Expression of Transformation-associated Protease(s) That Degrade Fibronectin at Cell Contact Sites

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ABSTRACT Virus-transformed fibroblasts show an increased production of proteases as well as loss of extracellular adhesive proteins. To determine whether these transformation-associated events are related, we investigated the capacity of Rous sarcoma virus-transformed cells (embryonic chick fibroblasts and mouse BALB/c 3T3) to degrade fibronectin by using a novel cross-linked protein substratum: fluorescence-labeled or radiolabeled fibronectin covalently linked to the surface of a fixed gelatin film. In serum-containing medium, the coupled fibronectin was not released when incubated without cells, and only a small amount was released when incubated with nontransformed cells. However, when transformed cells were seeded on the radiolabeled fibronectin-coupled substratum, there was a threefold increase in the time-dependent release of radioactivity into the medium. The released material was characterized as peptides with molecular sizes of <30,000 daltons. Correspondingly, growth of transformed cells on the rhodamine-fibronectin substratum resulted in the appearance of discrete negative fluorescent spots beneath the cells and along their migratory paths, whereas a uniform fluorescent carpet was detected with nontransformed cells. The release of radioactivity was partially inhibited by protease inhibitors, including α_2-macroglobulin, leupeptin, and benzamidine, but the negative fluorescent spots appeared unaffected by any of these inhibitors. However, both the release of radiolabeled peptides and the appearance of fluorescence-negative spots were inhibited by 1,10-phenanthroline at concentrations that did not affect cellular attachment and protein synthesis, thus supporting a role for proteases in localized degradation of fibronectin substratum. These fluorescence-negative spots coincided with sites of fibronectin disappearance as judged by indirect labeling with antibodies to cellular fibronectin. In addition, immunofluorescent analyses showed a correlation between vinculin localization and the negative fibronectin spots found under transformed cells, indicating that degradation occurs at cell substratum contact sites. These results can be correlated with other transformation-associated phenotypic changes, and are discussed in terms of the invasion of tumor cells into the extracellular matrix.

Fibronectins are large extracellular glycoproteins that promote the adhesion and spreading of fibroblastic cells on collagenous substrata (see references 17, 29, 36, and 37 for reviews). Oncogenic transformation of cultured cells by viruses causes a pleiotropic change in cellular properties including loss of fibronectin, reduced adhesion, rounded morphology, and loss of cytoskeletal organization. Because all of these changes can be reverted to some extent by the addition of fibronectins (2, 35, 39), it has been suggested that loss of fibronectin may be important for producing the transformed phenotype and may be associated with detachment and subsequent invasion of malignant cells in vivo (11, 37). Possible explanations for the loss of fibronectin in transformed cells include (*a*) reduced synthesis (1, 24, 31), (*b*) reduced binding (15, 20, 33), (*c*) increased rates of degradation (13, 19), or some combination of these three possibilities. In particular, loss of fibronectin has been related to increased production of cell surface proteases in transformed cells (7, 14, 16, 22, 25). These proteases

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may provide a mechanism for cells to detach their membrane from the fibronectin matrix at existing attachment sites, i.e., the extracellular matrix (ECM)[1] contacts in spread fibroblasts (9).

In this paper, we address the following questions: (*a*) Does Rous sarcoma virus (RSV)-transformation increase the expression of proteolytic activities on the cell surface? (*b*) Is fibronectin degraded by these proteases? (*c*) Are such activities localized at cell contact sites? To answer these questions, we have developed a novel substrate for cell surface proteases that involves the covalent linkage of fluorescence-labeled or radiolabeled fibronectin to the surface of cross-linked gelatin substrata (8, 9). Irreversible coupling of fibronectin, or other proteins, to such a surface prevents lateral interaction and translocation of the proteins by contact with the cell membrane, as shown with a coating method (3), during the assay. Fluorescence-labeled fibronectin substrata also permit direct visualization of fibronectin degradation under the ventral surface of the cells. In using the fluorescence and radiolabeled probes, we have demonstrated the existence of transformation-sensitive protease(s) that may be involved in the local degradation of fibronectin at ECM contact sites.

MATERIALS AND METHODS

Cell Culture: Primary cultures of embryonic chick fibroblasts (ECF) were prepared from 10-d-old White Leghorn embryos (Truslow Farms Incorporated, Chestertown, MD), and infected by a wild-type, Schmidt–Ruppin (SR) strain or temperature-sensitive strain (ts68) (19) of RSV as previously described (24). Cells were cultured in Dulbecco's modified Eagle medium containing 4,500 mg/liter of glucose, 10% fetal calf serum, 10 μg/ml gentamycin (Grand Island Biological Co., Grand Island, NY), and 2 mM glutamine. Cells were passed every 2–3 d with trypsinization, and were usually plated at a density of 5×10^4 cells per 35-mm tissue culture dish. In some experiments, BALB/c mouse 3T3 cells were infected with the SR wild-type strain of RSV and were cultured as described above.

Fibronectin Coupled to Gelatin Film: Confluent tertiary cultures were used for urea extraction and purification of cellular fibronectin as previously described (5, 38). To prepare metabolically labeled cellular fibronectin, we cultured ECF in media containing [U-^{14}C]leucine (4 μCi/ml, sp act 353 mCi/mmol) or [^{35}S]cysteine (100 μCi/ml, sp act 934 mCi/mmol) for 24 h before purification of cellular fibronectin as described (5). Bovine plasma fibronectin was purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). Fibronectin was conjugated to tetramethyl rhodamine isothiocyanate (Research Organics, Inc., Cleveland, OH), and conjugates with a fluorophore-to-protein ratio of 5:1 were separated on DEAE-cellulose columns for use in the present studies (8).

Purified fibronectin, rhodamine conjugates of fibronectin, or metabolically labeled fibronectin was covalently linked to the surface of a fixed gelatin film according to a previously described procedure (8). Briefly, the gelatin solution (heat-denatured bovine type I collagen, purchased from Sigma Chemical Co., St. Louis, MO) was first coated on either No. 1 glass coverslips or 96-well microtiter plastic plates and the film was fixed with 4% glutaraldehyde overnight. The free aldehyde groups on the surface of fixed gelatin film were further coupled to fibronectin, usually at the concentration of 0.1 mg/ml, followed by quenching and masking of nonspecifically sticky sites with 3% bovine serum albumin plus 0.1 M glycine. To monitor released radioactive product of fibronectin in the medium, [^{35}S]fibronectin was coupled to the substratum at a concentration of 0.2 mg/ml. Controls for coupling of fibronectin included (*a*) reacting with bovine serum albumin instead of fibronectin in parallel incubations, (*b*) first quenching with glycine, followed by absorption of fibronectin to the fixed gelatin surface (in that fibronectin has high affinity to unfixed gelatin), and (*c*) removing noncovalently bound fibronectin with 4.5 M urea. The amount of coupled fibronectin was estimated by adding a known amount of [^{35}S]fibronectin, and measuring the amount of unbound protein.

Immunofluorescent Labeling and Microscopy: Antibodies, secondary antibody conjugates, and procedures for immunolabeling of cells have been described elsewhere (9). Labeled cells were observed with a Zeiss Photomicroscope III (Carl Zeiss, Inc., New York) equipped with epifluorescence. A Plan-neofluar 25/0.8 phase objective (Carl Zeiss, Inc., New York) was routinely used for low magnification observations of fibronectin clearing and a Planapo 63/1.4 objective (Carl Zeiss, Inc.) for high resolution studies on single cells.

Determination of Proteolytic Activity: To analyze degraded products of ^{14}C- or ^{35}S-labeled fibronectin-coupled substrata, we collected aliquots of culture media at various time periods after cellular attachment, and determined radioactivity by liquid scintillation spectrometry. In addition, the radioactivity remaining associated with the substratum was determined after Triton X-100 (1%) solubilization of the cell monolayer, and medium incubated without cells was used to estimate the nonspecific release of the radiolabeled product. Cells were cultured on the cross-linked gelatin film coated on the well of flexible assay plates (Falcon 96-well microtiter plates—Micro Test III, Becton, Dickinson Labware, Oxnard, CA). After removal of media, the cell layer grown on fibronectin substrata was lysed in 1% Triton X-100 and the solution was collected. The substratum was then removed by cutting off the bottom of the well. The molecular size of digestion products released into the medium was determined by gel filtration chromatography on a Sephadex G-50 column (Pharmacia Fine Chemicals, Piscataway, NJ) (12).

Enzymes and Enzyme Inhibitors: To examine the nature of putative proteases, we seeded cells in the complete medium containing each of the following inhibitors at various concentrations indicated in Table I: aprotinin, α_1-antitrypsin (α_1-proteinase inhibitor), 1,10-phenanthroline, soybean trypsin inhibitor, pepstatin, and benzamidine from Sigma Chemical Co. (St. Louis, MO); α_2-macroglobulin, dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone dihydrochlorine (DGGACKD), D-phenylalanyl-prolyl-arginine chloromethyl ketone (PPACK), 2-mercaptomethyl-3-guanidinoethyl thiopropanioc acid (Plummer's inhibitor), guanidinoethylmercaptosuccinic acid (GEMSA), and ZINCOV inhibitor from Calbiochem–Behring Corp. (La Jolla, CA), and leupeptin from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

The possibility that fluorescence-negative spots in substrata are due to masking by other ECM components was examined by treatment of these substrata with the following enzymes: collagenase from Calbiochem–Behring Corp., hyaluronidase and chondroitase ABC from Seikagaku Kogyo Co. (Tokyo, Japan), and *Flavobacterium* heparanase (a gift from Dr. L. Culp, Case Western Reserve University, Cleveland, OH).

RESULTS

Attachment and Spreading of Transformed Cells on Fibronectin Substrata

ECF and their transformed counterparts attached to the fibronectin-coupled, cross-linked gelatin substratum within 15 min after plating, and their attachment was similar to that of ECF grown on plastic dishes. Maximal spreading on the fibronectin substratum was achieved by 6 h after plating as compared to that of 18 h on plastic surface.

To determine whether cellular transformation by RSV results in increased expression of proteolytic enzymes at the cell surface or in the extracellular milieu capable of degrading fibronectin, we first investigated the effect of cell monolayers on the rhodamine-labeled fibronectin substrata by visualizing fibronectin degradation under the ventral surface of the transformed cells. Normal and wild-type SR strain virus-transformed ECF were seeded on the rhodamine-fibronectin substratum at the confluent density of 5×10^4 cells per dish (35 mm) to restrict cell migration. Under these condition fluorescence-negative (black) spots began to appear on the substratum of the transformed cell culture within 6 h as shown in Fig. 1*B*. The fluorescence-negative areas increased in size during the next 18 h (Fig. 1*D*), but increased in number only slightly (cf. Fig. 1*A* and *B* with *C* and *D*). However, when seeded at subconfluent density, the black spots increased in number and in size (Fig. 1*E–H*). These results suggest that the localized degradation of fibronectin substrata occurs either directly under the cells or along their migratory pathway.

Transformed cells have been shown to produce elevated levels of various membrane and soluble proteases (7, 14, 16, 22, 25, 26, 27). These proteases may be involved in overall

[1] *Abbreviations used in this paper*: ECF, embryonic chick fibroblasts; ECM, extracellular matrix; RSV, Rous sarcoma virus; SR, Schmidt-Ruppin strain; ts68, temperature-sensitive strain.

TABLE I

Effects of Protease Inhibitors on Degradation of Fibronectin-coupled Substrata by SR-infected ECF

Inhibitors	Concentration	Effect on cellular attachment and spreading	Toxicity* (protein synthesis)	Fibronectin clearing‡	Radioactivity released§
None (control)	—	None	None	+	100%
Soybean trypsin inhibitor	100 μg/ml	None	ND	+	ND
Leupeptin	0.5 mM	None‖	None	+	65%
Benzamidine	2 mM	None	None	+	54%
Aprotinin	200 μg/ml	None	ND	+	ND
Pepstatin	0.3 mM	None‖	ND	+	ND
α_2-Macroglobulin	0.3 μM	None	None	+	85%
1,10-Phenanthroline	0.3 mM	None	None	−	56%
α_1-Antitrypsin	0.2 mM	None	ND	+	ND
DGGACKD	0.3 mM	None‖	ND	+	ND
PPACK	0.4 mM	None	ND	+	ND
Plummer's inhibitor	0.4 mM	None	ND	+	ND
GEMSA	0.9 mM	None	ND	+	ND
ZINCOV inhibitor	0.7 mM	None‖	None	+	100%

DGGACKD, dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone dihydrochlorine; *PPACK*, D-phenylalanyl-prolyl-arginine chloromethyl ketone; *GEMSA*, guandinoethylmercaptosuccinic acid; *ND*, not determined.

* Toxicity was determined by the reduction of ^{14}C-leucine incorporation.

‡ Fibronectin clearing by the cells at 6 h culture that showed similar negative spots as in Fig. 1*B* was considered positive degradation (designated +); lack of negative spots on substrata was considered inhibition of degradation (designated −).

§ Radioactivity released from the fibronectin substrata was determined as percentage of total counts released. Results are from triplicate walls.

‖ In the presence of these inhibitors cells are more spreaded than the control. All assays in this table were performed at 6 h after cell plating in media containing 10% fetal calf serum.

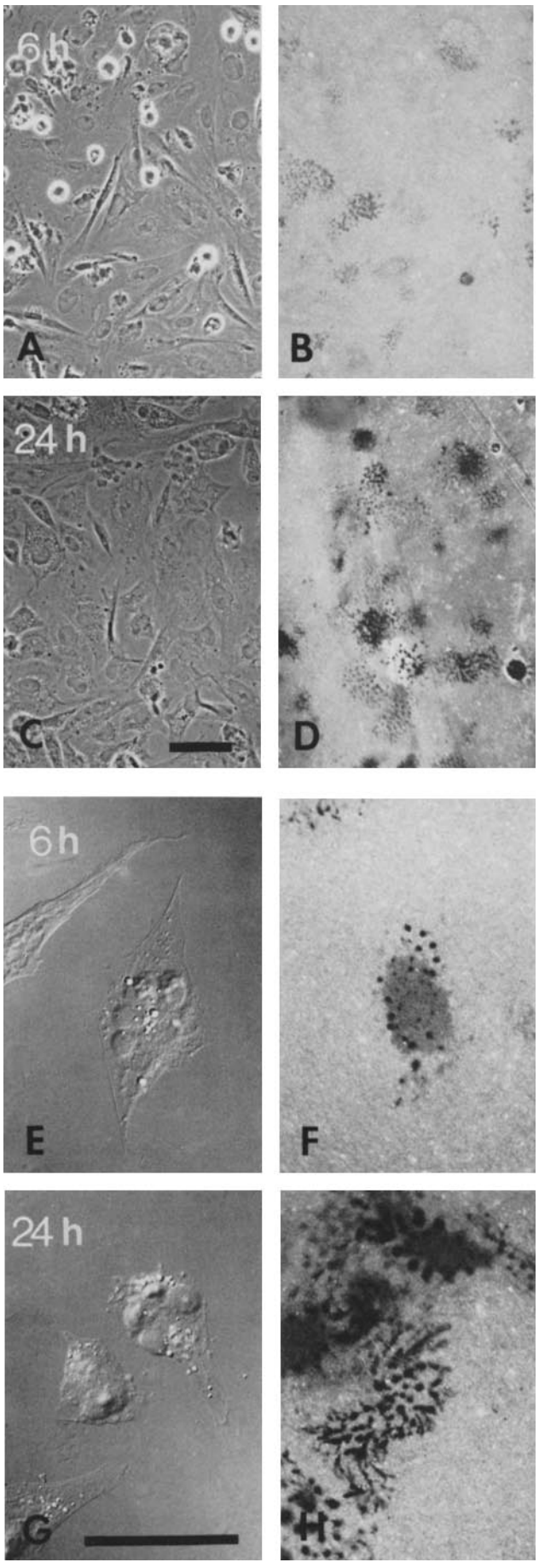
removal of fibronectin from areas surrounding black spots. Accordingly, we examined the difference in the intensity of fluorescence of the fibronectin substrata as a function of time of cell incubation. Measurements of the diffuse fluorescence using the automatic spot reading of Zeiss Photomicroscope III indicated a twofold reduction in fluorescent intensity from 6- to 24-h cultures. Thus, the general removal of fibronectin from the surface of the substratum may involve a number of different proteases that are distinct from those responsible for the localized degradation characterized by the appearance of black spots (Fig. 1).

Localized degradation of fibronectin by transformed cells may not be restricted to the exogenous protein, inasmuch as the cell-associated fibronectin may also be degraded. To investigate this possibility, cells grown on rhodamine-fibronectin substrata were immunolabeled with fluorescein-antibody conjugates directed against both cellular and substratum fibronectin. The labeled specimens were observed with a high-resolution × 63 objective using alternative rhodamine and fluorescein filter settings. The results shown in Fig. 2 reveal a uniform substratum fluorescence under the normal ECF (Fig. 2*B*) indicating no detectable proteolytic activity; however, discrete black spots were detected on the rhodamine-labeled fibronectin substratum of transformed cells (Fig. 2*E*), indicative of fibronectin degradation at these areas. In addition, fluorescence-negative spots on the fibronectin substratum coincided with clearing sites of extracellular fibronectin fibers deposited by cells as shown by indirect labeling with antibodies to fibronectin (Fig. 2*F*). These negative immunofluorescent spots are not due to masking by proteoglycan and hyaluronic acid (21), because treatment of substrata with enzymes specific for these ECM components produced no apparent effect on the appearance of the negative immunofluorescent spots (see below for more details). It is also important to note that the present method of covalent linkage of fibronectin to the substratum surface clearly demonstrated the retention of coupled fibronectin at focal adhesion sites formed between cells and the substratum (cf. Figs. 2 and 4). In contrast, fibronectin coated on the glass surface by the method of Avnur and Geiger (3) was removed from focal adhesion sites by both normal and transformed ECF.

When mouse 3T3 cells and SR-transformed 3T3 cells were tested for fibronectin clearing, negative spots were found under transformed cells but not under the nontransformed cells (results not shown). Thus, the appearance of negative spots on fibronectin substrata directly corresponded to transformation by RSV.

Initial Sites of Fibronectin Clearing under the Transformed Cells

When ECF were infected with the SR ts68 and grown at the permissive temperature (37°C), >40% of the infected cells produced spots of fibronectin clearing as found for ECF infected with wild-type virus and grown under similar conditions (Figs. 1 and 2). However, direct demonstration of initial sites of fibronectin clearing under the cells requires the use of ts68-infected cells. To further investigate the time course of expression of the proteolytic activity under the transformed cells, the ts68-infected ECF were seeded on the rhodamine-fibronectin substratum at the nonpermissive temperature (41°C) for 3 h to allow for maximal spreading; the cultures were then shifted to the permissive temperature (37°C) for various periods of times before microscopic examination. Within 1 h after the temperature shift (Fig. 3*A–C*), negative fluorescent spots appeared on the rhodamine-fibronectin substrata (Fig. 3*B*), and became more evident after 2 h (Fig. 3*E*, *H*). In contrast, a uniform carpet of fluorescence was observed in cultures grown at the nonpermissive temperature. Furthermore, indirect immunofluorescent labeling of these cultures with fluorescent antibodies, directed against fibronectin, revealed that these negative spots coincided with areas depleted of the immunoreactive protein (cf. Fig. 2*F* and Fig. 3*F*). These fibronectin-depleted zones could be detected directly



beneath the cell body by 1 h after the temperature shift (Fig. 3*C* and *F*), and peripheral to the cell after 3 h (Fig. 3*I*) suggesting that sites of fibronectin clearing correspond to sites of detachment of the cell margin.

Clearing of Fibronectin Substrata at Sites of Cell Contacts

It was recently shown by immunofluorescence (4) and immunoelectron microscopy (9) that vinculin, a protein of M_r 130,000, is concentrated at the sites of cell contact on the cytoplasmic side of the plasma membrane of cultured fibroblasts. To determine whether removal of fibronectin also occurred at sites of cell contact upon transformation, we immunolabeled vinculin in transformed cells to see if this protein co-localized with regions of fibronectin removal from the substratum. For these studies, ECF were grown on rhodamine-fibronectin substrata, lightly fixed with paraformaldehyde, permeabilized, and immunolabeled with fluorescein-antibody conjugates directed against vinculin to show the correlation between indirect vinculin labeling and direct fibronectin removal. By comparing the immunolabeling patterns of vinculin and rhodamin-fibronectin in ts68-infected ECF, grown at the permissive and nonpermissive temperatures for 3 h, we found that the ECM contact sites were present at the ventral surface of normal cells and showed positive labeling for both vinculin and fibronectin (Fig. 4*A* and *B*). However, the majority of the transformed cells had lost the ECM contact sites and exhibited a cluster of small patches that were immunolabeled for vinculin and coincident removal of fibronectin (Fig. 4*D* and *E*). Such a cluster of vinculin labeling, previously termed "rosettes" (11), is located near the ventral surface of the cell, usually partly under the nucleus. On the extracellular face of the rosette, fibronectin labeling was usually removed from the substratum (arrows in Fig. 4*D–E*). Thus, clearing of fibronectin substrata on the ventral surface of the cell appears to occur at sites of rosette contacts in transformed cells.

Analysis of Fibronectin Degradation

To quantitate fibronectin degradation, we seeded cells on radiolabeled fibronectin substrata and the release of radioactivity into the culture media was monitored. The time course of release of radioactivity into the medium (Figs. 5 and 6) correlated with the number and size of negative fluorescent spots described above (Fig. 1). In medium containing 10%

FIGURE 1 Time course of clearing of fibronectin-coupled substrata under SR-infected ECF grown for 6 h (*A* and *B*, and *E* and *F*) and 24 h (*C*, *D*, and *G*, *H*). The cells were seeded on rhodamine-fibronectin matrix coupled to cross-linked gelatin film at high cell density (5×10^4/35-mm dish) (*A*, *B*, *C*, and *D*) and at low density (5×10^3/35-mm dish) (*E*, *F*, *G*, and *H*). *A* and *C* are phase-contrast images and *E* and *G* are Nomarski differential interference contrast images. Black spots in fluorescent images (*B*, *D*, *F*, and *H*) indicate areas where rhodamine-fibronectin substrata have been digested and removed. Note that the black spots in confluent cultures (*B* and *D*) increase in size as a function of time. An average of 40% of the cells in 6 h culture (*A* and *B*) and in 24 h culture (*C* and *D*) shows evidence of fibronectin clearing. In sparse cultures (*F* and *H*), increased number and size of black spots are seen along the migratory pathway of the cells (*H*). Bar, 50 μm. (*A–D*) × 200; (*E–H*) × 480.

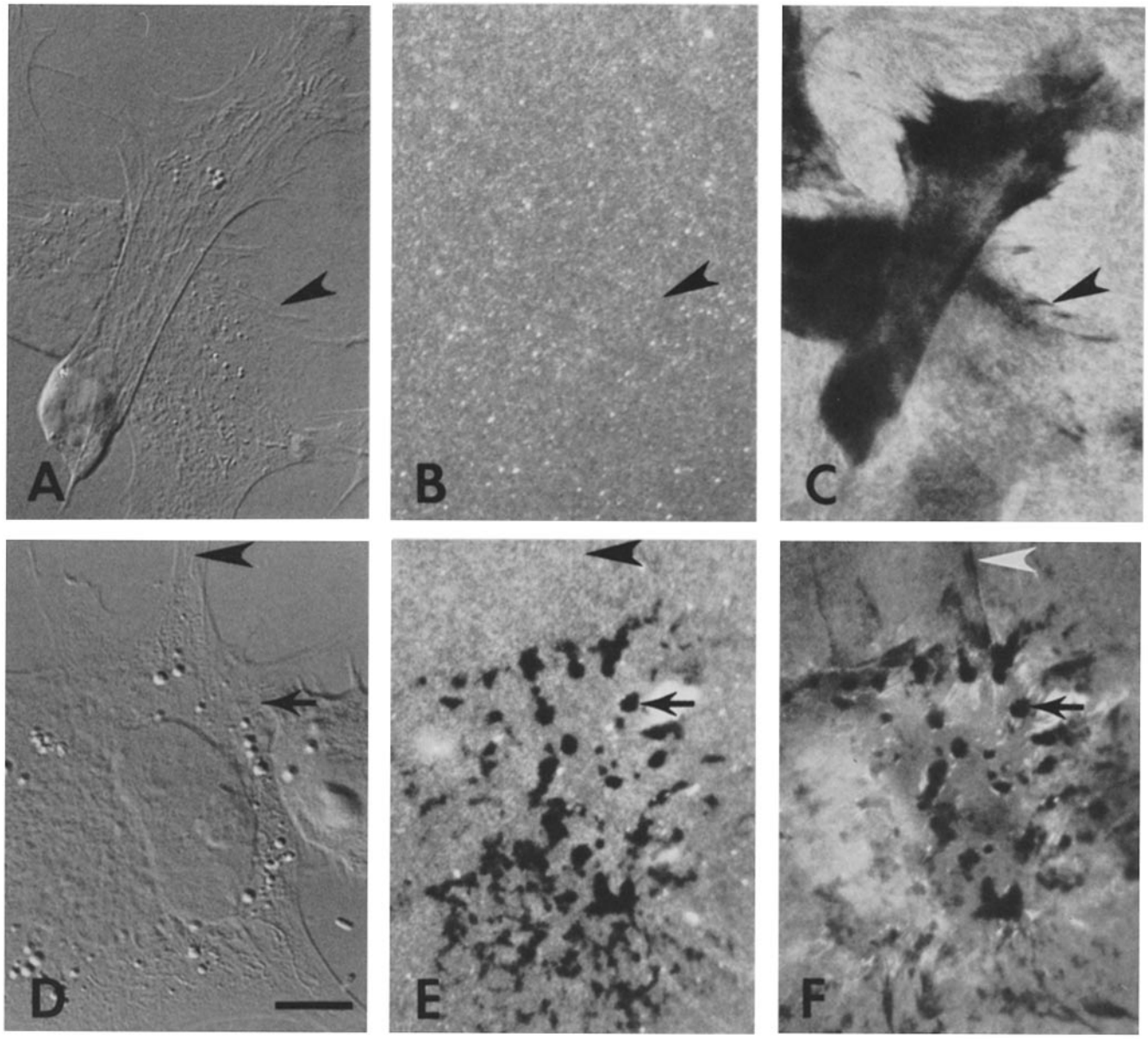


FIGURE 2 High resolution micrographs of fibronectin substrata under uninfected (*A*, *B*, and *C*) and SR-infected ECF (*D*, *E*, and *F*). *A* and *D* show the differential interference contrast images of the same cells; *B* and *E* are fluorescent images of rhodamine-fibronectin substrata; and *C* and *F* are fluorescent images of same cells and substrata using fluorescein-antibody conjugates directed against both cellular and substratum fibronectin. Under the normal cells a uniform pattern of fluorescence was observed on the fibronectin substratum (*B*). Under transformed cells, discrete black spots (shown by arrow in *E*) were detected on the labeled fibronectin substratum (*E*), and these spots coincided with sites of clearing of extracellular fibronectin fibers deposited by cells as shown by indirect labeling with antibodies to fibronectin (shown by arrow in *F*). In contrast with localized sites of fibronectin clearing, focal adhesion sites of both normal and SR-infected ECF were inaccessible to antibody labeling and showed negative streaks (shown by arrowheads in *C* and *F*) which corresponded to areas where fibronectin was present (shown by arrowheads in *B* and *E*). Bar, 10 μm. × 1,200.

fetal bovine serum, the initial release of radioactivity from the fibronectin substrata was detectable by the 5th hour in transformed cultures of 3T3 (Fig. 5) and ECF (Fig. 6). By 48 h 33% of radioactive fibronectin originally coupled to the substratum was released into the medium (Figs. 5 and 6); whereas, in the nontransformed cultures (Figs. 5 and 6), <10% of the radioactivity was released under similar conditions. Some degraded material seems to be endocytosed, inasmuch as cells lysed with 1% Triton X-100 released an amount of radioactivity equivalent to ~10% of the total radioactivity found in the medium.

When the media of normal and transformed cells were analyzed by Sephadex G-50 gel filtration, threefold more [^{35}S]cysteine counts were found in the included volume of the column with media derived from transformed cells versus normal cells. In addition, most of the released counts were not precipitable by 10% trichloroacetic acid, consistent with the above suggestion that fibronectin fragments released into media by transformed ECF have M_r of <30,000 daltons. The degradation of the fibronectin substratum occurred only in the presence of cells in that conditioned media containing 10% fetal bovine serum were without effect for up to 48 h at

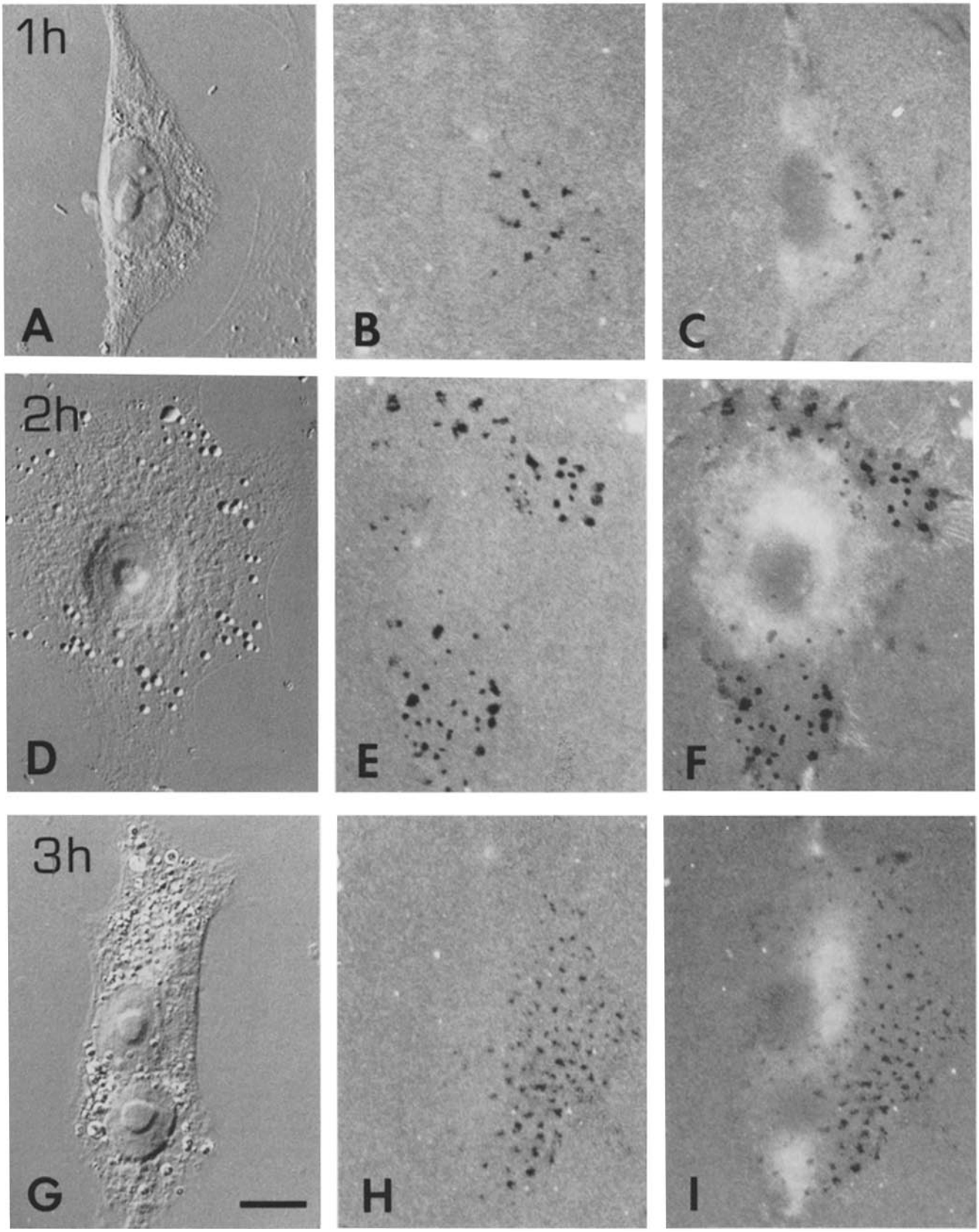


FIGURE 3 Demonstration of initial sites of clearing of fibronectin matrix under ts68-infected ECF grown at the permissive temperature (37°C) for 1 h (*A*, *B*, and *C*), 2 h (*D*, *E*, and *F*), and 3 h (*G*, *H*, and *I*) after temperature shift from 41°C. The cells were seeded on rhodamine-fibronectin layer coupled to cross-linked gelatin film (*B*, *E*, and *H*) and were indirectly labeled with fluorescein-antibodies to cellular and substratum fibronectin (*C*, *F*, and *I*). Differential interference contrast images of the same cells are shown in the left most panels (*A*, *D*, and *G*). Negative fluorescent spots on rhodamine-fibronectin substrata (*B*, *E*, and *H*) represent areas where fibronectin has been cleared. Indirect labeling with fluorescein-antibodies to fibronectin (*C*, *F*, and *I*) shows the absence of fibronectin corresponding to the fluorescence-negative spots indicated in *B*, *E*, and *H*. These spots appear under the cell body immediately after shift to the permissive temperature (*C* and *F*) and peripheral to the cell in older cultures (*I*). Bar, 10 μm. × 1,200.

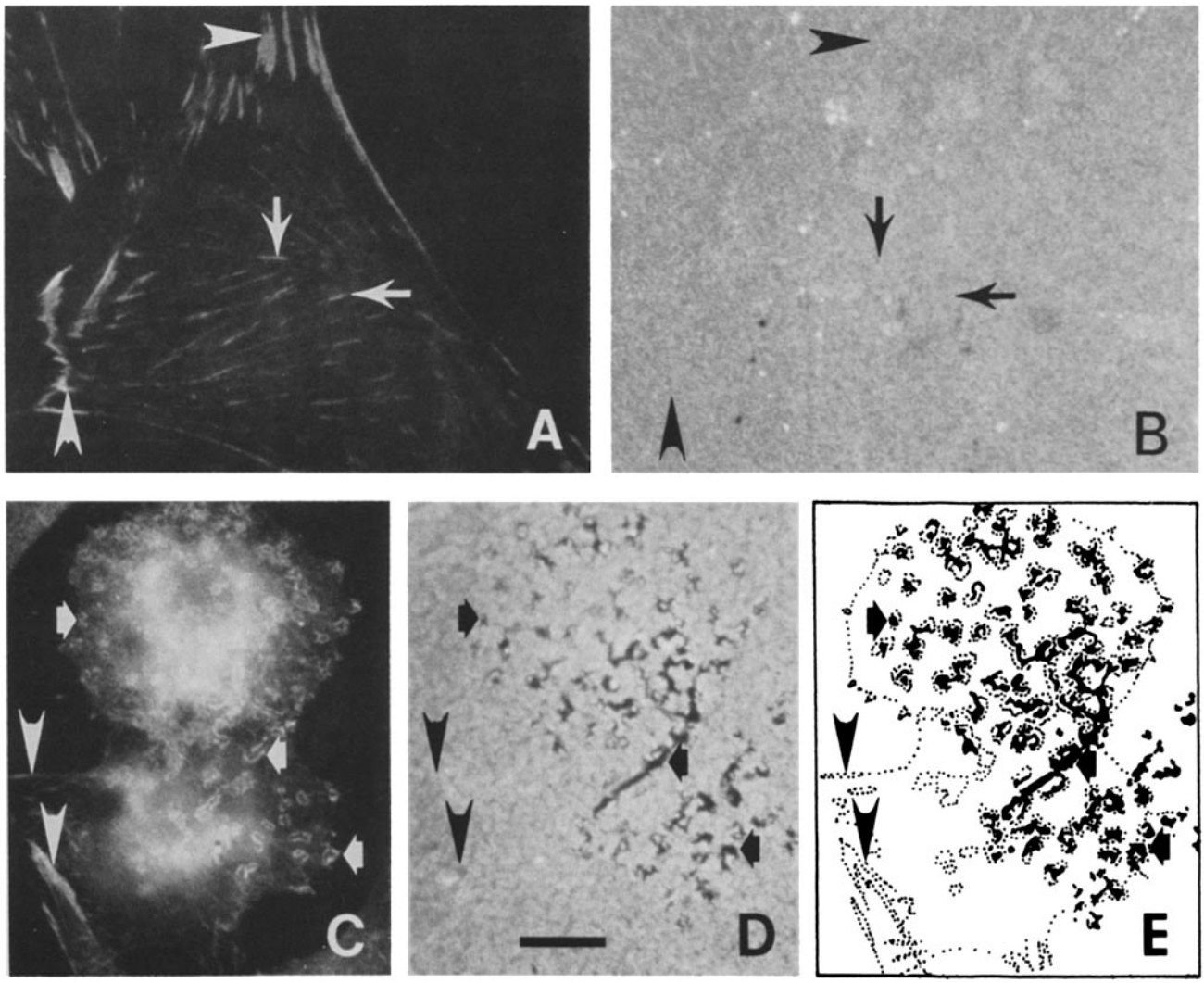


FIGURE 4 Clearing of fibronectin substrata occurred at transformation-associated, vinculin-rich cell contact sites. (*A* and *C*) Vinculin localization using indirect fluorescein-antibody labeling on ts68-infected ECF grown at the nonpermissive temperature (41°C) for 6 h (*A*) and the cell 3 h after shift to the permissive temperature (37°C) (*C*). (*B* and *D*) The rhodamine-fibronectin substratum under the same cells shown in *A* and *C*, respectively. (*E*) The overlay tracing of vinculin localization (shown as dotted outline) and degraded fibronectin spots (shown as solid black) in the transformed cells indicated in *C* and *D*. The identically positioned arrowheads in the normal cells (*A* and *B*) and in the transformed cells (*C–E*) point to the focal adhesion sites that label intensely for vinculin but do not remove fibronectin covalently coupled to substrata. The identically positioned arrows in the normal cell (*A* and *B*) point to ECM contacts that lie under the ventral surface of the normal cells, and label for vinculin in the form of streaks (arrows in *A*) but do not show fibronectin removal (arrows in *B*). ECM contacts are rarely detected in transformed cells, however, rosette contacts indicated by the identically positioned arrows in *C–E* are found in transformed cells that label intensely for vinculin, and produce negative fibronectin spots (*E*). Some negative fibronectin spots are seen peripheral to the transformed cells (*E*), indicating sites where cells migrated away. Under some rosette contacts in *E* that label for vinculin, there is no detectable black spots suggesting that these spots are newly formed rosettes and thus degraded spots are yet to be detected. Bar, 10 μm. × 1,200.

37°C.

Because fibronectin was initially coupled to the gelatin film, the possibility arises that collagenase activity may account for the release of some of the bound fibronectin. Accordingly, we examined the role of collagenase-like activity in matrix degradation by transformed cells. Replacement of rhodamine-fibronectin conjugates with rhodamine-gelatin conjugates that were coupled to the cross-linked gelatin film did not produce negative fluorescent spots under transformed ECF for up to 48 h in culture; neither did fluorescence-negative spots appear when the cross-linked gelatin film was directly conjugated to rhodamine. Another possibility is that the negative immunofluorescent spots could be due to masking by other secretory molecules such as proteoglycan and hyaluronic acid (21). To test this possibility, we treated the transformed cell cultures with the enzymes hyaluronidase (25 TRU/ml), chondroitase ABC (0.1 U/ml), or *Flavobacterium* heparanase (0.02 U/ml) (21) individually or in combination, and then performed indirect antibody labeling for fibronectin. These enzymes produced no apparent effect on the appearance of the negative spots under transformed cells, suggesting that a transformation-associated protease, expressed at cell contact sites, is involved in localized degradation of the adhesion protein fibronectin.

Effects of Protease Inhibitors on Fibronectin Degradation

To provide additional support for the involvement of protease(s) in localized degradation of fibronectin substrata, we examined effects of various protease inhibitors on the formation of fluorescence-negative spots and the release of radioactive fragments using concentrations of inhibitors that did not significantly affect cellular attachment and spreading nor amino acid incorporation (Table I). These effects of inhibitor treatment were evaluated at 6 h after cell plating as shown in Fig. 1*A* and *B*; >90% of the cells were viable, as determined by trypan blue exclusion (not shown) and [^{14}C]leucine incorporation (Table I). In addition, none of the inhibitors had any major morphological effect on cellular attachment and spreading on the fibronectin substrata.

It is also significant that leupeptin and benzamidine, inhibitors of plasminogen activator activity (25, 26), decreased the degradation of fibronectin substrata when determined by the release of radioactivity into the culture medium containing 10% fetal bovine serum, but had no discernible effects on the formation of the black spots. Of the various inhibitors tested (see Table I), only 1,10-phenanthroline inhibited formation of fluorescence-negative spots and the release of radioactivity into the culture medium. Also, the inhibitory effect of 1,10-phenanthroline was not reversed by removing it from the culture medium as expected of a metal chelator. The concentration of the inhibitor (60 μg/ml) had no effect on cellular attachment and spreading, nor on protein synthesis. These results suggest that a transformation-associated metalloendoprotease is expressed at cell-substratum contact sites capable of degrading fibronectin.

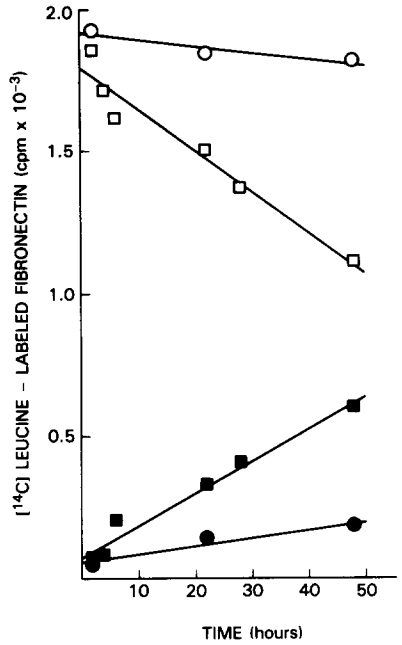


FIGURE 5 Time course of release of radioactive peptides from [^{14}C]fibronectin-coupled substrata by 3T3 cells (●, ○) and SR-infected 3T3 cells (■, □). Open circles (○) and squares (□) indicate the substratum counts and solid circles (●) and squares (■) show the medium counts. Cell suspensions were seeded at concentration of 5 × 10^4 cells/ml so that the cell layer reaches confluency at 6 h. Each data point represents the average of triplicate samples (SD <15%). The curves are fit by linear regression analysis with a correlation coefficient >0.95.

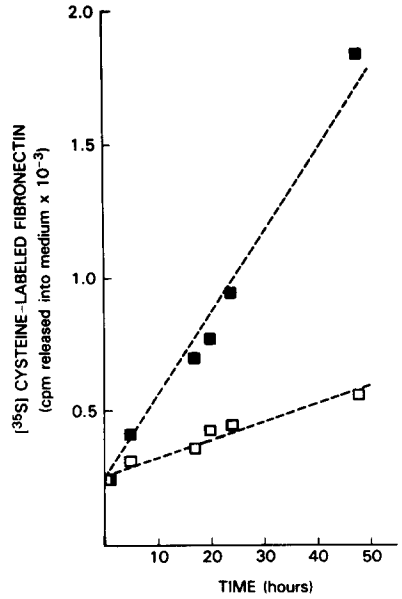


FIGURE 6 Time course of release of radioactive peptides from [^{35}S]fibronectin substrata by ECF (□) and SR-infected ECF (■). Cell density used is identical to that in Fig. 5. In this experiment, excess [^{35}S]cysteine-labeled fibronectins were used to react the crosslinked gelatin film on wells of 96-well microtiter plates. An average of 6 × 10^3 cpm of [^{35}S]fibronectin were remained on each well at the time of seeding cells of which about 3.6 × 10^3 cpm was estimated as covalently coupled counts (see Materials and Methods for details). Each data point represents the mean of triplicate samples (SD <15%). The curves are fit by linear regression analysis with a correlation coefficient >0.97.

DISCUSSION

In view of the characteristically different adhesive properties of normal and transformed cells, it is probable that important insights into the mechanisms of cell transformation will come from a knowledge of the molecular nature of extracellular adhesive proteins, such as fibronectin, and of the changes in adhesive proteins that are produced by transformation-induced cell surface proteases. Fibronectin, an extracellular adhesive glycoprotein of fibroblasts and other cells, is usually absent or found in reduced amounts on the surface of transformed cells (references see 17, 29, 36, and 37 for reviews). In this article, we have shown that highly localized degradation of extracellular fibronectin occurs at sites of the rosette contacts to the substratum. In that vinculin may be phosphorylated by the transforming gene product of RSV-(pp60^{src}) (30) and both vinculin and pp60^{src} are located at the rosettes (11, 23), our observation that the appearance of the rosettes and fibronectin degradation occur within 3 h after shift of ts68-infected ECF is consistent with the notion that the rosettes and their activity are among the early events of transformation (6). Localized degradation of fibronectin at the rosette contacts could break attachments of the cell membrane to the substratum and could in turn play an important role in the rounding up of the cell (6), in the disorganization of the microfilament bundles (2, 35, 39), and in the decrease in cell-substratum adhesion (34).

The localized degradation of fibronectin at cell contact sites in cultures of transformed cells may be important in the regulation of detachment, migration, and subsequent invasion of cells into the connective tissue. It was recently demonstrated that cell surface proteases are involved in other proc-

esses, including trophoblast invasion into the maternal tissues during implantation (13), the invasion of tumor cells and migration of endothelial cells through the basement membrane (18), and myoblast fusion (10).

It has been suggested that cell surface proteases may also be responsible for degradation of extracellular fibronectin fibers and the morphological changes that accompany cell transformation; these include plasminogen activator or plasmin (25–27, 32), and other less well-defined cell surface proteases (7, 14, 22). Although the nature of the protease remains to be identified, previous workers (25–27) have reported that enhanced expression of a membrane-associated protease involved in plasminogen activation is closely correlative with RSV transformation. The present system of culturing transformed cells on fibronectin-coupled substrata may be useful in determining the nature of the protease(s) involved in localized degradation of cell surface fibronectin. Our finding that leupeptin and benzamidine, both inhibitors of plasminogen activator, blocked the release of radiolabeled degradative fragments, but had no discernible effect on localized degradation as shown by the fluorescence-fibronectin assay (Table I), cannot rule out the possibility that a membrane-bound form of plasminogen-activator is highly concentrated at sites of cell-substratum contact and is relatively inaccessible to inhibitors known to block its activity. Localization experiments using antibody directed against purified plasminogen activator would be required to test this possibility. However, the fact that transformed cells do not form negative spots on rhodamine-gelatin substrata appears to rule out a role for gelatinases. Inhibitor experiments in this report show that only 1,10-phenanthroline inhibits both overall and localized fibronectin degradation.

The negative spots on fibronectin substrata observed may be due to localized protease secretion by transformed cells, to membrane proteases on the cell surface, or to endocytosis. The fact that fibronectin clearance occurs only at contact sites, and that media conditioned by transformed cells do not digest fibronectin matrix argues against a secretory product and favors membrane-associated protease. However, lysis of the cell layer with detergents releases digested fibronectin, suggesting that some of degraded products were in fact internalized. The specific removal of fibronectin at rosette-contact sites by transformed cells is the result of membrane-associated proteolytic activity rather than to a nonenzymatic mechanism of removal as previously reported for noncovalently coupled fibronectin on glass surface (3, 9). The results obtained with nontransformed cells indicate that the appearance of rosette contacts and degraded fibronectin spots is directly related to transformation and not to alterations in the physiological state of the cell.

The rosettes associated with the majority of RSV-transformed cells are implicated as surface protrusions emanating from the ventral surface forming close contacts with the substratum (11, 23). They do not appear to be simply the residual adhesion of RSV-transformed cells to their substrata (11, 23, 28). We have shown that the rosettes detected by immunolabeling of vinculin are associated with sites of fibronectin degradation at the ventral surface of the cell. This suggests a possible role for these structures in the invasion of RSV-transformed cells into their extracellular matrices. For example, the redistribution of vinculin upon transformation may directly reflect intracellular molecular events that change the expression and localization of the cell surface proteases involved in the penetration of cell surface protrusions into the extracellular matrix. Further work is required (*a*) to characterize the protease(s) responsible for fibronectin degradation, and (*b*) to determine if the rosettes represent defined ultrastructural entities that are involved in invasive processes and if they are also present in other neoplastic cells involved in malignant spreading.

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