Journal of Supramolecular Structure 5:239 (191)–255 (207) (1976)

Molecular Composition and Origin of Substrate-Attached Material From Normal and Virus-Transformed Cells

Lloyd A. Culp

Department of Microbiology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

The proteins and polysaccharides which are left adherent to the tissue culture substrate after EGTA-mediated removal of normal, virus-transformed, and revertant mouse cells (so-called SAM, or substrate-attached material), and which have been implicated in the cell-substrate adhesion process, have been characterized by SDS-PAGE and other types of analyses under various conditions of cell growth and attachment. The following components have been identified in SAM: 3 size classes of hyaluronate proteoglycans; glycoprotein C_0 (the LETS glycoprotein); protein C_a (a myosin-like protein); protein C_b (MW 85,000); protein C_1 (MW 56,000, which is apparently not tubulin); protein C_2 (actin); proteins C_3–C_5 (histones) which are artifactually bound to the substrate as a result of EGTA-mediated leaching from the cell; and proteins C_c, C_d, C_e, and C_f. The LETS glycoprotein (C_0) and C_d appear in newly-synthesized SAM (which is probably enriched in "footpad" material – "footpads" being focal areas of subsurface membranous contact with the substrate) in greater relative quantities than in the SAM accumulated over a long period of time (which is probably enriched in "footprint" material – remnants of footpads left behind as cells move across the substrate). C_0 and C_d turn over very rapidly following short radiolabeling periods during chase analysis. The SAM's deposited during a wide variety of cellular attachment and growth conditions contained the same components in similar relative proportions. This may indicate well-controlled and coordinate deposition of a cell "surface" complex involving the hyaluronate proteoglycans, the LETS glycoprotein, actin-containing microfilaments with associated proteins, and a limited number of additional proteins in the substrate adhesion site. Evidence indicates that SAM is the remnant of "footpad" vesicles by which the cell adheres to the substrate and that EGTA treatment weakens the subsurface cytoskeleton, allowing these footpad vesicles to be pinched off from the rest of the cell. Three different models of cell-substrate adhesion are presented and discussed.

Key words: substrate, adhesion, footpad, microfilaments, protoglycans, glycoprotein

BACKGROUND

The mechanism(s) by which normal and malignant cells adhere to artificial tissue culture substrates has been of long-standing interest because of the stringent anchorage dependence of normal cells for such a substrate for growth and survival in vitro and the notable anchorage independence of malignant cells [see reviews by Taylor (1), Weiss (2)

and Curtis (3)]. Malignant cells move much more aggressively across the substrate than do normal growth-controlled cells (4), and exhibit a facility for underlapping neighboring cells while continuing to divide as substrate-independent cells (5). It is also apparent that the mechanism of cell-substrate adhesion is more experimentally approachable than that of cell-cell adhesion because the substrate surface is a static entity and can be effectively manipulated to enrich for portions of the surface membrane which interact with it. It was imperative then to remove cells from the substrate with reagents that (a) minimized damage of cell surface proteins and polysaccharides, and (b) enriched for the portion of the cell surface involved in the substrate adhesion site for subsequent removal by stronger reagents. The basic question being attacked is whether normal and malignant cells (in our case virus-transformed cells) have different mechanisms for adhesion to artificial plastic or glass substrates because of quantitative or qualitative differences in the cell "surface" molecules involved.

CELL REMOVAL

Culp and Black (6) demonstrated that treatment of substrate-bound BALB/c 3T3 (the A31 clone), SV40*-transformed 3T3 (the SVT2 clone), and Con A-selected revertant cells with the Ca^{++}-specific chelating agent EGTA resulted in efficient cell removal while leaving a small portion of cellular protein and polysaccharide tightly bound to the substrate, the so-called substrate-attached material (SAM) (7). Normal and revertant cells deposited much more of this material than the "wild-type" transformed cells, indicating an interesting quantitative correlation with the more adherent (and therefore less mobile) normal and revertant cells. In general, approximately 1–4% of the cell's protein content and 3–9% of its polysaccharide content remained substrate-bound, depending on the cell type being analyzed.

Is SAM actively involved in the substrate adhesion process, or is it artifactually bound to the substrate subsequent to natural secretion of macromolecules into the medium during growth or subsequent to EGTA-mediated leakiness from intracellular compartments (6)? A variety of experimental approaches have now been used in our laboratory to indicate that most of the components in SAM may be direct participants in the cell-substrate adhesion process, although definitive proof for their precise molecular role is still lacking. Pulse-chase analysis of the metabolic source of SAM (8) and autoradiography experiments to determine its topographical relationship with regard to the location and movement of cells on the substrate (9) indicated that SAM had not resulted from secretion of components into the medium followed by nonspecific binding to the substrate, but that SAM was some portion of the cellular "footpads" on the underside surface by which the cell adheres to the substrate (10, 11).

*Abbreviations: BB, bromphenol blue dye marker; Con A, concanavalin A; CSP, cell surface protein as defined in (20); EDTA, (ethylenedinitrilo) tetraacetic acid; EGTA, ethylenebis (oxyethylenenitrilo) tetraacetic acid; EM, electron microscopic; GAG, acidic glycosaminoglycans (formerly referred to as mucopolysaccharides); GAP, glycosaminoglycan-associated proteins; LETS, large-external-transformation-sensitive glycoprotein as defined in (19); MEM × 4, Eagle's minimal essential medium supplemented with 4 times the concentration of vitamins and amino acids; MSV, murine sarcoma virus; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SV40, Simian virus 40.

BIOCHEMICAL CHARACTERIZATION

Efficient SAM removal from the substrate required treatment with alkali or sodium dodecyl sulfate, an indication of the tenacity by which this material is bound (7). Subsequent analysis by Terry and Culp (7) demonstrated by a variety of methods that the polysaccharide was principally hyaluronic acid after long-term radiolabeling of SAM during cell growth in medium containing radioactive glucosamine (14) on plastic or glass substrates. Minor amounts (less than 5% of the total GAG) of sulfated glycosaminoglycans were also found (7, 12, 13).

Evidence for selective enrichment in SAM of hyaluronic acid from the wide variety of cell surface polysaccharide-containing moieties is given in Fig. 1 showing the resolution of 3 size classes of glucosamine-radiolabeled GAG polysaccharide in the 5% well gel after slab SDS-PAGE analysis of SAM (Fig. 1–called GAG-1, -2, and -3). SAM contained different relative proportions of the 3 GAG size classes when compared to the membrane preparation. While SAM contained very little glycoprotein identifiable in the 8% separating gel, the enriched surface membranes contained a wide variety of glycoproteins [Fig. 1; (16)], as well as hyaluronic acid (15). Both SV40- and MSV-transformed BALB/c 3T3 cell SAM's contained the same triplet of GAG – a large amount of GAG-1 with smaller and equivalent amounts of GAG-2 and GAG-3 (17). Normal and transformed hamster Nil-B SAM's, on the other hand, displayed a predominant GAG-1 band and a decreased amount of GAG-3 relative to GAG-2 as compared with the murine SAM's.

When SAM was analyzed by SDS-PAGE after long-term or short-term growth in radioactive sulfate to determine the distribution of the minor sulfated-GAG components (which are not readily detectable by glucosamine radiolabeling), 90% of the radioactivity co-electrophoresed with GAG-1 and 10% with GAG-3 – a distribution pattern very different from the glucosamine-radiolabelling pattern. This indicates heterogeneity in the molecular composition of these "dissociated" complex polysaccharides which appear to be proteoglycans (see below).

Gel filtration indicated tenacious binding of some of the protein in SAM to the GAG polysaccharide as large proteoglycan complexes (7). Unless indicated otherwise, the following studies apply to SAM prepared from plastic-grown cells. To further resolve the biochemical complexity of proteins in leucine-radiolabeled SAM, slab SDS-PAGE analysis was performed after reduction of disulfide linkages. The separating gels of Fig. 2 indicate that SAM is an enrichment from surface membrane preparations of 3 protein bands in the 5% well gel (Fig. 2A) which co-electrophorese with the 3 GAG bands observed in Fig. 1, and are therefore called "glycosaminoglycan-associated proteins" (GAP-1, -2, and -3)†, corresponding to GAG-1, -2, and -3 bands, respectively (17). The 3 size classes of proteoglycan have different ratios of protein: polysaccharide with GAG-1 having the lowest ratio and GAG-3 having the highest. Compared with surface membrane preparations, SAM is also enriched in proteins C_1, C_2, C_3–C_5, C_0, and C_a–C_f (Fig. 2A and 2B).

Quantitation of the amounts of the major protein components was achieved by measuring peak areas after micro-densitometric tracing of autoradiograms or autofluorograms (Table I). BALB/c 3T3, SVT2, and Con A revertant SAM's contained a similar

†Proteins GAP-1 and GAP-3 were previously called "S_1" and "S_2," respectively (31). The GAP proteins have consistently co-electrophoresed with the GAG polysaccharides during analysis in many different gel systems.

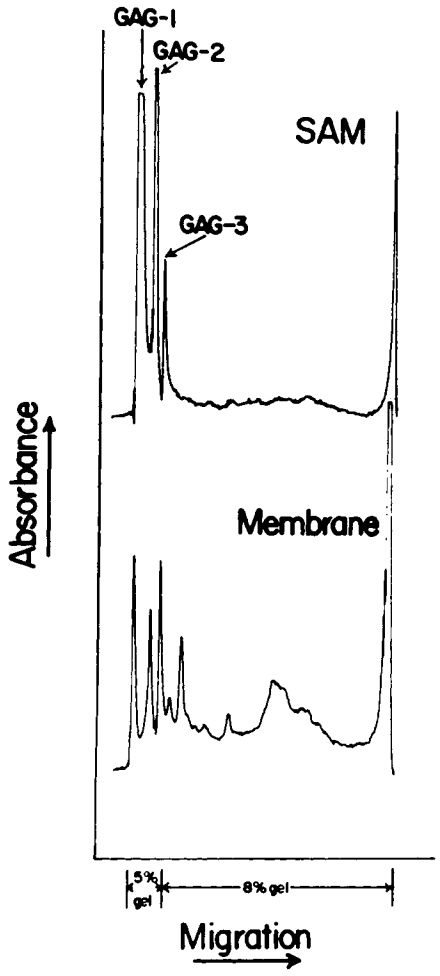

Fig. 1. Gel electrophoretic analysis of polysaccharide-containing components from 3T3 substrate-attached material and a preparation of surface membrane. BALB/c 3T3 cells were grown over a 72-hr period from a very sparse density until 75% of the plastic substrate was covered with cells in MEM X 4 supplemented with 0.5 μ Ci/ml D-[1-^{14}C] glucosamine. Cells were then removed by gentle shaking at 37°C for 30 min in 0.5 mM EGTA (in PBS), followed by gentle pipetting. The substrate surface was rinsed twice with PBS and once with distilled water; the substrate-attached material (SAM) was removed by shaking at 37°C in 0.2% SDS (in H_2O) for 30 min and prepared for gel electrophoresis as described previously (31). Enriched surface membranes (Membrane) of the EGTA-released cells were prepared by a modification (16) of the Brunette and Till method (18). Five thousand cpm of radioactive SAM or Membrane were loaded in adjacent wells of an SDS-PAGE slab gel and electrophoresed at 40 mA/gel for 6 hr. The gel was stained, infiltrated with PPO, dried, and fluorographed by the quantitative method of Laskey and Mills (39). The autofluorogram was then scanned with a Joyce-Loebl microdensitometer to generate scans of Absorbance vs Migration distance. The protease inhibitor, phenylmethylsulfonyl fluoride, was included in all extraction solutions (17).

ratio of C_1:C_2. SVT2 SAM was uniquely different from 3T3 or revertant SAM's in its much higher level of proteins C_3–C_5 and a somewhat elevated proportion of the GAP proteins.

Eight percent SDS-PAGE gels were used to better delineate the distribution of higher molecular weight SAM and membrane components (Fig. 2A). SAM is enriched in several membrane components – GAP-1, -2, -3 as described previously; C_0 (MW ~ 220,

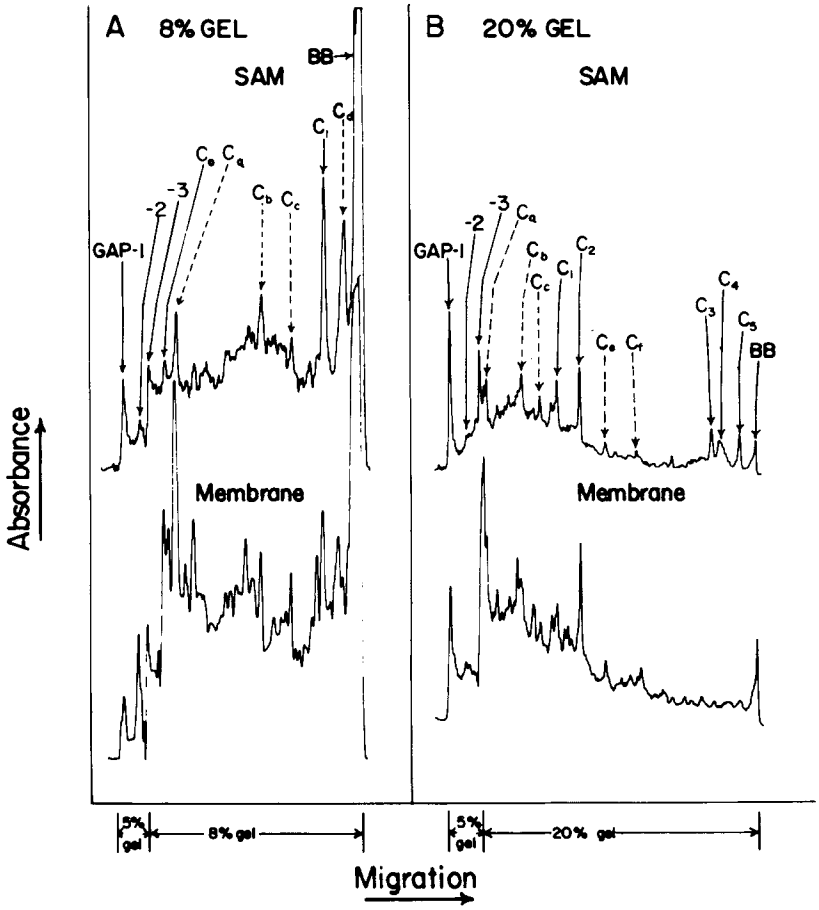

Fig. 2. Gel-electrophoretic analysis of protein-containing components from 3T3 substrate-attached material and preparations of surface membrane. BALB/c 3T3 cells were grown as described in the legend of Fig. 1, except that the medium contained 0.1 of the normal concentration of leucine and was supplemented with 0.5 μCi/ml of L-[U-^{14}C]-leucine. The substrate-attached material (SAM) and enriched surface membrane preparation (Membrane) were isolated and electrophoresed on the same slab SDS-PAGE 8% or 20% separating gels as described in the legend to Fig. 1; different preparations were used in experiments A and B. Ten thousand cpm of each sample was electrophoresed along with a variety of molecular weight markers. Major SAM proteins were identified as GAP-1, -2, -3, or C_1–C_5, while minor SAM proteins were identified as C_0 or C_a–C_f. BB = bromphenol blue front of the gel.

TABLE I. Ratios of Substrate-Attached Proteins From Normal and Virus-Transformed Cells[a]

Ratio (x:y)	Ratio value[b]											
Cell type →	3T3				SVT2				Revertant			
Expt. no. →	1	2	3	Av.[d]	1	2	3	Av.[d]	1	2	3	Av.[d]
GAP_S[c]:C_1	7.7	4.8	5.3	5.7	9.2	10.3	8.3	9.3	10.7	4.0	7.4	6.4
GAP_S[c]:C_2	3.7	2.5	2.1	2.7	2.9	6.9	3.2	3.9	3.2	2.4	4.6	3.2
GAP_S[c]:C_3	10.4	6.2	13.0	9.1	3.1	4.6	2.4	3.2	24.0	6.7	24.0	12.7
GAP_S[c]:C_4	10.5	7.6	17.2	10.6	2.6	5.1	3.4	3.5	37.0	10.2	34.0	15.3
GAP_S[c]:C_5	24.0	24.9	12.4	19.0	10.4	12.8	8.1	10.2	74.0	21.1	17.0	31.0
C_1:C_2	0.48	0.53	0.40	0.47	0.32	0.66	0.39	0.43	0.35	0.61	0.67	0.51
C_1:C_3	1.3	1.3	2.5	1.6	0.34	0.45	0.28	0.35	2.3	1.7	2.3	2.0
C_1:C_4	1.4	1.6	3.2	1.8	0.28	0.50	0.40	0.38	3.5	2.6	1.8	2.4
C_2:C_3	2.8	2.5	6.2	3.4	1.0	0.68	0.73	0.82	7.6	2.8	3.5	3.9
C_2:C_4	2.8	3.0	8.1	4.0	0.89	0.75	1.0	0.90	11.8	4.2	2.7	4.7

[a]Different preparations of leucine-radiolabeled substrate-attached material were electrophoresed and analyzed as described in the legend to Fig. 2. Peak areas were determined using autoradiograms of leucine-radiolabelled SAM which had accumulated during 72 hours of exponential cell growth on plastic substrate in medium containing ^{14}C-leucine. All samples were run on the same gel. Autoradiograms were traced with a Joyce-Loebl microdensitometer under conditions where the peak area was linearly proportional to the amount of radioactivity in the band as described previously (17).

[b]The ratio value was determined by dividing peak area of component X by peak area of component Y, where X and Y are specifically denoted in the ratio column.

[c]GAP_S represents the sum of the peak areas of GAP-1 + GAP-2 + GAP-3.

[d]These ratio values were determined by averaging the peak area data for 3 different preparations of each cell type and by calculating the ratio value of the averages of the indicated gel bands.

000); C_a (MW 200, 000); C_b (MW 85,000); C_c (MW 67,000); and C_1 (MW 56,000). Glucosamine-radiolabeling and extensive exposure to autoradiography film (to compensate for the overwhelming mass of GAG polysaccharide) indicated that C_0 is a glycoprotein, and that C_a, C_b, C_c, and C_1 are nonglycosylated proteins (as well as C_2 and C_3–C_5 as determined on 20% gels). 3T3, SVT2, and revertant SAM's contained these components in similar relative proportions after long-term radiolabeling – the ratio of C_0:C_a = 0.2–0.3, C_0:C_1 = 0.20, C_a:C_1 = 0.4. Swiss 3T3 SAM contained a much higher concentration of C_0 relative to C_a and C_1, while MSV-transformed BALB/c 3T3 SAM contained a lower concentration of C_0.

Various approaches were used to further characterize the major SAM proteins (17), the results of which are summarized in Table II. C_0 is the LETS (19) or CSP (20) glycoprotein because it (a) is glycosylated, (b) is the appropriate size (MW ~ 220,000), (c) is iodinatable with lactoperoxidase as a membrane component (16), (d) is very trypsin-labile as a membrane component, and (e) co-electrophoreses with hamster LETS‡. The sizable amounts of this component in the SAM from the transformant SVT2 may reflect the fact that these cells have a concentration of LETS in their surface membranes which is similar to the concentration found in BALB/c 3T3 cells (16). Protein C_a possesses many properties in common with the heavy chain of myosin: (a) co-electrophoreses with skeletal muscle myosin; (b) is not glycosylated; (c) is iodinatable with lactoperoxidase as

‡Hamster LETS was kindly provided by Dr. Richard Hynes.

TABLE II. Substrate-Attached Cell Proteins[a]

Cell protein	Apparent mol. wt.[b]	Properties
GAP-1, -2, -3	Large (bound to GAG)	Not collagenous
C_0	220,000	LETS glycoprotein (high turnover)
C_a	200,000	Myosin-like
C_x	175,000	Glass-bound glycoprotein
C_y	145,000	Glass-bound glycoprotein
C_b	85,000	?
C_c	67,000	?
C_1	56,000	May not be tubulin
C_d'	49,000	Glass-bound protein
C_d	48,000	Higher turnover
C_2	45,000	Actin
C_e	37,000	?
C_f	27,000	?
C_3	14,000	Histone
C_4	13,000	Histone
C_5	11,000	Histone

[a]Leucine-radiolabeled substrate-attached material was isolated as described in the legends to Figs. 1 and 2, and analyzed by a variety of techniques after SDS-PAGE separation, as described in the text, to partially characterize them. Major proteins are denoted with subscript numbers and minor proteins with subscript letters.

[b]Apparent molecular weights were determined by slab SDS-PAGE analysis using bovine serum albumin, actin, myoglobin, myosin, and E. coli β-galactosidase as markers from plots of the log of the molecular weight vs migration distance.

a membrane component, consistent with the recent membrane association of a myosin-like protein in nonmuscle cells (21, 22); and (d) is specifically immunoprecipitable from Triton-solubilized extracts of BALB/c SVT2 membrane preparations with an antibody prepared against highly purified mouse L cell myosin.** Although protein C_b (MW 85,000) is similar in size to α-actinin, it was well-separated from carrier porcine skeletal α-actinin after analysis on a 2-dimensional gel system described by O'Farrell (25)††,‡‡, an indication that it may not be α-actinin (other evidence will be required to determine if C_b is a unique α-actinin-like component as a functional moiety in SAM).

Although C_1 has an apparent molecular weight of 56,000 consistent with its being tubulin, several pieces of evidence indicate that it may not be tubulin. It migrates in SDS-PAGE gels as a slightly smaller protein than pig brain tubulin. C_1 does not incorporate any tryptophan after cell growth in medium containing radioactive tryptophan, which results in very effective radiolabeling of C_2 (actin – see below) and the GAP proteins. Stephens (23) has reported 4–5 tryptophan residues per 55,000 MW in tubulin, while Collins and Elzinga (24) have reported 4 residues per 45,000 MW for actin. Plastic substrates coated with 3T3 SAM do not bind ^{3}H-colchicine, which would be expected if the large amount of C_1 in SAM were indeed tubulin. Perhaps this protein is a component

**Pre-immune serum did not precipitate C_a from these extracts. The antibodies were kindly provided by Dr. Ira Pastan.

††Proteins were isoelectric-focused in a 2 mm disc gel as the first dimension, and subsequently electrophoresed in a slab SDS-PAGE gel as the second dimension.

‡‡Porcine skeletal α-actinin was kindly provided by Dr. Richard Robson of Iowa State University.

of the 100 Å beta-filaments which may be associated with the actin-containing microfilaments of the cell (32, 33).

Considerable evidence indicates that C_2 is actin. It co-electrophoresed with rabbit skeletal muscle actin in 1-dimensional SDS-PAGE gels and in a 2-dimensional gel system described by O'Farrell (25). Most of the radioactive peptides of C_2 generated by tryptic digestion co-migrated with rabbit skeletal muscle actin peptides during 2-dimensional analysis in a similar fashion to that described by Gruenstein, Weihing, and Rich (26).

A limited amount of information has been obtained on the nature of the GAP proteins. Proline radiolabeling of cells and analysis of SAM by SDS-PAGE revealed a comparable distribution of proline-labeled proteins to that observed with leucine labeling. This would suggest that none of the SAM proteins, particularly the GAP proteins, are collagen, which would be greatly enriched in proline.

Proteins C_3–C_5 appear to be histones since they (a) co-electrophorese with the 3 major bands of calf histone, (b) do not contain tryptophan [an amino acid not found in histones (27)], and (c) are very lysine-rich in comparison with the other SAM proteins. Several pieces of evidence indicate that histones are not "natural" components of substrate-adherent material but are leached from the cell during the EGTA treatment with resultant artifactual binding to the substrate, perhaps to the highly negatively-charged polysaccharide (17). In the first place, histones are not a normal constituent of surface membranes [(16), Fig. 2]. Secondly, they were not found in substrate-attached material after removal of 3T3 or SVT2 cells by scraping cells off the plastic with a rubber policeman, whereas the other SAM components were present in the same relative proportions as found in EGTA-resistant SAM. Finally, the amounts of C_3–C_5 varied considerably from preparation to preparation, while the other SAM components were present in similar relative proportions.

In addition to the radioactive cellular proteins identified in SDS-extractable SAM, a limited number of distinct serum proteins were resolved in these slab gels as Coomassie blue-stainable bands (17) which were not radiolabeled, and are listed in Table III. Protein S_6 co-electrophoresed with albumin. These proteins were extractable from plastic substrates which had been exposed to 10% donor calf serum in MEM X 4 and EGTA-treated to remove loosely adherent serum components (31). Proteins S_3–S_{11} were also identified after growth of normal or transformed cells to confluence and were present in the same relative concentrations as serum components extracted from "cell-free" substrates. Unfortunately, serum components S_1 and S_2 co-electrophoresed with GAP-1 and GAP-3, respectively, in SAM preparations. These serum components appear to play a critical role in the substrate adhesion process (31).

The data described above apply to SAM isolated after removal of normal or transformed cells from plastic substrates. When cells were grown on Brockway glass substrate in radioactive leucine or glucosamine precursors and subsequently removed by EGTA treatment, glass-bound SAM's contained the same distribution of proteins and polysaccharides described above for plastic-bound SAM's, except for a reduced content of C_1 (17). Three unique components were also prominent in glass-bound SAM – C_d', C_x, and C_y.

Other methods of cell removal have been used as a check on the specific origin of plastic-bound SAM components (17). EDTA treatment yields the same SAM components as identified above for EGTA treatment. Scraping cells off the substrate resulted in the same proportions of proteins reported above, but with no histone [scraped cells are minimally leaky compared to EDTA- or EGTA-treated cells (6)].

TABLE III. Apparent Molecular Weights of Substrate-Attached Serum Proteins[a]

Serum protein	Molecular weight
S_3	175,000
S'_3	140,000
S_4	85,000
S_5	73,000
S_6	65,000
S_7	49,000
S_8	29,000
S_9	27,000
S_{10}	23,000
S_{11}	11,000

[a]These are the approximate molecular weights determined by SDS-PAGE analysis using bovine serum albumin, actin, myoglobin, myosin, and E. coli β-galactosidase as markers. Proteins S_1 and S_2, which co-electrophoresed with GAP-1 and GAP-3, respectively, were too large to obtain reliable sizing information (31).

The specificity and origin of the deposition of these various substrate-attached proteins and polysaccharides have also been approached by studying the composition of SAM during a variety of cell attachment and growth conditions, since the previous data pertains to SAM deposited during exponential growth of cells for a 2- or 3-day period in radioactive precursor. This is also important in light of the fact that SAM probably results from 2 different processes – [1] natural deposition subsequent to cell movement across the substrate with resultant pinching-off of substrate-bound footpads at the posterior end of the cell, leaving so-called "footprints" of SAM behind (9); and [2] artificial separation of the cell from the substrate at points of immediate contact between the two via "footpads" during the EGTA treatment. "Footpads" (10, 11, 28) are localized blebs of surface membrane on the underside surface of the cell by which it adheres to the substrate, and whose distribution on the substrate is similar to that of glucosamine- or leucine-radiolabeled SAM detected autoradiographically (9).

Study of the accumulation of SAM as a function of growth indicated a proportionality between increased cell growth and increased accumulation of SAM (either glucosamine- or leucine-radiolabeled) until the substrate surface was completely covered with cells (8). At that point, SAM accumulation discontinued, even though transformed cells continued to grow and pile into dense layers. Similarly, Mapstone and Culp (29) measured stable attachment of EGTA-subcultured cells during the first hour of re-attachment to fresh substrate and deposition of leucine- or glucosamine-radiolabeled SAM. The kinetics of the cell attachment and SAM deposition processes were very similar.

The SAM deposited during the first hour of re-attachment of EGTA-subcultured cells has been examined by SDS-PAGE (17). The same proteins and polysaccharides were observed in similar relative proportions to those described in Figs. 1 and 2 for long-term growth samples, except for (a) a somewhat elevated amount of C_1, (b) a reduced amount of GAG-1, and (c) an increased amount of GAG-3 (Table IV).

TABLE IV. Distribution of Substrate-Attached Proteins and Polysaccharides Under Various Growth and Attachment Conditions[a]

Experiment type	Experimental conditions	Altered distribution[b] of:	
		Polysaccharide	Protein
(A)	Long-term radiolabeling during exponential growth of cells on plastic	GAG-1 > GAG-2 ≅GAG-3	Major amounts of GAP-1, GAP-3, C_1-C_5 and minor amounts of GAP-2 and C_a--C_f and C_0.
(B)	Long-term radiolabeling during exponential growth of cells on glass	Same as (A)	Decreased amount of C_1; major amounts of C'_d, C_x, and C_y
(C)	Long-term radiolabeling during exponential growth of cells on SAM-coated plastic	ND[c]	Increased amount of GAP-3 with a reduced amount of GAP-1
(D)	Attachment of Pre-radiolabeled cells to plastic	Major amount of GAG-3, with minor amounts of GAG-1 and GAG-2.	Increased amounts of C_1, C_0, and C_a
(E)	Attachment of Pre-radiolabeled cells to SAM-coated plastic	ND[c]	Increased amounts of C_d and GAP-1; decreased amount of GAP-3; otherwise, same as (D)
(F)	Chasing of leucine-radiolabeled cells (long-term radiolabeling) – plastic	ND[c]	Same as (A)
(G)	Short-term leucine-radiolabeling during exponential growth of cells – plastic	ND[c]	Preponderance of C_d with same distribution of other proteins as (A), except high proportion of C_0 to C_a or C_1
(H)	Chasing of short-term leucine-radiolabeled cells – plastic	ND[c]	C_0 and C_d disappear; similar amounts of GAP proteins, C_1, and C_2 turnover
(I)	Long-term radiolabeling during exponential growth until substrate saturated with cells[d] – plastic	Same as (A)	Same as (A)
(J)	Short-term leucine-radiolabeling of growth-inhibited 3T3 cells – plastic	ND[c]	Same as (G)
(K)	Chasing of (J)	ND[c]	Same as (H)

[a]BALB/c mouse 3T3 or SVT2 cells were grown as described under Experimental Conditions in medium containing radioactive leucine to assay for cellular SAM proteins or radioactive glucosamine to assay for glycosaminoglycan polysaccharides. At the end of the growth or attachment experiment, cells were removed by EGTA treatment and SAM removed by SDS treatment as described in the legends to Figs. 1 and 2.

[b]SAM proteins and polysaccharides were resolved in slab SDS-PAGE gels as described in Figs. 1 and 2. The patterns obtained from autoradiograms or quantitative autofluorograms of dried gels under the experimental conditions being used were compared to the patterns obtained in experiment (A) in which cells were grown in medium containing radioactive precursor for 72 hr during exponential growth of the cells; SAM was harvested in experiment (A) before 3T3 cells became growth-inhibited and before cells had covered 75% of the area of the substrate.

[c]Not done.

[d]3T3 cells have become density-inhibited in these experiments.

The results of analyses of SAM deposition during various attachment and growth conditions have been summarized in Table IV [also, (17)]. In general, the same proteins and polysaccharides were always found in similar relative amounts, except for a quantitative change in the amounts of 1 or 2 components under some conditions. These data suggest *coordinate deposition* of these components as a tightly-associated cell "surface" complex.

GROWTH ON AND ATTACHMENT TO SAM-COATED SUBSTRATE

If SAM is a result of "natural" deposition during movement of cells on the substrate as so-called "footprints," then other cells in the environment would presumably interact with this material. It was of interest to determine if coatings of SAM on substrates affected cell behavior by newly-attached cells. Culp (30) studied the kinetics of attachment of cells to glass substrates coated with 3T3 SAM (by previous growth to confluence of 3T3 cells and removal by EGTA treatment). EGTA-subcultured 3T3 or revertant cells attached to uncoated or SAM-coated substrates identically. SVT2 cell attachment, on the other hand, was positively stimulated. This effect can also be mimicked by substrate-bound serum components which have been partially characterized (31).

Mapstone and Culp (29) studied deposition of glucosamine- or leucine-radiolabeled SAM during these early attachment processes on substrates with or without SAM coatings, and found the kinetics of deposition by 3T3 or revertant cells unaffected. Deposition by SVT2 cells was quantitatively reduced by 30–35% during the entire attachment process to SAM-coated substrate, and this was shown not to be due to a pool of SVT2 cells which cannot themselves deposit SAM, but which might be able to attach via the pre-deposited SAM. All the cells in the attaching population seem to be affected.

The distribution of SAM proteins and polysaccharides deposited by these SVT2 cells attaching to coated substrates has been examined for the possibility of qualitative differences (17) by SDS-PAGE. This SAM contained the same distribution of proteins and polysaccharides as material deposited by cells attaching to uncoated substrate (Table IV). Thus, the inhibition effect by SAM coatings upon SAM deposition was a quantitative effect resulting in reduced deposition of all components by attaching cells.

Long-term growth of 3T3 or SVT2 cells on *nonradioactive* SAM-coated substrates did not generate differences in the patterns of *radioactive* SAM laid down by the growing cells (Table IV). Interaction of cells with SAM-coated substrate may be complicated by the focal distribution of SAM (9) and the availability of "SAM-free" areas of substrate.

Growth of SVT2 cells at *low densities* on 3T3 SAM-coated substrates uniquely affected their growth behavior (30), in that the cells became more epithelioid, resisted crawling over neighboring cells, and resisted movement away from the edge of the colonies. These effects were transient; eventually, cells at the center of colonies began to overlap and assumed the spindle-shaped morphology of transformed cells. Coatings of 3T3 SAM were not effective at inducing density-dependent inhibition of growth in *mass cultures* of SVT2 cells.

TURNOVER OF SUBSTRATE-ATTACHED PROTEINS AND POLYSACCHARIDES

Culp et al. (8) reported that SAM consisted of 2 different metabolic pools of proteins: a major portion of the protein radiolabeled over long periods of cell growth was stably bound to the substrate, while a pool of protein appearing in SAM during short

radiolabeling periods turns over with a half-life of 2–4 hours. The kinetics of turnover of this latter pool of protein are very similar to the kinetics of turnover of the small amount of sulfated-GAG found in SAM (13).

To determine if specific SAM components are more labile than others, SDS-PAGE analysis of SAM isolated during pulse-chase periods with radioactive leucine was performed [Fig. 3; (17)]. The 8% separating gel of well #1 (Fig. 3) indicates that after a 2 hr pulse of radioactive leucine during exponential growth of SVT2 cells GAP-1, -2, -3, C_0, C_a, C_b, C_c, and C_1 appear in SAM (also proteins C_2, C_e, and C_f identified in 20% gels), as well as a very large amount of protein C_d (MW 48,000) which is observed as a minor component during long-term radiolabeling of cells [Fig. 2; (17)]. Short pulses have generated proportions of SAM proteins GAP-1, -2, -3, C_a, C_1, and C_2 which are comparable to those seen with SAM deposited during long-term labeling (Table I); however, the proportion of C_0 (the LETS glycoprotein) to C_a or C_1 is tenfold higher than in long-

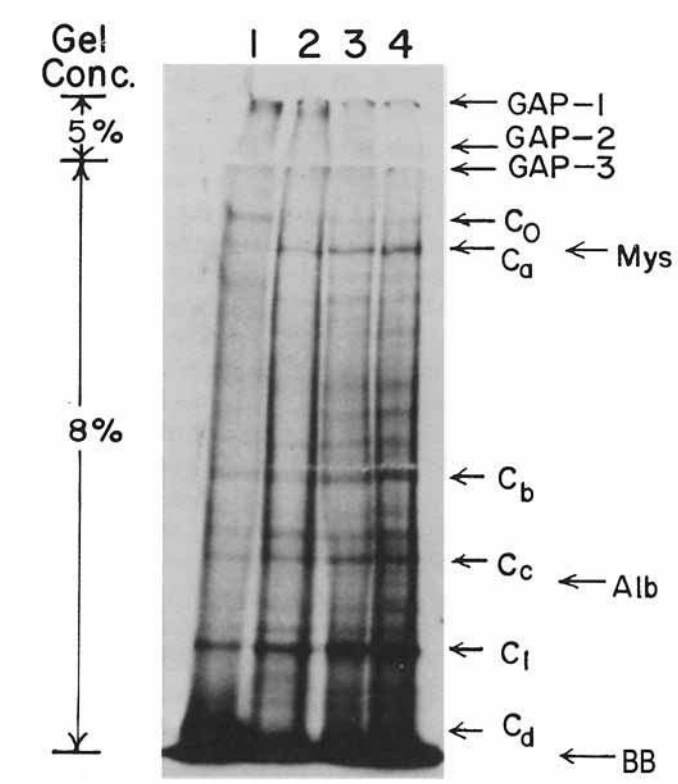

Fig. 3. Metabolic behavior of leucine-radiolabeled SAM proteins during PULSE-CHASE analysis. BALB/c SVT2 cells were radiolabeled during exponential growth in medium containing 2 μCi/ml L-[U^{-14}C]-leucine (and 0.02 of the normal concentration of leucine) for 2 hr (samples in wells #1 and 2) or 48 hr (samples in wells #3 and 4). At the end of these labeling periods, SAM was isolated as the PULSE samples, while other batches of cells were chased for 24 hr with medium containing the normal concentration of nonradioactive leucine; these SAM samples will be called the PULSE-CHASE samples (wells #2 and 4). SAM samples were prepared and electrophoresed on an 8% SDS-PAGE slab gel as described in the legend to Fig. 1; 10,000 cpm of each sample was applied to adjacent wells of the same slab gel. The samples are: well #1, 2-hr PULSE; well #2, 2-hr PULSE-24-hr CHASE; well #3, 48-hr PULSE; well #4, 48-hr PULSE-24-hr CHASE. Mys = myosin; Alb = bovine serum albumin; BB = bromphenol blue front.

term-radiolabeled preparations (compare 2-hr PULSE with 48-hr PULSE in Table V). This indicates that the LETS content in "footprint" SAM (9) which would be enriched by longer radiolabeling periods, is considerably less than in "footpad" SAM, which would be enriched by shorter radiolabeling periods during newly-synthesized footpad interaction with the substrate during cell movement.

When cells which have been pulsed for 2 hr are chased with nonradioactive leucine for 24 hr [70% of the radioactive SAM protein is lost (8)], well #2 (Fig. 3; also Table V) indicates that C_0 (the LETS) has almost completely disappeared from SAM, as well as protein C_d (17). In this particular autofluorogram, identical amounts of SAM radioactivity were electrophoresed; when comparisons are normalized for the 70% loss of radioactivity during the chase period, it has been determined (17) that small and relatively equivalent amounts of the GAP proteins, C_a, C_b, C_c, C_1, and C_2 also turn over (chasing for periods greater than 24 hr demonstrated no more turnover). The turnover of these proteins, particularly C_0 and C_d, did not generate new bands in the 8% or 20% separating gels. Presumably, these components were "sloughed" into the medium or "re-ingested" into other cell compartments. Similar metabolic phenomena were displayed by 3T3 and Con A revertant SAM's.

Chasing of cells for 24 hr after 48 hr of leucine radiolabeling resulted in minimal loss of radioactive protein in SAM (8) and with no appreciable differences in the distribution of proteins as determined with SDS-PAGE gels (compare well #3 of Fig. 3 which is the 48-hr pulse sample, with well #4 which is the chase sample, as well as the data of Table V). Even the small amount of C_0 (LETS) is conserved during the chase period, indicating relative metabolic stability of all the SAM components which have accumulated over a long period of time.

CONCLUSIONS

Substrate-attached material has been shown to be an enrichment of hyaluronate proteoglycans which have been resolved into at least 3 size classes, the LETS glycoprotein, and a number of components associated with submembranous microfilaments—the myosin-like protein, actin, and a few unidentified proteins. Although the histones appear to be

TABLE V. Pulse-Chase Analysis of the LETS Glycoprotein in Substrate-Attached Material[a]

Ratio (x:y)	Ratio value[b]			
	2-hr pulse	2-hr pulse; 24-hr chase	48-hr pulse	48-hr pulse; 24-hr chase
C_0:C_a	2.8	0.10	0.23	0.19
C_0:C_1	0.21	0.02	0.04	0.03
C_a:C_1	0.09	0.17	0.19	0.22

[a]Exponentially-growing populations of SVT2 cells were pulse-radiolabeled with ^{14}C-leucine for 2 hr or 48 hr, after which SAM was isolated to yield the indicated PULSE samples. Two additional batches of cells were chased with medium containing nonradioactive leucine for 24 hr after the 2- or 48-hr pulses; SAM was isolated from these batches as the PULSE-CHASE SAM samples. SAM's were then electrophoresed on 8% gels and autofluorographed as described in the legend to Fig. 3. Peak areas of specific bands on the same autofluorogram were determined with a Joyce-Loebl microdensitometer (17).

[b]The ratio value was determined by dividing peak area of component X by peak area of component Y, where X and Y are specifically denoted in the ratio column.

artifactually bound to the substrate as a result of EGTA-mediated leakiness of cells, considerable evidence indicates that the other components are not "leached" from the cell during natural growth or cell removal which would result in random binding to the substrate. [1] SAM is topographically located on the substrate at sites of direct cellular contact with the substrate in focal pools whose density is similar to that of cellular footpads (9). This evidence has been substantiated by observation of SAM with the scanning electron microscope as vesicular pads whose size and density on the substrate are very comparable to cellular footpads (28). [2] There is coordinate deposition of these various proteins (except for the histones) and polysaccharides during a wide variety of cell attachment and growth conditions (17). [3] When cells have saturated the substrate during growth, SAM deposition discontinues even though transformed cells continue to pile and even though all cell types continue to secrete large amounts of protein and polysaccharide into the medium of cultures (8). [4] The kinetics of deposition of the same proteins and polysaccharide during re-attachment to fresh substrate (after separation of cells from EGTA-solubilized material by centrifugation) is almost coincidental with the kinetics of stable cell attachment (29); this would not be expected if EGTA-solubilized proteins were binding to the substrate artifactually.

Several models of cell-substrate adhesion are being considered in light of the data which have been accumulated thus far, emphasizing the coordinate deposition of the components listed above as perhaps a cell "surface" complex involving high molecular weight proteoglycans, the LETS glycoprotein which may span the membrane (38), and surface-associated actin-containing microfilaments which have been shown to be plentiful at sites of cell-substrate and cell-cell adhesion in 3T3 and revertant cells, but diminished in transformed cells (32–35). The model in Fig. 4A proposes that actin-containing microfilaments penetrate the membrane and interact with proteoglycans directly to form the anchorage mechanism. EGTA treatment might weaken the filament array leaving a pool of filaments bound to the substrate while the membrane of the cell is released. There are several problems with this model: [1] no good evidence has been obtained that filaments ever penetrate the surface membrane of the cell; [2] this model would not explain how LETS, a reasonably well-defined membrane component, becomes substrate-associated, since lipid-bilayer material would not remain substrate-bound according to this model; [3] actin-filaments appear to be bound to the inner surface of the membrane of brush border microvilli by interaction with α-actinin (36); and [4] the vesicular-appearing nature of SAM as pinched-off footpads in scanning electron micrographs (28).

A more likely model (Fig. 4B) proposes that actin-containing microfilaments are linked to the inner surface of the membrane via components such as a presumptive α-actinin, the "surface-localized" myosin-like component, and/or the LETS glycoprotein as intramembranous components, interacting with a bed of proteoglycan composing the glycocalyx of the cell. These proteoglycans may then interact with specific serum components bound to the substrate (31). EGTA treatment might then weaken submembranous cytoskeletal materials with resultant pinching off of the footpad at the retraction fiber, leaving a footpad vesicle with tightly bound extrasurface and cytoskeletal elements involved in the adhesion site. Revel et al. (10) proposed a similar model for trypsin-mediated release of the cell from the substrate. Scanning EM analyses of EGTA-treated cells and of SAM itself (28) support this model. Substrate adhesion might therefore involve 2 different levels of organization of "surface" components – direct chemical interaction of cell surface proteins and/or polysaccharide with serum components on the substrate,

followed by reorganization of subsurface microfilaments to generate a firmly-bound cell surface lattice; such a 2-step process was observed for attaching cells by Mapstone and Culp (29).

A modification of the model in Fig. 4B is shown in Fig. 4C, whereby the high molecular weight proteoglycans are not direct mediators of the adhesion process but are "modulators" of the tenacity of adhesion by being adjacent to the sites of direct cell surface interaction with the substrate. Other transmembrane components, such as the large LETS or perhaps myosin-like proteins, would then be the direct participants in binding to the serum components. Consistent with the latter model are the trypsin-resistant variant CHO cells isolated by Atherly et al. (37) which have specifically-diminished hyaluronic acid synthesis and which adhere very tenaciously to the substrate. A variety of experimental approaches will be required to differentiate among these prospective molecular models of cell-substrate adhesion. Consideration must also be given to the similarity in turnover of SAM-containing LETS, protein C_d, and the sulfated GAG.

Virus-transformed cells are uniquely different from normal or revertant cells in several respects. [1] They deposit less SAM protein and polysaccharide quantitatively, although the qualitative composition of the SAM's and their metabolic behavior are very

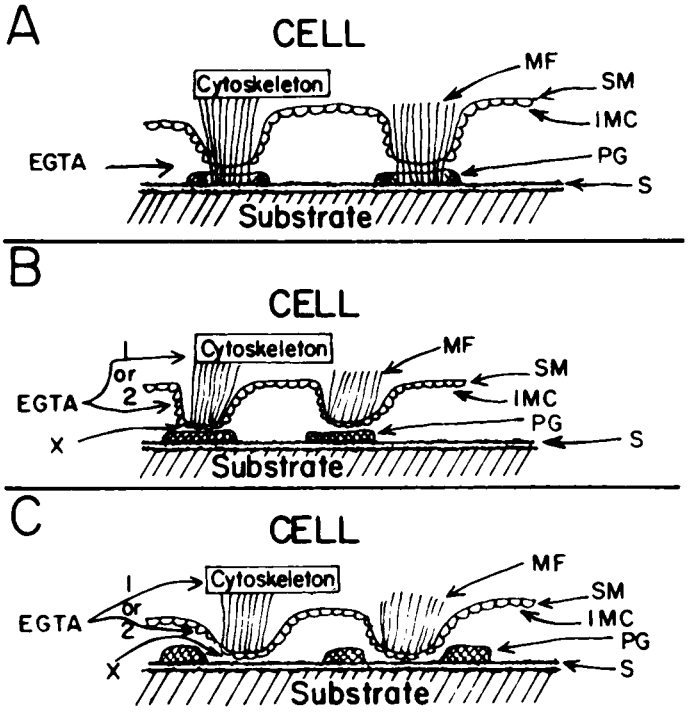

Fig. 4. Possible models of cell-substrate adhesion. Three different models are presented with various roles being assigned to the hyaluronate proteoglycans, the LETS glycoprotein, microfilaments, and the layer of adsorbed serum protein with various sites of EGTA action (sites 1 or 2) being proposed. The following abbreviations are used: MF, actin-containing microfilaments including associated proteins; SM, the surface membrane of the cell; IMC, integral membrane components (including components called X which may include the LETS glycoprotein and/or the myosin-like component); PG, the hyaluronate proteoglycans; and S, substrate-bound serum proteins.

similar. [2] Transformed cell attachment and colony morphology are uniquely affected by SAM-coated substrates. [3] The deposition of SAM by transformed cells attaching to SAM-coated substrate is uniquely inhibited. Much more evidence will be required to determine if subtle qualitative differences in their SAM's do exist. This is particularly true of the high molecular weight proteoglycans which are by far the major components and whose characterization to date has been minimal; the complexity of these materials may provide tremendous chemical versatility in altering the cell's social behavior.

Other methods of cell removal from the substrate, isolation of substrate adhesion variants, and re-addition of purified surface membrane components should prove valuable in further identifying which of these various components are critically important in the substrate adhesion process and whether they play direct roles or act as modulators. In any case, evidence gathered thus far indicates that substrate adhesion may be mediated by a complex array of specific cell and serum macromolecules.

ACKNOWLEDGMENTS

The author expresses appreciation for excellent technical assistance to Mrs. Josefina Buniel; and for funding to the National Cancer Institute (research grant 5-R01-CA-13513), the American Cancer Society (research grant BC-217 with partial support from the Ohio division), and the National Institutes of Health (training grant 5-T01-GM-00171). The author is a Harry H. Pinney Fellow in Cancer Research at Case Western Reserve University and a Career Development Awardee of the National Cancer Institute (1-K04-CA70709).

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