CELL-SUBSTRATUM ATTACHMENT AND CELL SURFACE HYALURONATE OF ROUS SARCOMA VIRUS–TRANSFORMED CHONDROCYTES

YUKO MIKUNI-TAKAGAKI and BRYAN P. TOOLE. From the Developmental Biology Laboratory, Medical Services, Massachusetts General Hospital, and the Departments of Medicine and Anatomy, Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT

Hyaluronate is associated with the cell surface of cultured Rous sarcoma virus–transformed chondrocytes. Detachment of these cells from their substratum by a variety of reagents is accompanied by release of 75–100% of this hyaluronate into solution. Treatment of the cells with 200 U/ml protease-free *Streptomyces* hyaluronidase at 37°C causes release of >90% of the cell surface hyaluronate and complete cell detachment. Treatment with a lower concentration of *Streptomyces* hyaluronidase (30 U/ml) at 25°C or a corresponding activity of testicular hyaluronidase gives similar results, but only in the presence of 1 mM EGTA. Treatment with the lower activities of either hyaluronidase or with 1 mM EGTA alone releases only ~45% of the cell surface hyaluronate and does not cause significant cell detachment.

It is concluded that there are two populations of cell surface hyaluronate differing in their accessibility or their resistance to dissociation from other components of the cell surface. It is proposed that the less readily released fraction is located between the transformed chondrocyte surface and substratum and is necessary for their interaction.

Rous sarcoma virus (RSV) transformation of chick embryo chondrocytes results in a dramatic decrease in synthesis of chondroitin-6-sulfate-proteoglycan and an increase in synthesis of hyaluronate (18, 20, 22, 24). A large proportion of the chondroitin sulfate-proteoglycan produced by normal chondrocytes accumulates extracellularly in the cell layer. In the case of RSV-chondrocytes, however, the major glycosaminoglycan (GAG) is hyaluronate, and the cell surface retains an almost constant amount of this polysaccharide. The cell surface hyaluronate is rapidly and continuously shed into the medium and replaced by the cell (18).

Associated with these biochemical changes are morphological and behavioral changes. The transformed cells lose the polygonal, flattened morphology and immobility of normal chondrocytes and grow more rapidly than the normal cells. They are spindle shaped and are more readily detached from their substratum. We are investigating the role of cell surface hyaluronate and its constant turnover in these changes. In this communication, we examine the effect of removal of cell surface hyaluronate on attachment of RSV-chondrocytes to their substratum.

MATERIALS AND METHODS

Preparation of Cell Cultures

Cultures of chondrocytes were prepared from SPF (specific pathogen free) Cofal-negative chick embryos (Avian Supply Co., Norwich, Conn.) and transformed with the Prague strain of Rous sarcoma virus as reported previously (18).

The transformed chondrocytes were plated at 10^4 cells per well (17 × 16-mm well, Linbro 24-well dishes, Flow Laboratories, Inc., Rockville, Md.) in 0.33 ml of Ham's F-12 medium (Grand Island Biological Co., Grand Island, N. Y. [GIBCO]) supplemented with 7% fetal calf serum and 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., fraction V) under which

conditions the cells attached to the substratum. Medium was changed every 48 h.

Isotope Incorporation and Cell Detachment

Medium was changed 24 h before labeling, which was then carried out for 12 h in 0.5 ml of the above-described fresh medium containing 30 μCi/ml of sodium [^{14}C]acetate (54 mCi/mmol, New England Nuclear, Boston, Mass.) or 10 μCi/ml [^{3}H]leucine (51.6 Ci/mmol, New England Nuclear). After incubation, the radioactive medium was removed and the cultures were washed three times with warm Hanks' balanced salt solution without calcium and magnesium (CMF-HBSS). Then 0.5 ml of the same solution containing various reagents was added to each well, and the cells were incubated for an appropriate time at 25° or 37°C. Duplicate wells were prepared for analysis at each time point. Additional wells for each experimental condition were prepared in the same manner but without radioactive materials. These were used for counting of cell numbers in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.) after dilution with 10 ml of CMF-HBSS containing 1 mM EDTA and flushing several times through a pipette. Reagents used for the treatment of cells were as follows: EGTA; bovine pancreas TPCK-trypsin, 205 U/mg (Worthington Biochemical Corp., Freehold, N. J.); hyaluronidase from *Streptomyces hyaluronlyticus nov.*, 2,500 U/mg (Seikagaku Kogyo Co., Ltd., Tokyo, Japan); hyaluronidase from bovine testis, 15,000 U/mg (Leo, Helsingborg, Sweden); and crude collagenase from *Clostridium histolyticum*, 402 U/mg (Worthington Biochemical Corp.). All reagents were dissolved in CMF-HBSS.

Isolation and Analysis of Hyaluronate and Protein

After incubation with each of the above reagents, three fractions were prepared for analysis. Soluble material, termed "supernate" or "supernatant fraction", released during the treatments was separated from suspended cells by centrifugation. The cell pellet is designated "detached cells." Cells plus any other material remaining adhered to the dish are termed "attached cells." The latter material was prepared for analysis either by further treatment of the dishes with 0.25% trypsin containing 1 mM EDTA (GIBCO) or by scraping in 4 M guanidinium HCl with 50 mM Na acetate buffer, pH 5.8, for hyaluronate or protein analyses, respectively. These treatments left no significant amounts of either labeled material on the dishes. Each fraction was then precipitated with 3 vol of cold ethanol containing 1.3% K acetate in the presence of carrier hyaluronate or bovine serum albumin. Labeled protein was measured directly in these precipitates after dissolving in 50 mM Tris HCl buffer, pH 7.2, and addition of Hydrofluor scintillation fluid (National Diagnostics Inc., Parsippany, N. J.). The amount of labeled hyaluronate was determined, after pronase digestion (1 mg/ml, in 0.1 M Tris buffer, pH 8.0, at 60°C for 12 h) and reprecipitation with ethanol, by chondroitinase ABC digestion (0.1 U per 0.2 μmol uronic acid in 50 mM Tris buffer, pH 7.5, at 37°C for 16 h), thin-layer chromatography and autoradiography of the disaccharide products as reported previously (18). Hyaluronate-containing supernatant fractions obtained after hyaluronidase treatments were further digested directly with chondroitinase ABC, lyophilized, and then analyzed by thin-layer chromatography.

We defined cell surface hyaluronate as that released into solution from saline-washed cells by treatment with 0.25% trypsin for 20 min at 37°C, and we processed it for analysis in the same way as that described above.

RESULTS

Correlation of Cell Detachment with Loss of Cell Surface Hyaluronate

The amount of hyaluronate released on detachment of RSV-chondrocytes from their substratum by a variety of reagents was measured. Cells were prelabeled with [^{14}C]acetate and washed as described in Materials and Methods, then treated at 37°C with 10 mM EGTA, crude bacterial collagenase (200 U/ml), or 0.25% trypsin until complete cell detachment was observed. The three fractions, supernatant, detached cells, and attached cells (which is identical to dish-associated materials in this experiment, because all the cells became detached), were prepared and hyaluronate content was measured as described in Material and Methods. Table I*A* shows that the bulk of the cell surface hyaluronate was released into the supernatant fraction as a result of each of the treatments. In the case of control cultures with CMF-HBSS alone, no significant cell detachment occurred within 90 min.

Except for the trypsin digestion, no cell detachment or significant cell deformation was observed for normal chondrocyte cultures under these conditions.

Cell Detachment as a Result of Specific Removal of Cell Surface Hyaluronate

RSV-chondrocytes prelabeled with [^{14}C]acetate were treated with hyaluronidases to see whether removal of hyaluronate resulted in cell detachment. From Table I*B* it can be seen that 200 U/ml of *Streptomyces* hyaluronidase, which is specific for hyaluronate (21), caused the cells to detach completely within 20 min at 37°C.

In addition, it was found that lower activities of this enzyme (30 U/ml at 25°C) caused detachment in 100 min, but only if 1 mM EGTA was also added to the medium (Table II). A similar result was obtained with testicular hyaluronidase, which degrades hyaluronate and the chondroitin sulfates. Neither the low concentration of hyaluronidase nor 1 mM EGTA alone gave rise to significant detachment above that occurring spontaneously in CMF-HBSS, even though 40–50% of the cell surface hyaluronate was released into solution by each of these treatments. It should be noted that,

TABLE I

Release of Cell Surface Hyaluronate and Detachment of RSV-transformed Chondrocytes

Reagent	Time for complete detachment at 37°	Distribution of hyaluronate*		
		Supernate	Detached Cells	Attached Cells
	min		%	
A. Trypsin (0.25%)	20	100	0	0
EGTA (10 mM)	70	76	7	17
Crude bacterial collagenase (200 U/ml)	90	84	14	2
B. Streptomyces hyaluronidase (200 U/ml)	20	92	8	0

* Results are expressed as percentages of cell surface hyaluronate, which comprises approximately 75% of total cell-associated hyaluronate. The remaining 25% is assumed to be intracellular.

of this 40–50%, approximately half (i.e., 25% of the total cell surface hyaluronate) was shed on incubating in the CMF-HBSS buffer alone. However, in combination, *Streptomyces* or testicular hyaluronidase plus 1 mM EGTA caused release of 88 and 73% of cell surface hyaluronate, respectively, accompanied by >90% cell detachment. Thus the inclusion of 1 mM EGTA appears to have increased the efficiency of hyaluronate removal from the cell surface and, in turn, of cell detachment. The increased hyaluronate removal is presumably the result of an increase in accessibility of substrate to enzyme, as the EGTA did not stimulate enzyme activity per se when tested against hyaluronate or chondroitin sulfate as substrates.

The above results indicate that removal of a large proportion of cell surface hyaluronate from RSV-chondrocytes causes detachment from their substratum.

Time-Course of Hyaluronate Removal and Cell Detachment

The time-courses of release of prelabeled, cell-associated hyaluronate and of cell detachment were compared to obtain additional evidence that these two parameters were closely correlated. Because these events were slower at low concentrations of hyaluronidase in 1 mM EGTA than at the higher enzyme concentrations, the former condition was used for a more precise comparison. Prelabeled cells were incubated for various times under each condition, then the distribution of hyaluronate in supernatant, detached cell and attached cell fractions was measured. This distribution is shown in Fig. 1. It can be seen that, at all times, almost 100% of the hyaluronate is either released into solution or remains associated with the attached cells, except in the late stages of detachment caused by *Streptomyces* hyaluronidase plus 1 mM EGTA, where 25–30% is associated with the detached cells.

TABLE II

Effect of Removal of Hyaluronate on Cell Detachment

Reagent	Cells detached	Hyaluronate released into supernate*
	%	%
CMF-HBSS	14	25
Streptomyces hyaluronidase (30 U/ml in CMF-HBSS)	27	48
Testicular hyaluronidase** (320 U/ml in CMF-HBSS)	21	40
1 mM EGTA in CMF-HBSS	21	48
Streptomyces hyaluronidase (30 U/ml in 1 mM EGTA/CMF-HBSS)	98	88
Testicular hyaluronidase** (320 U/ml in 1 mM EGTA/CMF-HBSS)	93	73

* Results are expressed as percentage of cell surface hyaluronate released in 100 min at 25°C.

** 320 U/ml testicular hyaluronidase is equivalent in activity to 30 U/ml of *Streptomyces* enzyme under these conditions.

In Figs. 2 and 3, the rates of release of cell surface hyaluronate into the supernatant fraction are compared with the rates of cell detachment for each condition. It is clear that treatment with either hyaluronidase (at these low concentrations) in the absence of EGTA or with 1 mM EGTA without enzyme caused similar amounts of hyaluronate to be released into solution and that this amount never exceeded 50%. Moreover, the amount of hyaluronate released quickly ap-

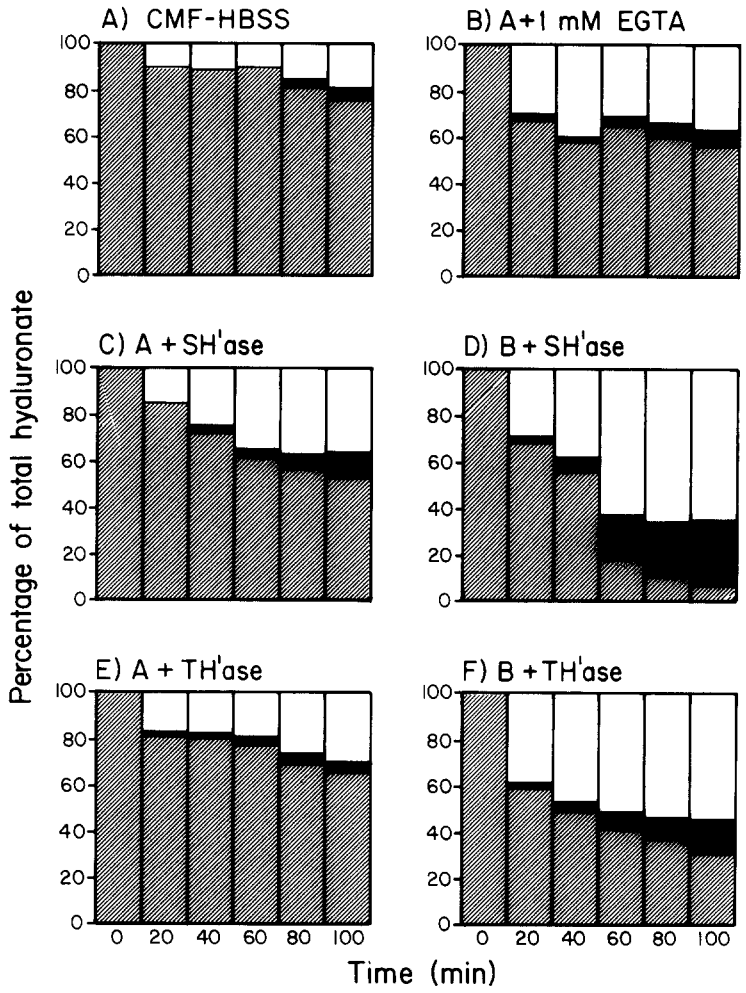

FIGURE 1 Time-course of changes in distribution of cell surface hyaluronate during treatment at 25°C with: (*A*) CMF-HBSS, (*B*) CMF-HBSS plus 1 mM EGTA, (*C*) CMF-HBSS plus 30 U/ml *Streptomyces* hyaluronidase (SH'ase), (*D*) CMF-HBSS plus 1 mM EGTA plus 30 U/ml SH'ase, (*E*) CMF-HBSS plus 320 U/ml testicular hyaluronidase (TH'ase), (*F*) CMF-HBSS plus 1 mM EGTA plus 320 U/ml TH'ase. 30 U/ml of SH'ase is equivalent in activity to 320 units per ml of TH'ase in CMF-HBSS using hyaluronate as substrate. The rates of cell detachment obtained in this series of experiments are given in Figs. 2 and 3. Open areas, supernatant fractions; hatched areas, detached cells; striped areas, attached cells.

proached a plateau, indicating that continuous slow release of hyaluronate was not occurring. Each of these treatments gave rise to only 10–20% detachment of cells. However, when either enzyme was combined with 1 mM EGTA, virtually all of the cell surface hyaluronate was released and all of the cells became detached. This indicates a strong correlation between cell detachment and release of the ~50% cell surface hyaluronate that remained.

Effect of Cell Detachment by Hyaluronidase on Protein Distribution

Possible contamination by protease in the hyaluronidases used above was checked by the method of Lin et al. (16) using methylated bovine serum albumin as a substrate. Under conditions where <1 ng TPCK-trypsin/ml was readily detectable, no activity was obtained in the *Streptomyces* hyaluronidase solution used for detachment

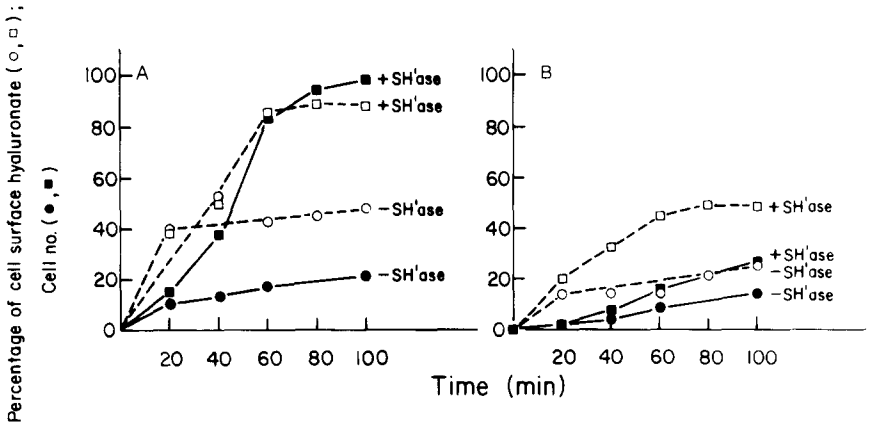

FIGURE 2 Comparison of rates of release of cell surface hyaluronate (open symbols) and cell detachment (closed symbols) during treatment at 25°C with 30 U/ml *Streptomyces* hyaluronidase (SH'ase) in (*A*) the presence and (*B*) the absence of 1 mM EGTA.

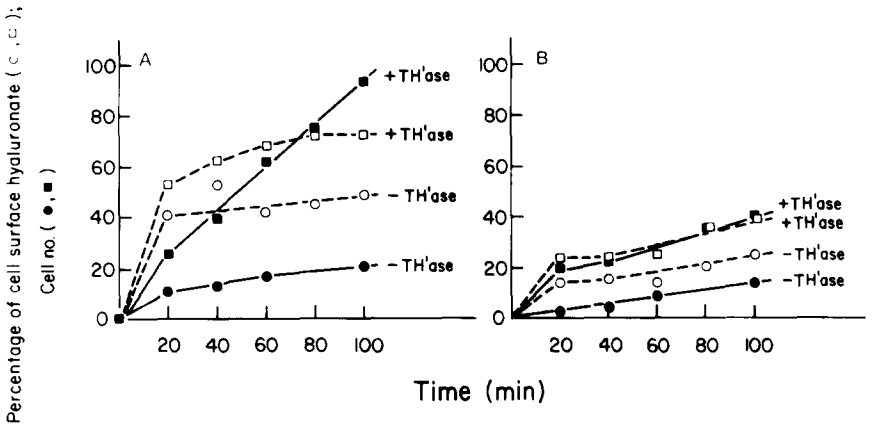

FIGURE 3 Comparison of rates of release of cell surface hyaluronate (open symbols) and cell detachment (closed symbols) during treatment at 25°C with 320 U/ml testicular hyaluronidase (TH'ase) in (*A*) the presence and (*B*) the absence of 1 mM EGTA.

and <1 ng in the testicular hyaluronidase solution. No cell detachment was obtained with TPCK-trypsin at concentrations up to 200 ng/ml, under the conditions used in the experiments described above.

To test further the specificity of the enzyme treatments, cells were prelabeled with [^{3}H]leucine, treated with *Streptomyces* or testicular hyaluronidase plus 1 mM EGTA in CMF-HBSS (as described in the section above) and then analyzed for distribution of ^{3}H-protein in supernatant, detached cell, and attached cell fractions. Controls were performed with 1 mM EGTA in CMF-HBSS. In all cases, very small amounts of ^{3}H-protein were released into solution and no significant differences were obtained as a result of inclusion of either hyaluronidase. Similar amounts of label remained associated with detached and attached cells throughout the course of cell detachment (Fig. 4).

In addition, SDS polyacrylamide (6%) slab gel electrophoresis of the supernatant fractions revealed no unique bands in the hyaluronidase-treated samples (data not shown).

DISCUSSION

The major findings of this study are: (*a*) detachment of RSV-chondrocytes from their tissue culture substratum by a variety of methods results in

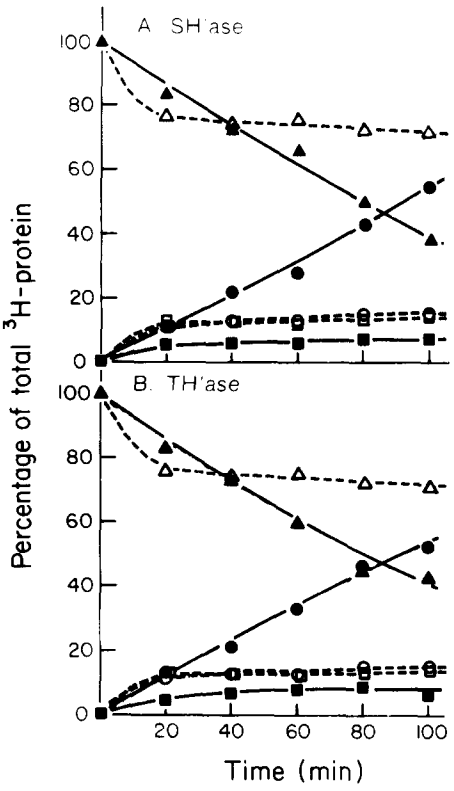

FIGURE 4 Distribution of [^{3}H]leucine-labeled protein during treatment at 25°C with (*A*) 1 mM EGTA (□, ○, △) or 30 U/ml *Streptomyces* hyaluronidase (SH'ase) plus 1 mM EGTA (■, ●, ▲) in CMF-HBSS, (*B*) 1 mM EGTA (□, ○, △) or 320 U/ml testicular hyaluronidase (TH'ase) plus 1 mM EGTA (■, ●, ▲) in CMF-HBSS. Squares, supernatant fraction; circles, detached cells; triangles, attached cells.

the release of most of the cell surface hyaluronate into solution, and (*b*) specific removal of most of the cell surface hyaluronate leads to detachment of the cells. Moreover, kinetics studies have shown a clear correlation between the removal of cell surface hyaluronate and detachment of cells.

Treatment of cells with 1 mM EGTA or low activities of testicular hyaluronidase or *Streptomyces* hyaluronidase in CMF-HBSS caused the release of 40–48% of cell surface hyaluronate into solution but did not cause significant detachment. Approximately half of this fraction was shed into CMF-HBSS medium alone. However, combination of either hyaluronidase with 1 mM EGTA or use of higher hyaluronidase activity caused rapid loss of 73–100% of cell surface hyaluronate and complete cell detachment. We interpret these results to indicate that there are two populations of cell surface hyaluronate. One of these, ~45%, is loosely attached or easily accessible and thus removed by either enzyme or by 1 mM EGTA treatment. The second population, ~55%, is (*a*) deep within the glycocalyx and protected by more external macromolecules, (*b*) bound to other cell surface macromolecules that inhibit the action or access of the hyaluronidases, or (*c*) trapped or bound between the cell and substratum. Treatment with 1 mM EGTA evidently facilitates access of the hyaluronidases to this latter population, which in turn is necessary for continued attachment of RSV-chondrocytes to the substratum. Another possible interpretation would be that hyaluronidase removes a population of hyaluronate that prevents access of EGTA to calcium bound to a protein that is in turn important for cell attachment. This is unlikely, however, because (*a*) the higher concentration of *Streptomyces* hyaluronidase caused complete detachment in the absence of EGTA and (*b*) with low enzyme activity, EGTA treatment led to a significant increase in hyaluronate removal, presumably the result of increased access of hyaluronidase to a usually less-accessible population of cell surface hyaluronate.

The experiments presented here do not distinguish between a direct involvement of hyaluronate in the binding of cells to their substratum and an indirect role (e.g., stabilization or retention of some other binding agent such as fibronectin, which has been shown to contain a specific binding site for hyaluronate [39]). However, that no detectable degradation or release of protein was obtained as a specific result of the enzyme treatments that caused cell detachment suggests that hyaluronate is directly involved. Loss of fibronectin as a result of removal of hyaluronate is unlikely for the following reasons: (*a*) mature chondrocytes in culture do not accumulate detectable fibronectin at their surface or in the extracellular matrix (7); (*b*) RSV transformation of fibroblasts causes a decrease of fibronectin rather than an increase (1, 12); and (*c*) SDS polyacrylamide gel electrophoresis of [^{3}H]leucine-labeled proteins, as described under Results, did not give a detectable band corresponding to standard fibronectin (data not shown).

The most likely explanation of our results is that the less accessible fraction of hyaluronate is organized in a specific manner between the lower

surface of the cell and the substratum, binding them together. The accessibility of this fraction may also relate to its metabolic status, because we have shown previously that the cell surface hyaluronate of RSV-chondrocytes is constantly shed into the medium and replaced by the cell (18). We have recently characterized binding sites for hyaluronate on the surface of various cultured cell lines (37) but no information is yet available concerning the nature of a putative binding site on the substratum. In this regard, however, Culp and coworkers (6, 29) have shown that hyaluronate is a component both of the "footprints" left behind by migrating cells and the "footpads" that remain adhering to the substratum after cell detachment by EGTA. Also, Turley and Roth (36) have demonstrated glycosylation of substratum-bound hyaluronate by migrating simian virus (SV)–transformed 3T3 cells, possible mediated by cell surface glycosyltransferases. Kraemer and Barnhart (14) have shown that cell surface hyaluronate synthesis increases in response to attachment of Chinese hamster ovary (CHO) cells to the substratum. All of these findings point to a role for hyaluronate in cell-substratum interactions.

Several observations by other investigators suggest that increased hyaluronate levels are associated with weakened adhesion of cells to their substrata. For example, actively dividing cells are known to become rounded and adhere less strongly to their substrata than nondividing cells (28, 30). Cultures of growing cells also produce increased amounts of hyaluronate (5, 15, 19, 31). In addition, a CHO cell-line variant that is more resistant to detachment by trypsin or EGTA than the parent line has been isolated and shown to produce much lower levels of hyaluronate (2). The reverse is true of another variant that is more readily detached than the parent line (4). Also, many virally transformed cells in culture are known to be more weakly adherent to their substratum than their parent counterparts, e.g., the RSV-chondrocytes studied here, RSV-fibroblasts (38), SV-3T3 cells (9), and polyoma-transformed baby hamster kidney (BHK) cells (27). In the case of the chondrocytes and fibroblasts, transformation by RSV has been shown to cause greater production of hyaluronate (3, 13, 18). This is also the case for SV-transformed human fibroblasts (11). However, this situation is not straightforward, because SV-3T3 cells produce less hyaluronate than 3T3 cells, and polyoma-transformed BHK cells produce less than BHK cells (10; see footnote 1). Possibly the actual amount of hyaluronate produced may be a less important factor than its organization. In this regard, metabolic experiments with 3T3 and SV-3T3 cells suggest that the concentration of hyaluronate specifically located in cell adhesion sites (or footpads) may increase in association with cell detachment as compared with initial attachment (26). However, there has not yet been any direct demonstration of hyaluronate involvement in cell substratum adhesion for cells other than the RSV-chondrocytes studied here.

In light of the above discussion and the data we have presented, we propose that when cells or localized areas of the cell surface become loosely adherent to the substratum (e.g., during mitosis, during the localized detachments and reattachments associated with cell movements, or on transformation), hyaluronate is involved in cell surface–substratum binding. This interaction would be weaker and would involve different ligands than when cells are functioning under more stable circumstances. The involvement of hyaluronate in this phenomenon may in turn be important in the control of (*a*) cell interactions and cell movements within the hyaluronate-enriched extracellular matrices characteristic of early stages of development of many embryonic tissues (8, 23, 25, 32–34) and (*b*) tumor cell invasion (17, 35).

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[1] C. B. Underhill and B. P. Toole, unpublished data. We have confirmed the results of the study by Hamerman et al. (10) showing that SV-3T3 cells produce less hyaluronate than 3T3 cells. In addition, we have shown (*a*) that this difference occurs in the cell-associated and medium fractions and (*b*) that polyoma-transformed BHK cells also produce less hyaluronate than BHK cells.

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