TWO FUNCTIONALLY DISTINCT POOLS OF GLYCOSAMINOGLYCAN IN THE SUBSTRATE ADHESION SITE OF MURINE CELLS

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ABSTRACT

Footpad adhesion sites pinch off from the rest of the cell surface during EGTA-mediated detachment of normal or virus-transformed murine cells from their tissue culture substrates. In these studies, highly purified trypsin and testicular hyaluronidase were used to investigate the selective destruction or solubilization of proteins and polysaccharides in this substrate-attached material (SAM). Trypsin-mediated detachment of cells or trypsinization of SAM after EGTA-mediated detachment of cells resulted in the following changes in SAM composition: (a) solubilization of 50–70% of the glycosaminoglycan polysaccharide with loss of only a small fraction of the protein, (b) selective loss of one species of glycosaminoglycan-associated protein in longterm radiolabeled preparations, (c) no selective loss of the LETS glycoprotein or cytoskeletal proteins in longterm radiolabeled preparations, and (d) selective loss of one species of glycosaminoglycan-associated protein, a portion of the LETS glycoprotein, and proteins C_d (mol wt 47,000) and C_e' (mol wt 39,000) in short term radiolabeled preparations. Digestion of SAM with testicular hyaluronidase resulted in: (a) almost complete solubilization of the hyaluronate and chondroitin sulfate moieties from long term radiolabeled SAM with minimal loss of heparan sulfate, (b) solubilization of a small portion of the LETS glycoprotein and the cytoskeletal proteins from longterm radiolabeled SAM, (c) resistance to solubilization of protein and polysaccharide in reattaching cell SAM which contains principally heparan sulfate, and (d) complete solubilization of the LETS glycoprotein in short term radiolabeled preparations with no loss of cytoskeletal proteins. Thus, there appear to be two distinct pools of LETS in SAM, one associated in some unknown fashion with hyaluronate-chondroitin sulfate complexes, and a second associated with some other component in SAM, perhaps heparan sulfate. These data, together with other results, suggest that the cell-substrate adhesion process may be mediated principally by a heparan sulfate–LETS complex and that hyaluronate-chondroitin sulfate complexes may be important in the detachability of cells from the serum-coated substrate by destabilizing LETS matrices at posterior footpad adhesion sites.

When normal or SV40[1]-transformed murine cells are detached from their tissue culture substrate by treatment with the Ca^{++}-specific chelating agent EGTA, they leave a small pool of protein and polysaccharide strongly adherent to the substrate, so-called substrate attached material (SAM) (12, 36, 6, 10). SAM is probably similar to the "microexudates" described previously from ellipsometric (37, 46, 47, 31) and electron microscope (34, 53) investigations. SAM-coated substrates were subsequently shown to affect the reattachment kinetics (6), the morphological spread and movement (6), and the growth behavior of virus-transformed cells (6, 47). These studies, as well as others (reference 13; reviewed in reference 10), provided indirect evidence that substrate-attached material was important in the substrate adhesiveness of cells. This conclusion has now been confirmed by autoradiography (7) and scanning electron microscope (36) experiments which establish that SAM is composed of virtually intact footpads which (a) mediate adhesion of cells to the serum-coated (40, 46) substrate (18, 33), (b) are pinched off from the rest of the cell surface during EGTA treatment, and (c) remain firmly bound to the substrate. Therefore, SAM represents a considerable enrichment of one class of cellular adhesion site and thus is suitable for appropriate structure-function analyses, and is particularly relevant for determining the adhesive differences between normal and malignant cells (12, 38).

SAM from normal and SV40-transformed BALB/c 3T3 cells was originally shown to contain the glycosaminoglycan hyaluronic acid (42). In addition, Rollins and Culp (manuscript submitted for publication) have subsequently identified siza-

[1] Abbreviations used in this paper are: CIg, serum-contained cold-insoluble globulin which is very similar in composition and function to the LETS glycoprotein (51, 10); CSP, avian cell surface protein (48–50); EGTA, ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetate; GAG, acidic glycosaminoglycan; GAP, glycosaminoglycan-associated protein (8); LETS, Large External Transformation-Sensitive glycoprotein (20); MEM × 4, Eagle's minimal essential medium supplemented with a fourfold concentration of vitamins and essential amino acids; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline without divalent cations; SAM, substrate-attached material; SDS, sodium dodecyl sulfate; SV40, Simian Virus 40; TPCK, L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone.

ble pools of heparan sulfate and unsulfated and 4-sulfated chondroitin, but little of the other GAGs, in this adhesive material. The rate of synthesis and cell-associated accumulation of these GAGs have previously been studied to some extent in the cells used in our studies (35, 5). There are only trace quantities of collagen in the substrate-attached material (11), which indicates that adhesion of these particular cell types to serum-coated substrates is not mediated by a collagenous matrix. SAM is also enriched in the LETS glycoprotein (20) (also commonly referred to as fibronectin in mammalian cells [44]), actin, myosin, and a protein which is probably the subunit protein of the 10-nm-diameter filaments of these cells (8, 9, 10). LETS has also been identified in SAM recently by immunofluorescent localization using a monospecific antibody (29). The GAGs, LETS glycoprotein, and the microfilamentous proteins were shown to be coordinately deposited in SAM under different conditions of cellular growth and attachment, suggesting that these components form a cell "surface" complex that mediates the adhesive bond (9, 10). The effects of readdition of avian CSP or LETS to deficient cells has also indicated the importance of this cell surface glycoprotein in adhesive functions (49, 50, 1). Cell variants defective in the synthesis of various polysaccharides have been shown to exhibit altered adhesiveness to tissue culture substrates (2, 32).

To learn something of the topographical accessibility and possible functional role of the various GAGs and the LETS glycoprotein in this adhesion site, we have studied the selective destruction and/or solubilization of these components with highly purified trypsin or testicular hyaluronidase. When cells have been treated in situ, some proteolytic enzymes have been shown to selectively destroy some cell surface components, particularly the LETS glycoprotein (20, 3, 44, 41, 45). Other studies (Cathcart and Culp, unpublished data) have indicated that the cytoskeletal proteins in SAM are enclosed within the pinched off membrane (see Rosen and Culp, [36]) and are therefore highly inaccessible to these enzymes. The results reported here indicate that there appear to be two functionally different pools of glycosaminoglycan in this adhesive material and two distinct pools of the LETS glycoprotein which may associate with different GAG classes.

MATERIALS AND METHODS

Cell Culturing and Radiolabeling

BALB/c 3T3 (A31 clone) and SV40-transformed 3T3

(SVT2 clone) cells were grown in plastic tissue culture flasks between their 10th and 25th passages in MEM × 4 supplemented with 250 U/ml penicillin, 250 μg/ml streptomycin, and 10% calf serum as described previously (6). Cells were free of Mycoplasma by assays described previously (6, 8).

To extensively radiolabel cellular proteins or polysaccharides, cells were inoculated into 100-mm-diameter plastic dishes (1.5 × 10⁶ 3T3 or SVT2 cells in 10 ml of medium) or 60-mm-diameter dishes² (0.5 × 10⁶ cells in 5 ml of medium), and were allowed to attach and spread for 24 h. Cell monolayers were rinsed twice with warm PBS and then refed with radiolabeling medium. For protein radiolabeling of 3T3 cells, this medium contained 1/10th the normal leucine concentration in MEM × 4 supplemented with 2 μCi/ml [4, 5 ³H]leucine (sp act 60 Ci/mmol); for SVT2 cells, it contained 1/25th the leucine concentration plus the same supplement. For polysaccharide analyses, complete MEM × 4 was supplemented with 2 μCi/ml of [1-³H]glucosamine (sp act 3.0 Ci/mmol). Cells were grown for an additional 48 h for subsequent enzymatic digestion experiments (final cell density in 100-mm dishes: 2.5-3.0 × 10⁶ 3T3 or 5–6 × 10⁶ SVT2 cells; final density in 60-mm dishes: 1–1.3 × 10⁶ 3T3 or 2.2-2.7 × 10⁶ SVT2 cells). These experiments will be referred to as long-term radiolabeled analyses.

To pulse-radiolabel cell populations, 3T3 or SVT2 cells were inoculated at the densities described above and allowed to grow for 48 h until ~70–80% of the dish surface was covered with cells. Cell monolayers were rinsed twice with warm PBS and refed with 3 ml of the appropriate radiolabeling medium described above, except that 5 μCi/ml [³H]leucine or 5 μCi/ml [³H]-glucosamine was used. Cells were incubated in this medium for 2 h before enzymatic analyses were performed. These experiments will be referred to as short-term radiolabeling analyses. None of the radiolabeling protocols used in these studies was deleterious to cell growth.

Cell Trypsinization

To identify and compare substrate-attached material after trypsin-mediated detachment of cells to SAM after EGTA-mediated detachment, cultures were radiolabeled with the appropriate precursor, rinsed twice with warm PBS, and then treated with a prewarmed solution of TPCK-trypsin in PBS. For concentration-dependence studies, 0–300 μg/ml was generally used for 15 min incubations at 37°C; for time course studies, 5 μg/ml was used for time points varying between 0 and 120 min. After trypsinization, cells were rinsed with PBS and incubated with a PBS solution of 100 μg/ml soybean trypsin inhibitor and 3-mM phenylmethyl sulfonyl fluoride (PMSF) for 5 min at 37°C to destroy proteolytic

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² 60-mm-diameter dishes were used for kinetic analyses and 100-mm-diameter dishes for preparative experiments with SDS-PAGE.

activity. Undetached cells were subsequently removed by a 15-min incubation of all dishes at 37°C with 0.5-mM EGTA in PBS followed by gentle pipetting to quantitatively remove cells. The SAM-coated dishes were rinsed three times with PBS and SAM quantitatively removed with 0.2% sodium dodecyl sulfate (SDS) (2 ml/60-mm dish; 5 ml/100-mm dish) by shaking at 37°C for 30 min for determination of radioactive content or for SDS-polyacrylamide gel electrophoresis (PAGE) analyses.

SAM Trypsinization

Radiolabeled cell populations in the appropriate dishes were rinsed twice with warm PBS, treated for 30 min at 37°C with 0.5 mM EGTA in PBS (2 ml/60-mm dish or 5 ml/100-mm dish) on a gyratory shaker, and gently pipetted to quantitatively remove cells. A variety of studies have indicated that contamination with whole cells is <0.01% (7, 9, 12, 36). The SAM-coated dishes were rinsed twice with warm PBS and then treated with the indicated concentrations of TPCK-trypsin (generally 0.25-10 μg/ml) in PBS for various periods of incubation, as described above. For kinetic analyses, the trypsinate at the end of the incubation was aspirated and diluted to 0.2% in SDS; an aliquot of 0.2 ml was analyzed to determine the amount of radioactive material solubilized using four separate dishes per individual concentration or time point. For SDS-PAGE, four 100-mm dishes per concentration or per time point were analyzed by decanting the trypsinate, rinsing the dish twice with PBS and incubating the dish with 5 ml of 100 μg/ml soybean trypsin inhibitor and 2-mM PMSF for 5 min at 37°C, and harvesting the SAM with 5 ml of 0.2% SDS. The trypsin-mediated effects noted in these studies were not observed when the enzyme was pretreated with soybean trypsin inhibitor or was added along with 100 μg/ml bovine serum albumin.

SAM Hyaluronidase Treatment

SAM-coated dishes were prepared as described above for kinetic or SDS-PAGE analyses. Dishes were incubated in varying concentrations of highly purified testicular hyaluronidase (0–50 μg/ml for 15 min at 37°C, using two different batches of enzymes with specific activities of ~7,500 U/mg) or for varying times (5 μg/ml at 37°C for 0-120 min). Samples of solubilized or resistant material were harvested as described for SAM trypsinizations for kinetic or SDS-PAGE analyses. Hyaluronidase-mediated effects reported here were not observed when enzyme was pretreated in a boiling water bath before addition to SAM-coated dishes or when 50 μg/ml purified hyaluronic acid was added along with the enzyme; co-addition of bovine serum albumin failed to inhibit hyaluronidase-mediated effects.

SDS-PAGE

Gel electrophoresis was performed as described previously (8) using 12% Ortec Inc., Oak Ridge, Tenn.

slab gels. In brief, a set of four 100-mm dishes was used for each concentration or time point of enzyme treatment. The SDS-solubilized SAMs were vacuum dialyzed to a 100 μl vol, dialyzed against sample buffer (15% glycerol, 0.2% SDS, 1% mercaptoethanol, 0.075 M Tris sulfate pH 8.4, and 0.001% bromphenol blue), and boiled for 10 min before SDS-PAGE. Equal amounts of radioactivity were electrophoresed, and all samples including undigested control preparations were run on the same slab gel in adjacent wells. Gels were then stained and autoradiographed directly or fluorographed as described previously (8) under conditions where time of exposure to the X-ray film was linearly proportional to the amount of radioactive material in the band. Autoradiograms and fluorograms were traced on a Joyce-Loebl microdensitometer, Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England.

Glycosaminoglycan Analysis

These procedures are described in detail by Rollins and Culp (manuscript submitted for publication). In brief, GAGs (after extensive pronase digestion to destroy all protein) were ethanol-precipitated three times from SDS-extracted and concentrated SAM preparations in the presence of carrier hyaluronic acid and chondroitin 4- and 6-sulfate. The polysaccharide was quantitatively digested with chondroitinase ABC to determine the amounts of radioactive hyaluronate- and chondroitin sulfate-derived Δ4, 5 unsaturated disaccharides subsequent to paper chromatography (52). Heparan sulfate was determined by a two-dimensional cellulose acetate electrophoresis method and by sensitivity to nitrous acid degradation before Sepharose 6B chromatography (24, 25, 27).

Materials

The following materials were obtained: plastic tissue culture dishes from Lux Scientific Corp., Newbury Park, Calif.; MEM × 4 from Grand Island Biological Co., Grand Island, N. Y.; calf serum from Kansas City Biologicals; [4, 5³H]leucine and [1-³H]glucosamine hydrochloride from Amersham/Searle Corp., Arlington Heights, Ill.; highly purified TPCK-trypsin, testicular hyaluronidase (HSEP), hyaluronic acid, and soybean trypsin inhibitor from Worthington Biochemical Corp., Freehold, N. J.; SDS, acrylamide, and bisacrylamide from Bio-Rad Laboratories, Richmond, Calif.; authentic standards of Δ4, 5 unsaturated disaccharides from Miles Laboratories Inc., Elkhart, Ind.; chondroitinase ABC or AC from Seikagaku Fine Chemicals, Inc., Tokyo, Japan; hyaluronate, chondroitin 4-sulfate and -6-sulfate from Sigma Chemical Co., St. Louis, Mo.; highly purified bacterial collagenase from Advanced Biofacture Corp., Lynwood, N. Y.

RESULTS

Cell Trypsinization

Initial interest was focused on whether trypsin-

mediated detachment of cells leaves more or less protein- and polysaccharide-containing SAM when compared to EGTA-mediated detachment. As shown in Fig. 1, trypsinization of SVT2 cells *in situ* left increasingly smaller pools of substrate-attached material as the enzyme concentration was increased and as compared to the EGTA-treated control population (0 trypsin concentration). At concentrations of 100 μg/ml or more, cells were completely detached with trypsin alone. The glucosamine-radiolabeled polysaccharide was much more labile to trypsinization off the substrate than was the protein, indicating that the labile polysaccharide is not required to maintain adherence of most of the protein to the substrate. Much of the protein in SAM has previously been shown to be cytoskeletal protein (8, 9). Trypsinization of 3T3 cells yielded similar data.
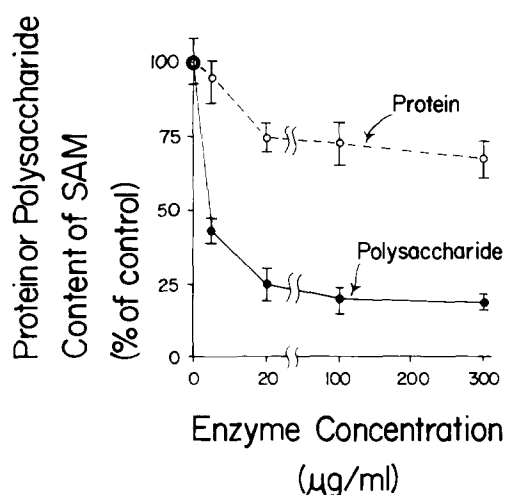

FIGURE 1 Substrate-attached protein and polysaccharide after trypsinization of cells *in situ*. SVT2 cells were grown in medium containing radioactive leucine or glucosamine (in separate experiments) for 48 h as described in Materials and Methods. Four dishes each were treated with the indicated concentration of TPCK-trypsin for 15 min at 37°C, followed by incubations in soybean trypsin inhibitor (plus PMSF) and 0.5 mM EGTA in PBS to remove persistently adherent cells as described in Materials and Methods. At trypsin concentrations of 100 or 300 μg/ml, all cells were detached during the trypsin treatment. The SAM was isolated by SDS treatment, an aliquot of which was assayed for content of radioactive *Protein* or *Polysaccharide*. These amounts were divided by the total amount of radioactive material from SDS-extracted untreated controls (EGTA-detached cells) and multiplied by 100 to obtain the percentage of SAM resistant to trypsinization. Simple averages of the four determinations are shown with error bars showing the range of the four determinations.

SDS-PAGE analyses were performed with SAM preparations from trypsin- or EGTA-detached cell populations to determine (a) if the same protein species remain substrate bound and (b) if specific species identified in EGTA-derived SAM, particularly the LETS glycoprotein (8, 9), are unusually labile to trypsinization of cells in situ. The fluorogram shown in Fig. 2 indicates that similar distributions of proteins are observed in SAMs from EGTA-treated or trypsinized cells, even at the highest trypsin concentration where complete cell detachment has occurred. These
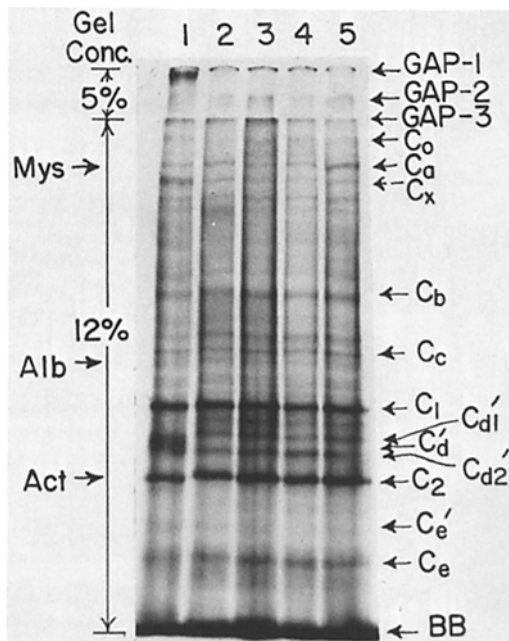

FIGURE 2 SDS-PAGE analyses of "SAM" from trypsinized cells. 3T3 cells were treated with the following concentrations of trypsin for fifteen min after growth for 48 h in radioactive leucine-containing medium, after which their SAM was isolated as described in Materials and Methods for electrophoretic analyses: well no. 1, 0 μg/ml trypsin; well no. 2, 5 μg/ml; well no. 3, 20 μg/ml; well no. 4, 100 μg/ml; well no. 5, 300 μg/ml. 10,000 cpm of each SAM sample was electrophoresed in adjacent wells of the same slab gel. The gel was dried and fluorographed as described in Materials and Methods. The fluorogram is shown. The following components in SAM have been partially or completely characterized (8, 9): GAP-1, -2, -3 are glycosaminoglycan-associated proteins; C_o = LETS glycoprotein; C_a = myosin; C_1 = subunit protein of 10-nm diameter filaments; C_2 = actin. The following markers were used: Mys = rabbit skeletal muscle myosin; Alb, bovine serum albumin; Act = rabbit skeletal muscle actin; BB = bromphenol blue marker dye.

cells were long-term radiolabeled; therefore, substrate-attached material being examined in these experiments is derived from both cellular footpad material and "footprint" material which cells leave behind as they migrate across the substrate (9, 10). Some features of these gels should be noted: (a) the LETS glycoprotein in SAM (C_o) is not unusually labile to trypsin relative to many of the other proteins; (b) the glycosaminoglycan-associated protein (GAP-1) (8) band is completely trypsin labile, correlating with the greater lability of the polysaccharide observed in Fig. 1 (most of which is GAG); (c) complete loss of minor protein C_d' (mol wt 49,000) with appearance of two discrete entities C_{d1}' (mol wt 50,000) and C_{d2}' (mol wt 48,000); and (d) partial loss of glycoprotein C_x which appears in different SAM preparations in variable amounts (8). These effects were noted at trypsin concentrations as low as 1 μg/ml. Similar observations were made using SVT2 cells.

Analyses by SDS-PAGE of SAMs were also performed using cell populations which had been pulsed with radioactive leucine for only 2 h to examine the lability of components in newly-formed footpads (33, 9). The prominently labeled C_d (mol wt 47,000) and C_e' (mol wt 39,000) proteins, which turn over rapidly during chase analyses of cells (8, 9), were completely eliminated from fluorograms at trypsin concentrations as low as 1 μg/ml, a concentration which has little effect on cell morphology during a 15-min incubation. The GAP-1 protein was also completely sensitive to removal under these conditions. The other proteins, including LETS (C_o) and the cytoskeletal proteins (C_a, C_1, and C_2), were again not selectively destroyed by trypsinization. These results suggest that integrity of C_d, C_e', and GAP-1 is not required to maintain the adhesiveness of these cells to the substrate, since they can be completely removed from SAM at very low trypsin concentrations which minimally solubilize (or digest) the LETS glycoprotein and cytoskeletal proteins[3] and minimally affect the morphology of cells.

SAM Trypsinization

We then designed experiments to test the tryp-

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[3] The following cytoskeletal proteins have been identified in SAM (8–10): C_a = myosin; C_2 = actin; C_1 = subunit protein of 10-nm-diameter filaments (4); and a small amount of α-actinin. Many of the unidentified proteins, including C_b–C_f, are not glycosylated, and some of these are presumably cytoskeletal elements.

sin lability of specific substrate-attached compo-
nents after prior removal of cells by EGTA treat-
ment. The solubilization of protein or polysaccha-
ride from SAM as a function of increasing trypsin
concentration is shown in Fig. 3 for long-term
radiolabeled SVT2 cells. A sizable amount of
polysaccharide (45%) and a smaller amount of
protein (15%) are labile to the lowest concentra-
tion of trypsin (0.25 μg/ml). At all trypsin concen-
trations, much more polysaccharide is released
than protein. 25% of the polysaccharide resists
solubilization, while an additional 15-20% of the
protein becomes somewhat more labile at higher
trypsin concentrations. Time course studies also
confirmed (a) the differences in lability of protein
and polysaccharide and (b) the resistance to solu-
bilization of 25-30% of the polysaccharide. Simi-
lar data were obtained for 3T3 cells. At trypsin
concentrations >100 μg/ml, all of the protein and
polysaccharide were eventually solubilized (data
not shown).

Analyses of short-term radiolabeled SAM prep-
arations established that (a) 60-70% of the poly-
saccharide was easily solubilized from the dish
while the remainder was relatively resistant and
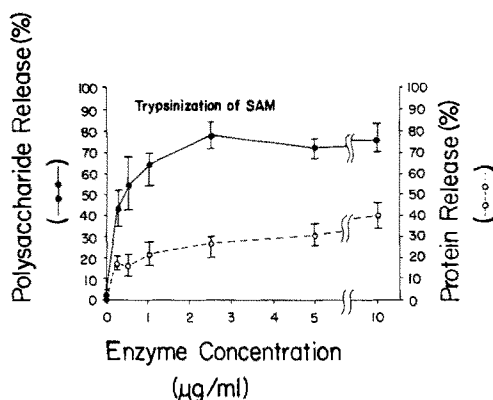(b) as much as 80% of the protein resisted

FIGURE 3   Trypsin-mediated solubilization of protein
or polysaccharide from SAM. SVT2 cells were grown
for 48 h in radioactive leucine-containing or glucosa-
mine-containing medium (in separate experiments).
SAM-coated dishes were obtained from these cultures
by EGTA-mediated detachment of cells and treated with
the indicated concentration of trypsin for 15 min at 37°C
as described in Materials and Methods. The amount of
solubilized radioactive material was determined; this
amount of radioactive material was divided by the
amount of radioactive material solubilized with SDS
using control dishes (EGTA-treated alone) and multi-
plied by 100 to obtain the percentage of total SAM-
containing solubilized protein or polysaccharide.

solubilization at trypsin concentrations as high as
20 μg/ml (data not shown). These results confirm
the results of cell trypsinization in situ, showing
that ~50-60% of the polysaccharide is not re-
quired to maintain the adherence to the substrate
of the majority of the protein in these adhesion
sites.

SDS-PAGE analyses were used to examine
selective destruction of specific protein entities
during trypsin treatment of SAM. In Fig. 4A, a
longterm radiolabeled and trypsinized SAM prep-
aration is compared to an untreated control. The
profiles look remarkably similar, except for (a)
selective loss of GAP-1 during trypsinization
(again correlating with the sizable loss of glycosa-
minoglycan), (b) selective reduction of band C_e',
and (c) no selective loss of the LETS glycoprotein
(C_o) or cytoskeletal proteins. These changes were
noted for trypsin concentrations varying from 0.25
to 10 μg/ml, indicating that GAP-1 and C_e' are
not required for adherence of the cytoskeletal
proteins and that all of the other components of
SAM are proportionally solubilized upon damag-
ing some unknown critical moiety.

Analyses of gels of short-term radiolabeled
preparations (Fig. 4B) indicated that GAP-1, the
prominently-labeled C_d, and C_e' are unusually
labile and that the cytoskeletal proteins (C_a, C_1,
and C_2), as well as C_b-C_f, are conserved in similar
proportion to those observed in untrypsinized
preparations. A portion of the LETS (C_o) was
labile at all trypsin concentrations, but the rest
was relatively resistant (Table I). Trypsinization
reduced the ratio of LETS to any of the cytoskel-
etal proteins by one-half, whereas the ratio of
cytoskeletal proteins to each other was relatively
unaffected. Profiles similar to the trypsinized sam-
ple in Fig. 4B were obtained at enzyme concentra-
tions varying between 0.25 μg/ml and 10 μg/ml.
In time-course studies using 1 μg/ml of enzyme,
incubations as short as 5 min were sufficient to
maximally solubilize GAP-1, C_d, and C_e'. Similar
results were obtained using 3T3 SAM-coated
dishes. These results are virtually identical to
those obtained with cell trypsinization.

Chemical analyses were then performed on
untreated or trypsinized SAM preparations from
long-term glucosamine-radiolabeled SVT2 cul-
tures to determine if some GAG species are
selectively resistant to solubilization. As noted in
Table II, untreated SAM contains appreciable
quantities of heparan sulfate, chondroitin, and
hyaluronic acid. After a trypsin treatment which
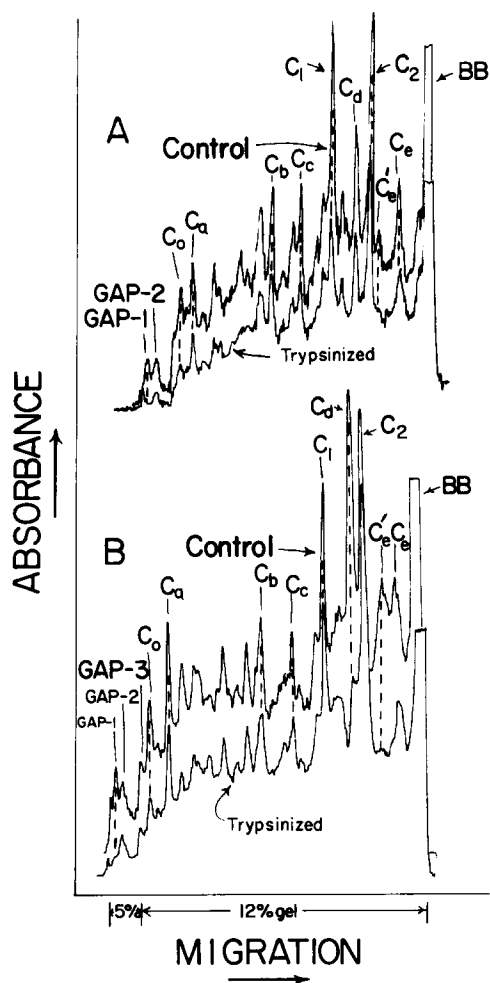solubilizes 46% of the polysaccharide and 10% of

FIGURE 4 SDS-PAGE analyses of trypsin-treated SAM. SAM was prepared from SVT2 cultures grown for 48 h (A) or 2 h (B) in radioactive leucine-containing medium. *Control* preparations received no trypsin, while *Trypsinized* preparations were treated with 5 μg/ml for 15 min at 37°C. Trypsin activity was destroyed with soybean trypsin inhibitor and PMSF and SAMs were isolated for SDS-PAGE analyses as described in Materials and Methods. 10,000 cpm of SAM of control (48 h), control (2 h), and trypsinized (2 h) samples were electrophoresed in adjacent wells of a slab gel, whereas only 6,000 cpm of the trypsinized (48 h) sample was used; the gel was fluorographed. Various proteins are identified in the legend to Fig. 2 or in the text. Treatment with trypsin concentrations varying between 0.25 and 10 μg/ml generated the pattern differences shown here.

the protein, the substrate-bound resistant material is considerably enriched in heparan sulfate, whereas much of the hyaluronate and chondroitin components have been solubilized. This indicates

that heparan sulfate, as well as LETS and the cytoskeletal proteins, is an integral component of this adhesive material while much, or perhaps all,

TABLE I

Relative Content of LETS and Cytoskeletal Proteins in Substrate-Attached Material

Ratio of components	Control	Trypsinized
C_0/C_a	0.59	0.29
C_0/C_2	0.24	0.091
C_1/C_2	0.49	0.47
C_a/C_2	0.41	0.34

SVT2 cells were pulse-radiolabeled for 2 h with medium containing radioactive leucine, EGTA-detached, and the SAM was treated with 0 (*Control*) or 5 μg/ml (*Trypsinized*) TPCK-trypsin at 37°C for 15 min as described in Materials and Methods. The SAM was then isolated by SDS treatment, after inhibition of protease activity, for SDS-PAGE analyses. The dried gel was fluorographed, and the fluorogram was traced with a Joyce-Loebl microdensitometer. Areas of the following specific peaks identified in the ratio column were determined: C_0 = LETS glycoprotein; C_a = myosin; C_1 = subunit protein of the 10-nm-diameter filaments; C_2 = actin. Experimental error in calculating peak errors varied from ± 5% for C_1 and C_2 to ± 9% of C_0.

TABLE II

Glycosaminoglycan in SAM Resistant to and Solubilized by Trypsin Treatment

	Fraction of total GAG (%)		
		Trypsin-treated	
GAG type	Control	Resistant	Soluble
Hyaluronic acid	14.0	4.4	34.4
Chondroitin-6-sulfate	2.4	1.0	5.1
Chondroitin-4-sulfate	13.6	12.2	26.4
Unsulfated chondroitin	18.0	2.3	22.0
Heparan sulfate	52.0	79.7	12.1
Total	100	100	100

SVT2 cells were grown for 48 h in radioactive glucosamine-containing medium and subsequently detached by EGTA treatment as described in Materials and Methods. One-half of the dishes were treated with SDS to remove SAM quantitatively for analysis of GAG content (*Control*). The other half were treated with 5 μg/ml TPCK-trypsin for 15 min at 37°C. At the end of the incubation, the *Soluble* material (46% of the radioactive material) was pooled and vacuum dialyzed to a small volume for GAG analyses. The dishes were rinsed twice with PBS containing protease inhibitors and SDS-extracted to obtain trypsin-*Resistant* material (53% of the radioactive SAM) for subsequent GAG analyses, which were performed as described in Materials and Methods and the legend to Table III.

of the other GAGs is not required to maintain adherence of cytoskeletal elements.

Hyaluronidase Treatment of SAM

The glycosaminoglycan content of SVT2 SAM under different cellular growth and attachment conditions is listed in Table III. Similar distributions were also observed in 3T3 SAM. Hyaluronic acid, chondroitin-4-sulfate, unsulfated chondroitin, and heparan sulfate were the principal components identified in long-term radiolabeled preparations, whereas heparan sulfate was the principal moiety found in newly-formed footpads from reattaching cells, further suggesting its importance as an intrinsic adhesive component. A series of

TABLE III

Glycosaminoglycan Content in SAM of SVT2 Substrate-Attached Material

GAG type	Total GAG (%)		
	Long-term	Re-attaching	Short-term
Heparan sulfate	52.0	82.0	38.0
Chondroitin-6-sulfate	2.4	0.6	2.6
Chrondroitin-4-sulfate	8.9	5.7	13.0
Dermatan sulfate	4.7	4.4	0
Chondroitin (unsulfated)	18.0	4.1	25.0
Hyaluronic acid	14.0	3.8	21.0

SVT2 cells were grown for 48 h (*Long-term*) or 2 h (*Short-term*) in radioactive glucosamine-containing medium and detached by EGTA treatment as described in Materials and Methods. The longterm radiolabeled cells were pelleted by centrifugation, rinsed once with PBS followed by recentrifugation, and resuspended in medium for inoculation into fresh dishes (*Reattaching*) for 2 h before cell removal by EGTA treatment. The SAMs from these three cell populations were harvested by SDS treatment as described in Materials and Methods. The glycosaminoglycan distribution was determined as described in Materials and Methods (in greater detail by B. Rollins and L. Culp, manuscript submitted for publication) for the hyaluronic acid and chondroitin classes by their quantitative breakdown to Δ4, 5 unsaturated disaccharides using chondroitinase ABC or chondroitinase AC enzymes or for heparan sulfate by a combination of two-dimensional electrophoresis on cellulose acetate strips or sensitivity to nitrous acid degradation (24, 25, 27). Terry and Culp (42) previously used alkali to extract and analyze polysaccharide from SAM on plastic substrates, and this treatment is not quantitative and selectively extracts hyaluronic acid from plastic-bound SAM (B. Rollins and L. Culp, unpublished data). SDS treatment of SAM-coated plastic quantitatively removes GAG for subsequent chemical analyses.

experiments were initiated to differentiate between the importance of the hyaluronate-chondroitin classes of GAG from that of heparan sulfate in maintaining substrate adherence of the LETS glycoprotein and the cytoskeletal elements by digestion of SAM with testicular hyaluronidase. The hyaluronate and chondroitin classes of GAG are susceptible to digestion with this enzyme whereas heparan sulfate is resistant.

In Fig. 5, the solubilization of protein and polysaccharide in SVT2 SAM is analyzed as function of enzyme concentration. With long-term radiolabeled preparations (Fig. 5A), 50% of the polysaccharide was solubilized along with only 10–15% of the protein. It is interesting to note that approx. one-half of the GAG in longterm radiolabeled SAM was hyaluronate plus chondroitin sulfate (Table III). The remainder of the protein and polysaccharide resisted solubilization. This suggested that adherence of much of the protein was not dependent upon the integrity of the susceptible hyaluronate-chondroitin classes of GAG. When short-term radiolabeled prepara-
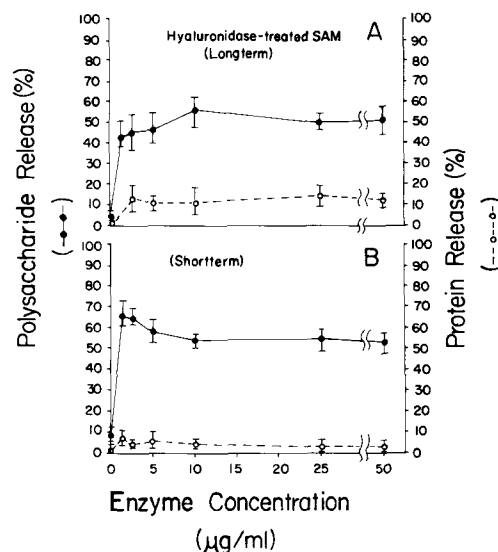

FIGURE 5 Hyaluronidase-mediated solubilization of protein or polysaccharide from SAM. SVT2 cells were grown for 48 (*A*) or 2 (*B*) h in radioactive leucine- or glucosamine-containing medium (in separate experiments to measure protein or polysaccharide release, respectively). SAM-coated dishes were obtained and treated with the indicated concentration of testicular hyaluronidase for 15 min at 37°C as described in Materials and Methods. Four separate dishes per datum point were used and the percentage of protein or polysaccharide solubilized by enzyme treatment was determined as described in the legend to Fig. 3.

tions were analyzed (Fig. 5 B), similar results were obtained with even less of the total protein and more of the polysaccharide being solubilized. This may correlate with the higher proportion of hyaluronate and chondroitin sulfate in short-term preparations when compared to long-term preparations (Table III). Similar observations were made with 3T3 cells.

Therefore, the solubilization of ~50–60% of the glucosamine-radiolabeled polysaccharide (Fig. 5) may reflect the content of susceptible hyaluronate or chondroitin species (Table II). To better define the nature of the GAGs solubilized or resistant to hyaluronidase treatment, chemical analyses were performed. The data of Table IV indicate that the vast majority of the hyaluronate and chondroitin classes were indeed solubilized by

TABLE IV

Glycosaminoglycan in SAM Resistant to and Solubilized by Hyaluronidase Treatment

| | Fraction of total GAG (%) | | |
| | | Hyaluronidase-treated | |
GAG type	Control	Resistant	Soluble
Hyaluronic acid	14		
Chondroitin sulfate*	34	> 4.0‡	94.0‡
Heparan sulfate	52	96.0	6.0
Total	100	100	100

SVT2 cells were grown for 48 h in radioactive glucosamine-containing medium and subsequently detached by EGTA treatment as described in Materials and Methods. One-half of the dishes were treated with SDS to remove SAM quantitatively for analyses of GAG content (control). The other half were treated with 5 μg/ml testicular hyaluronidase for 15 min at 37°C. At the end of the incubation, the *Soluble* material was pooled and vacuum dialyzed to a small volume for GAG analyses. The dishes were rinsed twice with PBS and SDS extracted to obtain hyaluronidase-*Resistant* material for subsequent GAG analyses. GAG analyses were performed as described in Materials and Methods and in legend to Table III.
* All of the various species of chondroitin sulfate identified in SAM (Table III) are pooled here.
‡ Since both hyaluronate and chondroitin sulfate are susceptible to digestion with hyaluronidase and since 77% of the hyaluronidase-solubilized GAG was dialyzable (and exceedingly difficult to analyze for specific moieties of hyaluronate and chondroitin sulfate classes), these two classes of GAG are classed together for comparison with heparan sulfate. Only 7% of the hyaluronidase-resistant material was dialyzable. Heparan sulfate chains are nondialyzable and resistant to hyaluronidase treatment.

digestion of long-term radiolabeled preparations and that the majority of the heparan sulfate resisted solubilization; therefore, the binding of heparan sulfate to the substrate in this adhesive material appears not to be dependent upon the other two classes of GAG.

SAM from reattaching cells (after prior EGTA-mediated detachment) contains principally heparan sulfate and only small quantities of hyaluronate and the chondroitins (Table III). Hyaluronidase treatment of this SAM liberated no more than 10–15% of total polysaccharide (Fig. 6), much less than the proportion of material from long-term radiolabeled SAM and again correlating with the content of hyaluronate and chondroitins. Very little protein was solubilized from reattaching SAM (data not shown). All of these data then indicate that adherence of much of the protein in SAM is not dependent on the hyaluronate and chondroitin classes of GAG, which are coordinately deposited in SAM (B. Rollins and L. Culp,
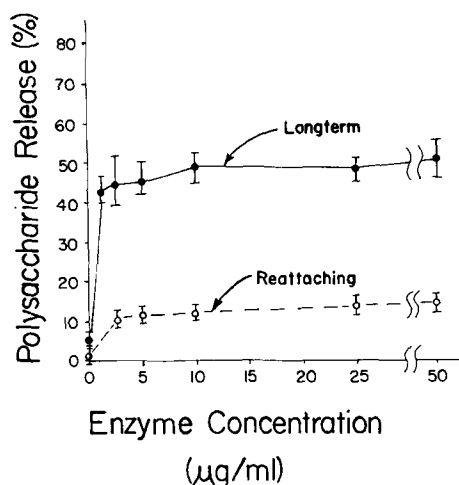

FIGURE 6 Hyaluronidase-mediated solubilization of polysaccharide from long-term-grown or reattaching cellular SAM. SVT2 cells were grown for 48 h in a radioactive glucosamine-containing medium. The cells were removed by EGTA treatment and the SAM from these cells was treated with the indicated concentrations of hyaluronidase as *Longterm* SAM as described in Materials and Methods. Meanwhile, the cells were pelleted by centrifugation, washed once with PBS, and allowed to reattach to fresh dishes in serum-containing medium (also supplemented with radioactive glucosamine). At the end of the 2 h, cells were removed by EGTA treatment, and the SAM (reattaching) was treated with hyaluronidase. The percentage of enzyme-solubilized polysaccharide was determined as described in Materials and Methods and the legend to Fig. 3.

manuscript submitted for publication), but may be dependent upon heparan sulfate.

SDS-PAGE analyses were performed before or after hyaluronidase digestion of SAM to determine if specific proteins were labile. The autoradiogram in Fig. 7 indicates that the protein resistant to hyaluronidase solubilization (wells nos. 2–4) exhibits patterns very similar to control preparations (well no. 1). The relative proportions of the LETS glycoprotein (C_o) and the various cytoskeletal proteins (C_a, C_b, C_c, C_1, and C_2) are conserved in the resistant material. The GAP
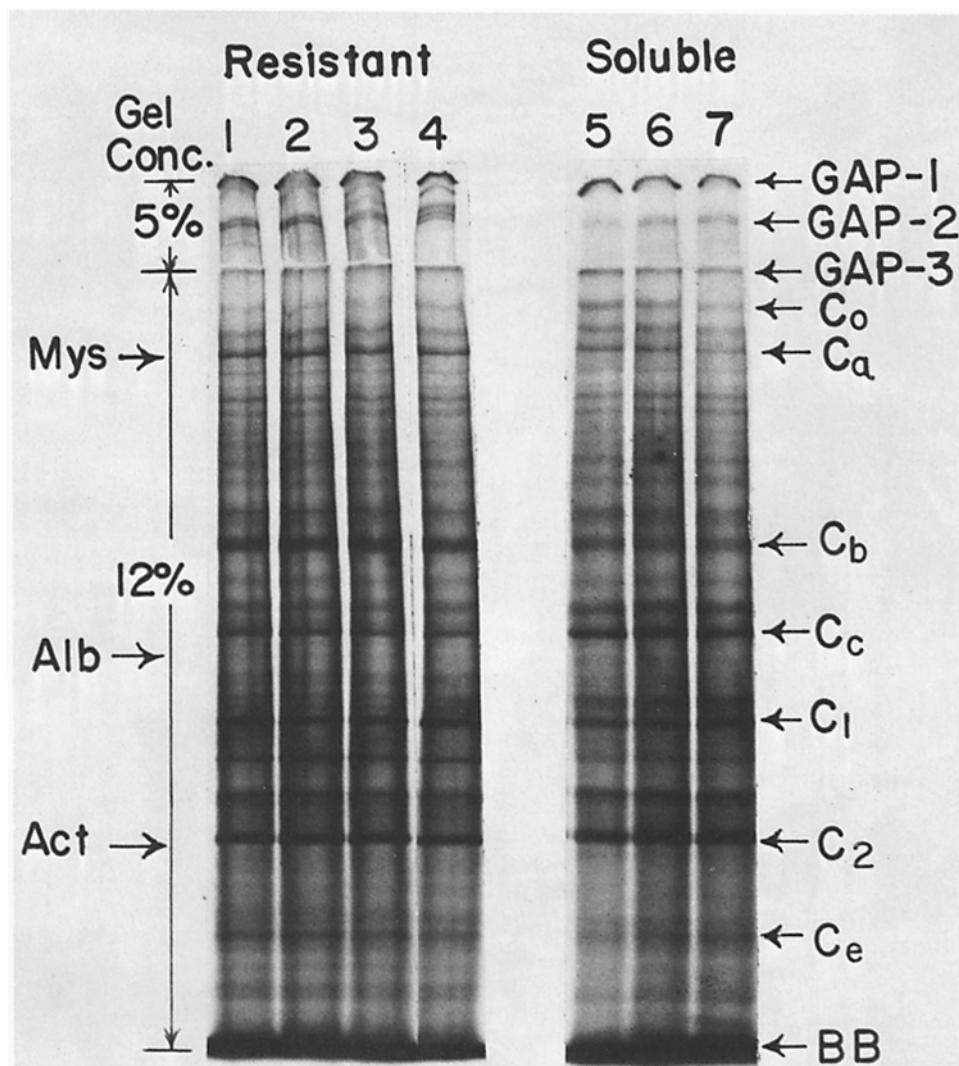

FIGURE 7   SDS-PAGE analyses of hyaluronidase-treated long-term radiolabeled SAM. SAM was prepared from SVT2 cultures grown for 48 h in [U-^{14}C]leucine-containing medium (0.5 Ci/mmol) and treated with hyaluronidase as described in Materials and Methods. The hyaluronidase-*Soluble* material was pooled, put in a boiling water bath for 5 min to destroy enzyme activity after addition of PMSF, concentrated by vacuum dialysis, and prepared for SDS-PAGE by described methods. The hyaluronidase-*Resistant* material was solubilized from the substrate in PMSF-containing SDS and prepared for SDS-PAGE as described in Materials and Methods. 10,000 cpm of each sample was electrophoresed in adjacent wells of the same slab gel and the dried gel was autoradiographed. The autoradiogram is shown with the following concentrations of enzyme used: wells no. 2 and 5, 2.5 μg/ml; wells no. 3 and 6, 10 μg/ml; wells no. 4 and 7, 50 μg/ml. Various proteins are described in the legend to Fig. 2 or in the text.

moieties in the stacking gel become more complex as a function of increasing hyaluronidase digestion (e.g., well no. 4) with the evolution of five or six distinct bands of radioactivity. Other approaches will be required to precisely define the chemical nature of these proteoglycan species. Interestingly, the hyaluronidase-soluble material, although containing only a small proportion of total SAM protein, contains all the species identified in resistant material with a slight enrichment of the LETS glycoprotein (C_o). Therefore, adherence of a small proportion of LETS and cytoskeletal components in SAM is dependent upon the integrity of some of the hyaluronate and/or chondroitin sulfate. These electrophoretic analyses were performed using equal amounts of radioactive material from all samples. If electrophoreses were performed on amounts of control, resistant, and soluble material per set number of cells according to the data of Fig. 6, it is readily apparent that only 5-10% of the LETS glycoprotein and the cytoskeletal proteins are solubilized with hyaluronidase, the remainder of the material being highly resistant at very high enzyme concentrations or extended periods of treatment.

Analyses of short-term radiolabeled preparations revealed several interesting facts. In Fig. 8, hyaluronidase digestion resulted in loss of all of the LETS glycoprotein (C_o) from resistant material (a "new" band of lower molecular weight was not observed). The GAP-1 band was also greatly diminished. The distribution of the cytoskeletal proteins in resistant SAM was not affected (data not shown). This was true for enzyme concentrations varying between 1 and 50 μg/ml (15 min incubations) and time points varying between 5 and 120 min (at 5 μg/ml). Electrophoresis of the soluble material generated a simple pattern with only four species identifiable – GAP species and LETS (C_o) (see Fig. 8), protein C'_{d2} (mol wt 48,000) and C'_{d1} (mol wt 50,000). None of the cytoskeletal protein was solubilized. Loss of LETS from SAM subsequent to hyaluronidase treatment was therefore not a result of proteolytic breakdown of this sensitive glycoprotein, since it could be quantitatively recovered from soluble extracts.

DISCUSSION

These studies on the composition of substrate-attached material after EGTA- or trypsin-mediated detachment of cells suggest that there may be a similar mechanism of action for the two reagents at the cellular level. Revel et al. (33)
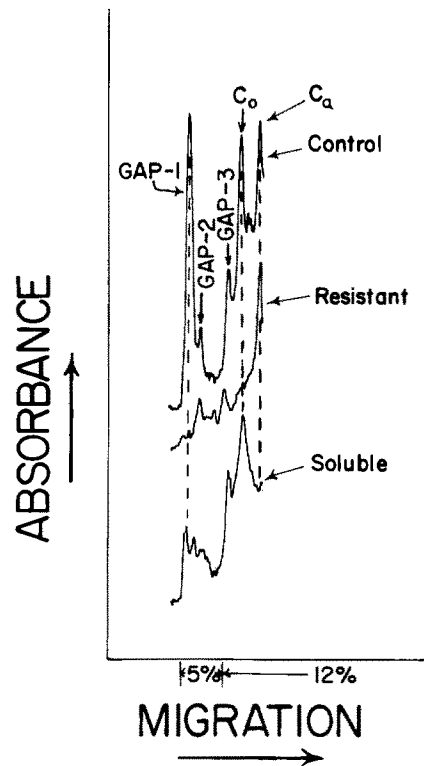

FIGURE 8 SDS-PAGE analyses of hyaluronidase-treated short-term radiolabeled SAM. SAM was prepared from SVT2 cultures grown for 2 h in radioactive leucine-containing medium. The SAM was treated with 0 (*Control*) or 5 μg/ml hyaluronidase, from which *Soluble* and *Resistant* fractions were prepared for SDS-PAGE as described in the legend to Fig. 7 or in Materials and Methods (there was no soluble material for analyses from control treatments). 10,000 cpm of each sample was electrophoresed, and dried gels were fluorographed. Tracings from only the high molecular weight regions of the fluorograms are shown. These electrophoretic differences were observed with hyaluronidase concentrations varying between 1 and 50 μg/ml.

using scanning electron microscopy identified cell-substrate adhesion sites called footpads as being somewhat resistant to trypsinization off the substrate. We have shown in this study that SAM from trypsinized cells (SAM_{try}) contains the same relative proportions of cytoskeletal proteins (C_a, C_1, and C_2), other unidentified proteins (C_b-C_f), and the LETS glycoprotein (C_o) as EGTA-resistant SAM (SAM_{EGTA}). Therefore, trypsin appears to disrupt surface membrane components in areas adjacent to adhesive bonds, which probably results in cytoskeletal disorganization by some unknown mechanism. This results in (*a*) cell round-

ing and retraction of the elastic surface membrane away from the adhesion site as observed by Rosen and Culp (36) and (b) persistence of a small pool of cytoskeletal elements which are membrane-associated in SAM subsequent to breakage of the retraction fibers during culture agitation. SAM$_{try}$ does differ from SAM$_{EGTA}$ by containing much less glycosaminoglycan polysaccharide — the amounts of this labile GAG correlating with the amounts of hyaluronate and chondroitin sulfate (Table III) in longterm radiolabeled SAM preparations and which were shown in other experiments to be selectively trypsin-labile (Table II). In the case of cell trypsinization, the material in the adhesion site exposed during enzymatic attack at the cell surface is presumably subjected to further proteolytic processes similar to those which occur during trypsinization of SAM directly.

Trypsinization of cells or SAM resulted in complete loss of the GAP-1 moiety, C$_d$ (mol wt 47,000), and C$_e'$ (mol wt 39,000) in SAM at very low enzyme concentrations or short time periods. The loss of these species occurred with minimal loss of the LETS glycoprotein and cytoskeletal proteins which suggests that these trypsin-labile entities are not critical mediators of adhesive bonds. (It should be noted here that we are referring to the bonds which hold the cellular SAM to the serum-coated substrate; adhesion of cells to the substrate seems to require appropriate cytoskeletal organization as well as SAM-serum-bonds [10]). The resistance of LETS to trypsin-mediated damage in the substrate adhesive material being examined here contrasts to its lability on the remainder of the cell surface as evidenced by loss of lactoperoxidase-iodinated material (21); however, only 10% of total cellular LETS remains in SAM and is unavailable to lactoperoxidase-catalyzed iodination reflecting probably its location in SAM as an intrinsic adhesive component (10). The loss of GAP-1 also correlates with the loss of much of the hyaluronate and chondroitin sulfate, probably the principal components in the GAG-1[4] band (reference 8; B. Rollins and L. Culp, unpublished data). This is supported by two other pieces of evidence: (a) SAM from reattaching cells contains much heparan sulfate and little

[4] SAM contains three size classes of glycosaminoglycan-containing proteoglycans which are separable in 5% Ortec SDS-PAGE gels, the largest being GAG-1 followed by GAG-2 and GAG-3; GAP-1, -2, and -3 are leucine-radiolabeled protein which are associated with the three GAG classes (8, 9).

hyaluronate and chondroitin sulfate, as well as a small amount of GAG-1 and GAP-1 and large amounts of GAG-3 and GAP-3 (8), and (b) trypsinization of glucosamine-radiolabeled SAM results in selective loss of GAG-1 (as determined by SDS-PAGE, L. Culp, unpublished data) as well as loss of hyaluronate and the chondroitins (as determined by chemical analyses, Table II).

These experiments have also identified two distinct pools of the LETS glycoprotein. Trypsinization of short-term radiolabeled SAM resulted in loss of approximately half of the LETS pool while the remainder was not selectively sensitive. Long-term radiolabeled SAM contained LETS which resisted selective loss relative to the cytoskeletal proteins, using a range of enzyme concentrations (0.25–10 µg/ml) which have previously been shown to selectively destroy LETS in other regions of the surface membrane (20, 21, 48, 3, 41, 45). These experiments do not establish whether trypsin-mediated solubilization of LETS results from direct enzymatic digestion of one portion of LETS in SAM or results indirectly from digestion of some unknown moiety with subsequent exposure of LETS to the enzyme. Hyaluronidase treatment results in complete solubilization of the LETS incorporated into SAM during a 2-h radiolabeling period (and no loss of radiolabeled cytoskeletal elements). There was solubilization of a small amount of LETS in long-term radiolabeled SAM, as well as solubilization of a small portion of all the cytoskeletal elements. These data suggest that newly-synthesized LETS in these adhesion sites, much of which is chasable from adhesive material (9), is associated with hyaluronate-chondroitin sulfate proteoglycan complexes (references 17, 19; B. Rollins and L. Culp, manuscript submitted for publication) and has not become associated with intramembranous cytoskeletal elements.

The prominence of the LETS glycoprotein in the substrate-attached material from a wide variety of cell lines, to the principal exclusion of most other cell surface glycoproteins, argues for its importance in cell-substrate adhesion processes (8, 9). Consistent with this is the demonstration that transformed cells become flattened and resist movement across the substrate when plated on SAM-coated substrates (6), that avian CSP is an agglutinin for erythrocytes (49), and that addition of CSP or LETS to virus transformed cells produces a flattened morphology and an increased resistance to detachment (50, 1).

What then does the adhesive LETS bind to? There are a number of experiments now which strongly suggest that a LETS: heparan sulfate complex may be the direct mediator of the adhesive bond to a substrate-bound receptor molecule in serum (40), perhaps a cold-insoluble globulin (reference 16; F. Grinnell, personal communication; R. Haas, B. Murray, and L. Culp, unpublished data). Solubilization of most of the hyaluronate and chondroitin sulfate from SAM, upon hyaluronidase or trypsin treatments, occurs with minimal solubilization of the LETS, heparan sulfate, and cytoskeletal proteins in long-term radiolabeled preparations. Reattaching cells deposit SAM with much heparan sulfate and LETS and little hyaluronate and chondroitin sulfate. Atherly et al. (2) have shown that trypsin-resistant variants of CHO cells synthesize very little hyaluronate but normal levels of heparan sulfate. Although CIg or LETS have been shown to bind to collagen (15, 22, 23, 30), there is virtually no collagen in the substrate-attached material of these murine cells (8, 11)[5]. Consistent with this is the inability of highly purified bacterial collagenase to solubilize any of the protein or polysaccharide from BALB/c 3T3 or SVT2 SAM (data not shown). CIg, the serum-contained form of LETS, has recently been shown to be a "lectin" which binds to the heparin class of glycosaminoglycans (39). Therefore, serious consideration should be given to an adhesion mechanism whereby cell surface LETS binds to heparan sulfate chains that are in turn bound to substrate-bound (collagen or tissue culture plastic) cold-insoluble globulin. The experiments reported here, although consistent with such a mechanism, do not provide direct evidence for it.

What function, then, do the hyaluronate and chondroitin sulfate classes of GAG play? These studies suggest that newly-synthesized LETS is associated with these complexes in adhesive materials, perhaps by binding to one type of GAG chain in these complexes. Several experiments indicate that these GAG classes may be important in detachability of cells. Reattaching 3T3 and concanavalin (Con) A revertant cells (6), which deposit very little hyaluronate and chondroitin sulfate in their SAM, are resistant to EGTA-mediated detachment from the substrate (B. Rollins and L. Culp, unpublished data); accumulation

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[5] Adhesion to either collagen or artificial tissue culture substrates appears to be mediated by serum-contained cold-insoluble globulin (10).

of these GAGs in SAM correlates with the acquisition of detachability by cells. Atherly et al. (2) have shown that trypsin-resistant variants of CHO cells synthesize very little hyaluronate and resist detachment from tissue culture substrates with chelating agents or trypsin. It is not clear how these complexes would destabilize the peripheral regions of footpad adhesion sites—perhaps by competing for binding to LETS with subsequent proteolytic release of these complexes into the medium or by disrupting the organization of a surface component (LETS?) whose stability in a surface matrix is required to maintain the integrity of cytoskeletal components. This latter possibility is suggested by the solubilization of a small pool of LETS and cytoskeletal elements upon hyaluronidase treatment of longterm radiolabeled SAM preparations.

In all the situations examined in our studies, the sensitivity to enzymatic attack of proteins and polysaccharides in SAM was identical for both BALB/c 3T3 cells and their virus-transformed counterparts. This would indicate that these results reflect fundamental properties of cells, be they normal or malignant cell types. However, subtle differences, such as alteration in the level of sulfation of the sulfated GAGs (35, 5, 43, 14) or the content of sulfate (24, 25, 43) or iduronic acid (28) in the heparan sulfates occurring upon virus transformation, would not have been detected with these approaches. Kraemer (26) has recently identified two different pools of heparan sulfate on the surface of CHO cells, one of which is released upon addition of heparin to cells; this raises the interesting question as to whether this exchangeable pool of heparan sulfate is bound to cell surface LETS. If LETS does bind to specific GAGs in adhesion sites, then the level of sulfation or iduronic acid content of heparan sulfate, in particular, may be quite important in determining the association constant between these two classes of molecules and/or their metabolic stability. Other experimental approaches and cell types, such as adhesion-altered variants which have been partially characterized (2, 32), will be required to relate behavioral differences, such as increased detachability and motility of virus-transformed cells, to specific cell "surface" molecular events.

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