cells that had not been treated with dexamethasone (data not presented).

Blasi et al. (7) have identified a 16–amino acid sequence in 54-kD urokinase that blocks the binding of [^{125}I]urokinase to A431 carcinoma cells. In our hands, this peptide, at high (0.1 mM) concentration, caused a 70% inhibition of specific binding of [^{125}I]urokinase–DIP to foreskin fibroblasts: 565 ± 277 cpm with the peptide, compared to 2,000 ± 222 cpm without the peptide (± indicating the range of duplicate measurements). Fig. 5 shows that the peptide prevented the reappearance of the strands of urokinase on dexamethasone-treated cells incubated with 54-kD urokinase.

In an attempt to completely eliminate the urokinase immunofluorescence that remained after the 48-h incubation of the cells with dexamethasone, the cells were incubated for 12 h in serum-free medium containing dexamethasone, subcultured by trypsinization, and further incubated in this medium for 48 h. This regimen resulted in the complete disappearance of urokinase-containing fibers. Brilliant dots, but no strands of urokinase immunofluorescence appeared on these cells when they were incubated with 54-kD urokinase. Urokinase strands failed to form on these cells even when they were cultured for 24 h in the presence of this ligand.

Numerous urokinase strands have recently been detected on HT 1080 fibrosarcoma cells (22). Because tumor cells in general tend to be less adhesive than normal anchorage-dependent cells and have reduced numbers of focal contacts (24), we sought to determine whether the urokinase strands on HT 1080 cells were accompanied by superimposed vinculin strands. Unlike the normal fibroblasts, HT 1080 cells that were stained for urokinase had a diffuse green glow, suggesting that urokinase was dispersed over the cells. In addition to this, the urokinase antigen present on HT 1080 cells was, as in the case of normal fibroblasts, distributed in discrete strands (Fig. 5). The strands differed from those of fibro-
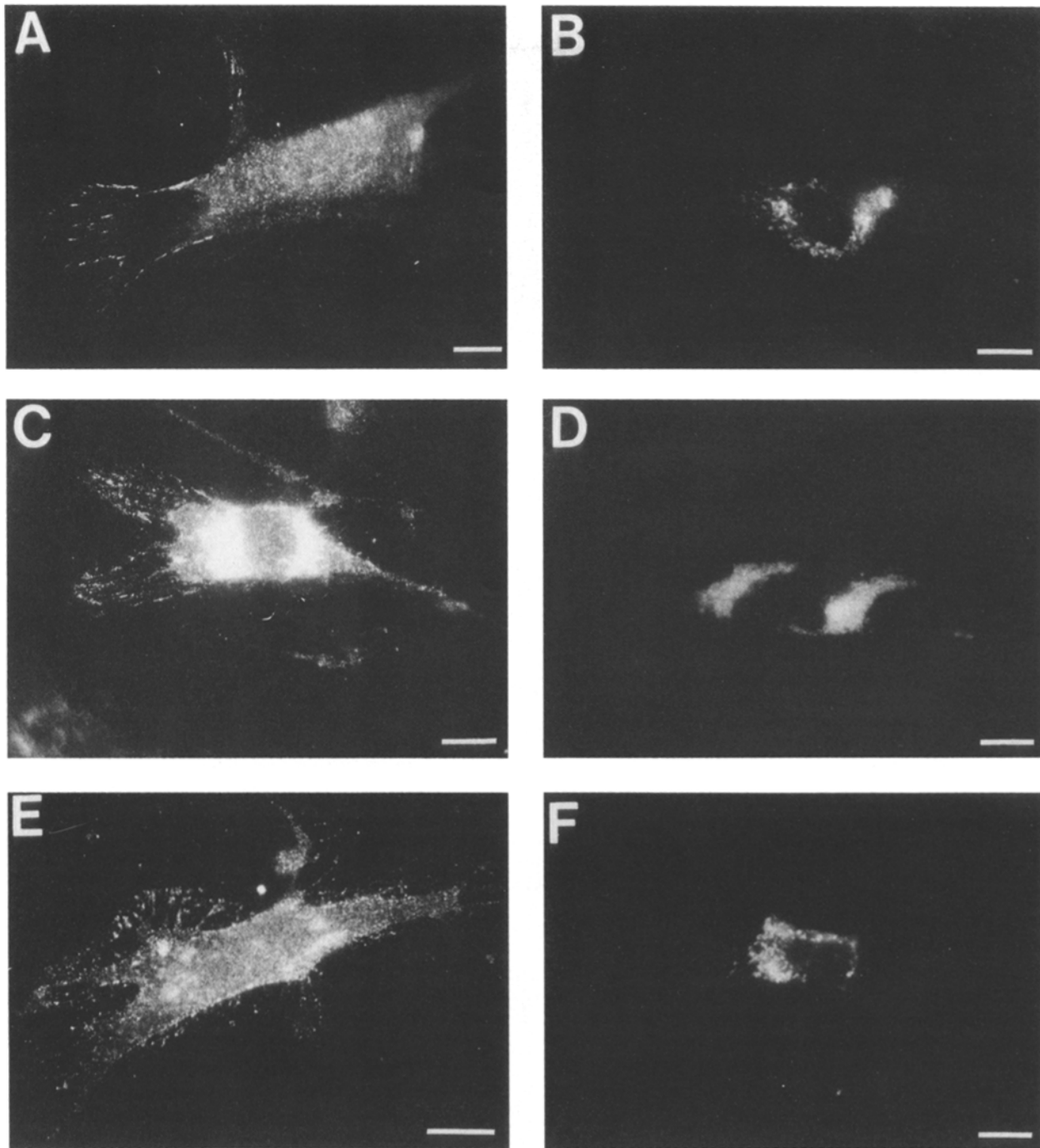
Figure 4. Disappearance of urokinase on dexamethasone-treated cells, and reappearance of urokinase strands after incubation of these cells with urokinase. Foreskin fibroblasts were seeded in 30-mm dishes (2 × 10⁴ per dish) in serum-containing medium. After 6 h, the cells were washed twice with and then cultured for 48 h in serum-free medium containing 0.1% BSA, 2 μg/ml human transferrin, and 4 μg/ml selenous acid (ATS medium) without (*A*) or with (*B-F*) dexamethasone (0.15 μM). Before fixation the dexamethasone-treated cells were incubated for 1 h on ice in PBS containing 1% BSA plus (*B*) no supplement, (*C*) 1.3 nM 54-kD urokinase, or (*D*) 1.3 nM 35-kD urokinase In *E* and *F*, the dexamethasone-treated cells were incubated for 15 min in 1% BSA/PBS, 1% DMSO without (*E*) and with (*F*) 0.1 mM "binding site peptide" (see Materials and Methods). 54-kD urokinase (1.3 nM final concentration) in 1% BSA/PBS was added and the coverslips were incubated for 1 h on ice before washes with PBS and fixation. Bars, 20 μm.

blasts in that they were more numerous and twisted, and extended inward closer to the centers of the cells. Vinculin strands were present in these cells but were very faint and were obscured by soluble vinculin. Examination of HT 1080 ventral surfaces by double immunolabeling clearly revealed vinculin strands superimposed on the strands of urokinase (Fig. 5).

Discussion

The present immunofluorescence study indicates that the
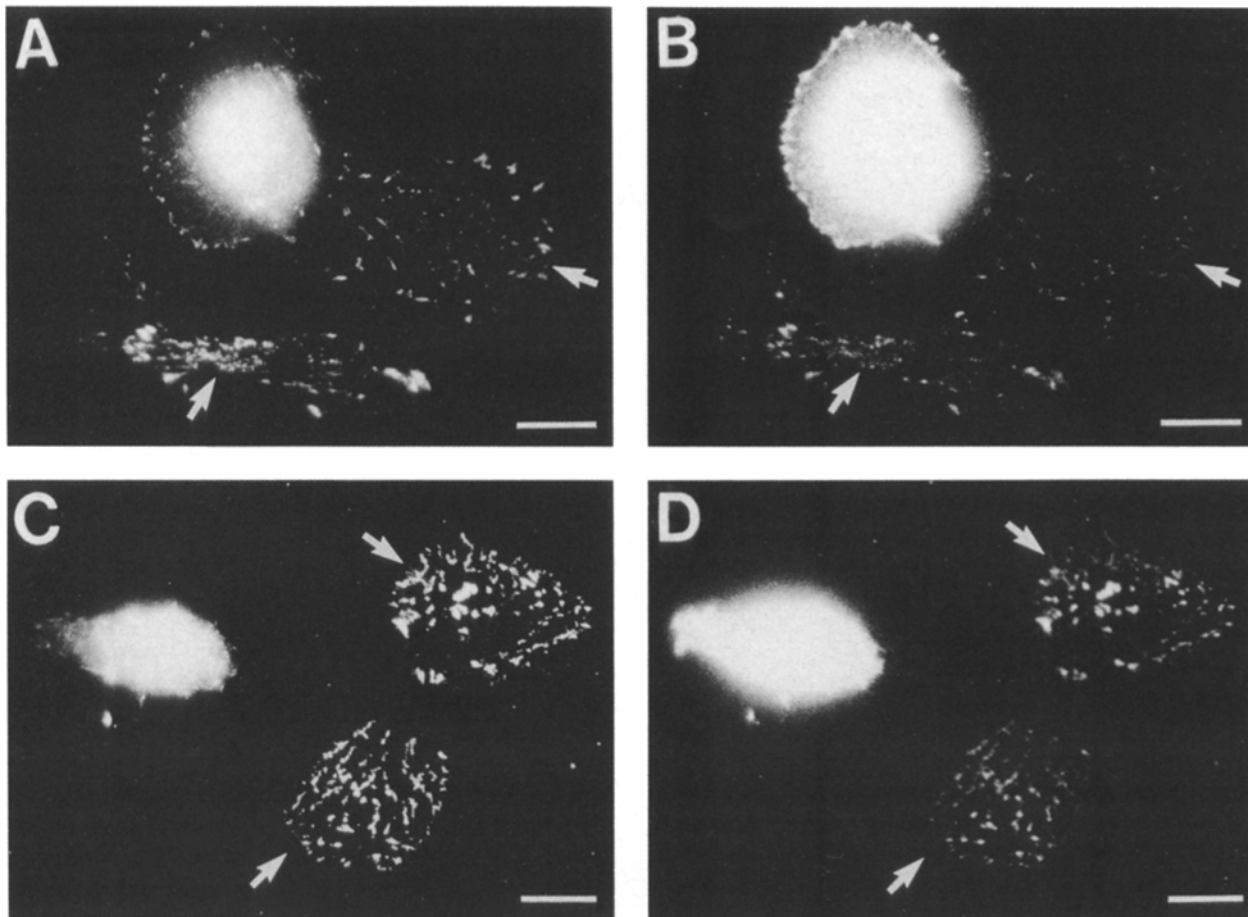
Figure 5. Urokinase and vinculin of fixed HT 1080 whole cells and ventral surfaces. Immunofluorescence staining was performed as described in Fig. 2. Ventral surfaces of HT 1080 cells were rinsed in 50 mM 2-(*N*-morpholino)ethane sulfonic acid containing 3 mM EDTA and 5 mM MgCl$_2$ (pH 6.0), incubated on ice for 2 min with the same buffer containing 1 mM ZnCl$_2$, and then rinsed with 20-30 jets of PBS before fixation. This procedure yielded coverslips that contained mostly ventral cell surfaces but also some intact cells. (*A* and *C*) Urokinase on an intact cell and ventral surfaces (*arrows*). (*B* and *D*) Vinculin of an intact cell and ventral surfaces (*arrows*). Bars, 20 μm.

urokinase on the surfaces of human fibroblasts is reversibly bound to "receptors" that are concentrated in discrete strands which are deposited at sites of focal contact between the cells and their substratum. The urokinase-binding sites that give rise to the urokinase strands could be identical to the recently described cell surface–binding sites for 54-kD [^{125}I]urokinase (3, 26, 29), because 54-kD but not 35-kD urokinase formed strands on dexamethasone-treated (urokinase-depleted) cells, and because a peptide fragment of 54-kD urokinase that blocks [^{125}I]urokinase binding to cells blocked this strand formation. However, one observation does not suggest this view. 54-kD urokinase did not convincingly form strands on cells that had been preincubated at pH 3 on ice, even though this preincubation procedure unmasks [^{125}I]urokinase-binding sites as it extracts exogenous cell surface urokinase (3, 4). It is possible that (*a*) even though the only fibroblast urokinase-binding sites detected by immunofluorescence were clustered in strands, these clustered sites actually represent the minority of the urokinase-binding sites, the rest being diffusely distributed, and (*b*) exposure to pH 3 selectively inactivates the clustered sites.

The urokinase strands were colocalized with strands of vinculin, an intracellular protein that is deposited at focal

adhesion sites (9). Cells actively move about on the surfaces of culture dishes, a process that must entail the repeated formation and dissolution of adhesive contacts with the (tissue matrix) substratum (1). The localization of cell urokinase at focal adhesion sites indicates that the enzyme may be poised to participate in the release of cells from adherent substratum proteins. That cells are capable of selectively hydrolyzing very small spots of extracellular matrix has been elegantly demonstrated by Chen et al. (10). Their work on virally transformed chick fibroblasts suggests that matrix destruction occurs precisely at sites of cell–substratum contact that underlay "rosettes" of cytoplasmic vinculin. However, in this system a metalloproteinase and not plasminogen activator seems to be responsible for the focal proteolysis (10).

Whether urokinase causes extracellular matrix destruction and promotes cell migration by direct hydrolysis of matrix proteins or by activating plasminogen is an open issue. The fastidious specificity of urokinase suggests the latter mechanism. However, Sullivan and Quigley (28) have demonstrated that the direct action of the avian counterpart of urokinase on substratum or cell surface proteins generates the characteristic transformed morphology of Rous sarcoma virus–transformed chick fibroblasts, and Quigley et al. (23) have

further shown that purified urokinase can directly cleave fibronectin. Although high (>µg/ml) concentrations of urokinase are required for this cleavage, the present results indicate that the local concentration of this enzyme at focal adhesion sites could be extremely high. The present work does not indicate whether the urokinase that is contained in strands is the single-chain ("proenzyme") form or the two-chain form. The latter is active. Whether the single-chain form is active under certain circumstances (16) or absolutely requires cleavage (by plasmin or certain other serine proteases) for activation is controversial (8, 16). Cubellis et al. have recently shown that single-chain urokinase that is allowed to bind to urokinase receptors on monocytes is susceptible to plasmin-mediated cleavage to two-chain urokinase (11).

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