Proteins, Proc. Rehovot Symp., 2nd 230.
Urry, D. W., Okamoto, K., Harris, R. D., Hendrix, C. F., and Long, M. M. (1976b), *Biochemistry 15,* (preceding paper in this issue).
Urry, D. W., Ohnishi, T., Long, M. M., and Mitchell, L. W. (1975b), *Int. J. Pept. Protein Res. 7,* 367.
Volpin, D., Urry, D. W., Cox, B. A., and Gotte, L. (1976), *Biochim. Biophys. Acta 439,* 253.

Electrophoretic Analysis of Substrate-Attached Proteins from Normal and Virus-Transformed Cells†

Lloyd A. Culp

ABSTRACT: The proteins which have been left tightly bound to the tissue culture substrate after ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA)-mediated removal of normal, virus-transformed, and revertant mouse cells and which have been implicated in the substrate adhesion process have been analyzed by slab sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Three size classes of hyaluronate proteoglycans were resolved in the 5% well gel; approximately half of the protein in the substrate-attached material coelectrophoresed with these polysaccharides—so-called glycosaminoglycan-associated protein (GAP). A portion of the GAP was shown to be highly heterogeneous and displaced from the polysaccharide by preincubation with calf histone before electrophoresis. The relative proportions of the proteoglycans varied in material deposited during a variety of cellular attachment and growth conditions. The remainder of the cellular protein in substrate-attached material was resolved as several major and distinct protein bands in 8 or 20% separating gels (a limited number of distinct serum proteins have also been identified as substrate bound). Protein C_0 (molecular weight 220 000) was a prominent component in the material from a variety of normal and virus-transformed cells and resembled the so-called LETS or CSP glycoprotein in several respects; protein C_a was myosin-like in several respects; protein C_2 was shown to be actin; and protein C_1 (molecular weight 56 000) does not appear to be tubulin. Histones were also present in most preparations of substrate-attached material, particularly at high levels in transformed cell material, and may result from EGTA-mediated leakiness of the cell and subsequent binding to the negatively charged polysaccharide. These substrate-attached proteins were (a) prominent in substrate-attached material from many cell types in characteristic relative proportions, (b) deposited by EGTA-subcultured cells during the first hour of attachment to fresh substrate, (c) deposited by cells growing on plastic or glass substrates (three additional components were also prominent in glass-attached material), and (d) deposited during long-term growth on or initial attachment to substrates coated with 3T3 substrate-attached material. Pulse-chase analyses with radioactive leucine indicated that these proteins exhibit different turn-over behaviors. These results are discussed with regard to the possible involvement of these substrate-attached proteins in the substrate adhesion process, with particular interest in the interaction of cytoskeletal microfilaments with other surface membrane components and with regard to alteration of substrate adhesion by virus transformation.

A variety of experimental approaches have been used to demonstrate that the growth and motility properties of normal and virus-transformed mammalian cells are considerably different on artificial substrates such as glass and specially treated polystyrene plastic. An understanding of cell–substrate adhesion at the molecular level should provide insight into the basic alterations of the cell surface which may characterize a malignant cell (Taylor, 1961; Weiss, 1962; Curtis, 1973). Microexudates "deposited" by cells onto the culture substrate have been detected by ellipsometric (Rosenberg, 1960; Poste et al., 1973) and electron microscopic (Yaoi and Kanaseki, 1972; Revel and Wolken, 1973) techniques. Particular interest is now being focused on identification of substrate-bound serum and cell surface components which mediate these adhesions (Takeichi, 1971; Revel and Wolken, 1973; Grinnell, 1974; Grinnell, 1975; Culp and Buniel, 1976).

Removal of cells from the tissue culture substrate with the Ca^{2+}-specific chelating agent EGTA[1] results in the persistence of cell-synthesized protein and polysaccharide on the substrate—so-called substrate-attached material, requiring treatment with alkali or $NaDodSO_4$ for efficient removal (Culp and Black, 1972a; Terry and Culp, 1974; Culp et al., 1975). Several experimental approaches (Culp and Black, 1972a; Culp, 1974, 1975; Culp et al., 1975; Mapstone and Culp, 1976) have indicated that this material is deposited during direct contact between the cell and the substrate and may mediate adhesion of cells to the substrate, perhaps via the

† From the Department of Microbiology, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106. *Received February 13, 1976.* This investigation was supported in part by U.S. Public Health Service Research Grant 5-R01-CA-13513 and an American Cancer Society Research Grant BC-217 (with partial support from the Ohio Division). 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).

[1] Abbreviations used are: BB, bromphenol blue dye marker; Con A, concanavalin A; tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; GAG, acidic glycosaminoglycans (formerly referred to as mucopolysaccharides); GAP, glycosaminoglycan-associated proteins; MEM X 4, Eagle's minimal essential medium supplemented with four times the concentration of vitamins and amino acids; MSV, murine sarcoma virus; mol wt, molecular weight; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; SV40, Simian virus 40; Temed, tetramethylenediamine.

footpads by which the cell adheres to the substrate (Revel et al., 1974; Rajaraman et al., 1974; Culp, 1975). Interest has been focused on biochemical characterization of this material and quantitative or qualitative differences in material from normal or transformed cells.

Terry and Culp (1974) identified the major polysaccharide component in substrate-attached material as hyaluronic acid in high molecular weight proteoglycan complexes, supporting the argument (Kraemer, 1971) for the importance of acidic glycosaminoglycans in cellular adhesion processes. The metabolic properties of the protein and polysaccharide in substrate-attached material were similar during long-term-growth experiments (Culp et al., 1975) or initial attachment experiments using EGTA-subcultured cells (Mapstone and Culp, 1976).

To further characterize the protein components of substrate-attached material, we have resorted to higher resolution analysis by slab polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$. By this technique, a limited number of cell "surface" proteins have been separated from the hyaluronate proteoglycans and characterized under a variety of growth and attachment conditions.

Materials and Methods

Cell Growth. Mouse fibroblast Balb/c 3T3 cells (clone A31), SV40-transformed A31 cells (clone SVT2), and Con A-selected revertant variant cells of SVT2 (Culp and Black, 1972b) were grown as described previously (Terry and Culp, 1974). Kirsten MSV-transformed A31 cells (free of helper murine leukemia virus; Stephenson and Aaronson, 1972) were provided by Dr. Stuart Aaronson. Use of density-inhibited hamster Nil-B, SV40-transformed Nil-B (SV-Nil-B clone 1), Swiss 3T3, and SV40-transformed Swiss 3T3 cells has also been described (Culp, 1974). All cells were grown in MEM X 4 supplemented with 10% donor calf serum, penicillin (250 units/ml), and streptomycin (0.25 mg/ml). Cells were incubated in humidified 5% CO_2–95% air at 37 °C. For experimental purposes cells were routinely grown in Lux plastic tissue culture dishes or Brockway glass bottles and were free of *Mycoplasma* according to the radiolabeling assay of Culp and Black (1972a).

Radiolabeling Procedures. For batch preparation of substrate-attached material, trypsinized cells (0.75×10^6 Balb/c or Swiss 3T3 cells; 0.5×10^6 Con A revertant or Nil-B cells; 1.0×10^6 SV-Nil-B cells; 1.4×10^6 MSV-3T3, SVT2, or Swiss SV3T3 cells) were inoculated into twelve 100-mm plastic tissue culture dishes containing MEM X 4. Six hours later, the medium was changed to one containing radioactive precursor. To radiolabel polysaccharides, 0.5 μCi/ml of D-[1-^{14}C]glucosamine (specific activity 55 mCi/mmol) was included. To radiolabel proteins, one of a variety of precursors was used as follows: (a) 0.5 μCi/ml of L-[U-^{14}C]leucine (specific activity 324 mCi/mmol) was added to medium containing a reduced concentration of leucine (20.8 mg/L for growth of 3T3, Con A revertant, and murine sarcoma virus-transformed 3T3 cells; 4.1 mg/L for growth of SVT2, Nil-B, and SV-Nil-B cells); (b) 2.0 μCi/ml of L-[^{35}S]methionine (specific activity 313 Ci/mmol) was added to medium containing a reduced concentration of methionine (6 mg/L); (c) 0.8 μCi/ml of L-[3-^{14}C]tryptophan (specific activity 50.7 mCi/mmol) was added to complete medium; (d) 5.0 μCi/ml of L-[U-^{14}C]proline (specific activity 200 mCi/mmol) was added to complete medium; or (e) 0.85 μCi/ml of L-[U-^{14}C]lysine (specific activity 240 mCi/mmol) was added to medium containing a reduced concentration of lysine (23.2 mg/L for growth of SVT2 cells). To radiolabel the sulfated GAG, 50 μCi/ml of $Na_2{}^{35}SO_4$ (specific activity 677 mCi/mmol) was added to complete medium. Unless indicated otherwise, all radiolabeling of cell populations was performed over a 72-h period during exponential growth (generally three to four generations) and was terminated before cells became density inhibited (approximately 70–80% of the substrate surface was covered with cells). None of the radiolabeling conditions used in these studies was deleterious to cell growth.

Isolation of Substrate-Attached Material. After incubation of cells for the appropriate length of time in medium containing radioactive precursor, medium was decanted and the cell layer was gently washed three times with PBS. Five milliliters of EGTA (0.5 mM in PBS) was added and the culture was incubated on a rotary shaker at 37 °C for 30 min to remove cells. The suspension of cells was gently pipetted over the surface of the dish to guarantee detachment of all cells. The substrate surface was then rinsed once with PBS and once with distilled H_2O. The substrate-attached material was extracted with 5 ml of 0.2% $NaDodSO_4$ (w/v in H_2O) during rotary shaking at 37 °C.

The extract from each batch of cells was concentrated by vacuum dialysis at room temperature to 100–300-μl volume (in general, less than 4–7% of the radioactive material was dialyzable during this concentration step), dialyzed against sample buffer (0.075 M Tris-sulfate pH 8.4, 1% mercaptoethanol, 0.2% $NaDodSO_4$, 15% glycerol, and 0.001% bromphenol blue) overnight at room temperature, and placed in a boiling water bath for 10 min before electrophoresis.

There were no effects upon the electrophoretic distributions reported in these studies by the presence of phenylmethylsulfonyl fluoride in the EGTA and the $NaDodSO_4$ extraction solutions, indicating minimal alteration of proteins by proteolysis. Electrophoretic profiles were stable upon storage of substrate-attached material in sample buffer (after boiling) at −20 °C for periods as long as 6 months.

Slab Polyacrylamide Gel Electrophoresis. Samples were electrophoresed on 12 × 16 cm slabs of polyacrylamide gel (1.5-mm thick) on a slab gel apparatus (Hoefer Scientific, San Francisco, Calif.) as described by Maurer and Allen (1972) and modified in ORTEC Application Note AN32A (ORTEC Inc., Oak Ridge, Tenn.). In brief, the well gel (or stacking gel) contained 5% acrylamide, 0.12% bisacrylamide, and 0.075 M Tris-sulfate, pH 8.4; the 20% separating gel contained 20% acrylamide, 0.1% bisacrylamide, and 0.375 M Tris-sulfate, pH 8.4; the 8% separating gel contained 8% acrylamide, 0.2% bisacrylamide, and 0.375 M Tris-sulfate, pH 8.4; the tank buffer contained 0.1% $NaDodSO_4$ and 0.065 M Tris-borate, pH 8.4. Electrophoresis was performed at room temperature for 6–7 h at a constant current of 40 mA/gel. Unless indicated otherwise, approximately 10 000 cpm of radioactive protein or 5000 cpm of radioactive polysaccharide was loaded per well. Gels were fixed, stained with Coomassie blue as described by Fairbanks et al. (1971), except that the destaining solutions did not contain Coomassie blue, dried under vacuum on a sheet of Whatman 3MM filter paper, and autoradiographed on Kodak Royal RP X-omat medical x-ray film (RP14) which was developed in a commercial automated Kodak X-Omat Processor located in the Radiology Department of Case Western Reserve University. Some gels were fluorographed for qualitative evaluation of banding patterns as described by Bonner and Laskey (1974).

Quantitation of bands in autoradiograms was performed after scanning with a Joyce-Loebl microdensitometer under conditions in which the peak area was linearly proportional to

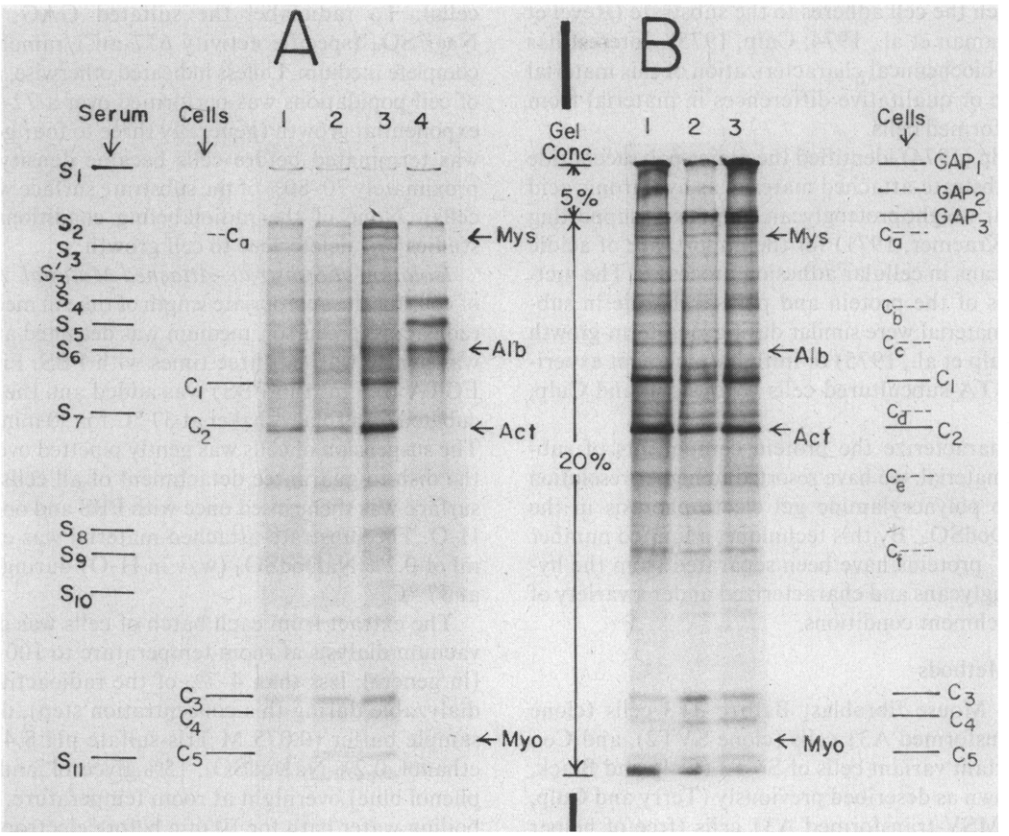

FIGURE 1: Substrate-attached serum and cell proteins. Substrate-attached material extracted with sodium dodecyl sulfate from plastic substrates was electrophoresed on 20% slab sodium dodecyl sulfate–polyacrylamide electrophoresis gels as described in Materials and Methods and stained with Coomassie blue (A) to detect serum and cell proteins or autoradiographed (B) to detect cell proteins after incorporation of [^{14}C]leucine during cell growth. Major serum (S) or cell (C) proteins are denoted with subscript numbers, while minor cell proteins are denoted with subscript letters; the major classes of cellular high molecular weight proteoglycans are denoted as GAP as described in the text. The following samples were electrophoresed: well 1, 3T3 substrate-attached material; well 2, SVT2 material; well 3, Con A revertant material; well 4, serum proteins adsorbed to plastic substrate over a 72-h period (Culp and Buniel, 1976). The following marker proteins were used: Mys, myosin; Act, actin; Alb, albumin; Myo, myoglobin. Different preparations of substrate-attached material and gels were run for experiments A and B.

the amount of radioactive material electrophoresed. The standard error of peak-area measurements varied from ±8% for small peaks to ±5% for large peaks. Wedges giving 0–1.0 or 0–3.0 optical density unit full-scale deflections were used in these studies. Comparative evaluation of data in these studies was performed in any particular experiment with samples electrophoresed in the same slab gel and exposed to the same piece of x-ray film.

Materials were purchased from the following sources: D-[1-^{14}C]glucosamine and L-[U-^{14}C]leucine from Amersham/Searle Corp.; L-[U-^{14}C]proline, L-[^{35}S]methionine, [^{35}S]Na_2SO_4, L-[U-^{14}C]lysine, and L-[3-^{14}C]tryptophan from New England Nuclear Corp.; acrylamide, bisacrylamide, Temed, EDTA, and EGTA from Eastman Organic Chemicals; Royal RP X-omat medical x-ray film from Eastman Kodak Co.; highly purified bovine serum albumin, Coomassie blue, and calf histone from Sigma Chemical Co.; rabbit skeletal muscle myosin and actin were generously supplied by Abbot Clark and Dr. Paul Vignos or Dr. Ray Lasek; pig brain tubulin from Dr. Ray Lasek; sperm whale myoglobin from Dr. Abram Stavitsky; sodium dodecyl sulfate from Matheson Coleman and Bell; MEM X 4 from Grand Island Biologicals Co.,; donor calf serum from Flow Laboratories; plastic tissue culture dishes from Lux Scientific Co.; 32-oz Brockway glass prescription bottles from Brockway Glass Inc.

Results

Initial Identification of Proteins and Polysaccharide. Figure 1A (wells 1–3) depicts the bands stainable with Coomassie blue for 3T3, SVT2, and Con A revertant substrate-attached material. Some of these stained bands coelectrophoresed with serum proteins (S_1–S_{11}) which are adsorbed to the plastic substrate in the presence or absence of cells (Culp and Buniel, 1976) and are sodium dodecyl sulfate extractable (well 4). One of these serum proteins (S_6) coelectrophoresed with bovine serum albumin. Cellular substrate-attached material also contained major proteins C_1–C_5 detectable by Coomassie blue staining (Figure 1A, wells 1–3) with some minor proteins such as C_a. These proteins were shown to be cell proteins via incorporation of radioactive leucine during exponential growth of cultures and detection autoradiographically (Figure 1B). Major proteins C_1–C_5 are the predominant moieties with several minor proteins such as C_a–C_f also appearing in these preparations. The relative quantities of these cell proteins detected autoradiographically (Figure 1B) after leucine incorporation were very similar to those detected by staining (Figure 1A).

Three other radioactive protein bands were also observed in the well gel as high molecular weight components (GAP-1, GAP-2, and GAP-3 in Figure 1B). These are termed "glycosaminoglycan-associated proteins" (GAP) because they coelectrophoresed with the only three major bands of glucosamine-radiolabeled polysaccharide observed in substrate-attached material (Figures 2-1 and 2-2) and previously identified as hyaluronic acid proteoglycans (Terry and Culp, 1974). The polysaccharide bands will be called GAG-1, GAG-2, and

GAG-3 (GAG, glycosaminoglycan), the locations of which correspond to GAP proteins 1, 2, and 3, respectively (Figure 2).[2] GAG-1 and GAP-1 after long-term radiolabeling of cells with glucosamine and leucine, respectively, were always the principle bands of proteoglycan (Figure 2). GAG-2 and GAG-3 were usually present in comparable quantities, whereas GAP-3 was usually present in much greater quantity than GAP-2. These data indicate varying ratios of protein–polysaccharide in the three bands of high molecular weight proteoglycans within one particular cell type.

Comparison of these electrophoretic patterns with patterns from enriched surface membrane fractions indicated considerable enrichment in substrate-attached material of the GAP proteins C_1, C_2, minor protein C_a–C_f, and the GAG polysaccharides (Vessey and Culp, manuscript in preparation); proteins C_3–C_5 have never been observed in membrane fractions.

Substrate-attached material has been shown to contain a low level of sulfated GAG (Terry and Culp, 1974; Roblin et al., 1975). Electrophoresis of sulfate-radiolabeled material revealed that greater than 90% of the radioactivity appeared in the GAG-1 band with the remainder of the radioactive material appearing in the GAG-3 band—a distribution pattern quite different from the glucosamine-radiolabeled GAG (principally hyaluronic acid).

Quantitation of Substrate-Attached Material. The quantitative distribution of radiolabeled proteins after long-term growth of cells in radioactive leucine and after electrophoretic separation is given in Table I for the major proteins. Approximately 50–60% of the protein in these extracts appeared in the high molecular weight proteoglycans with C_2 being the next major protein as determined by [^{14}C]leucine content. The SV40-transformed SVT2 cells were unique in their high proportion of proteins C_3–C_5 when compared with 3T3 and revertant cells. The ratio of the sum of the GAP proteins to C_1 or C_2 varied somewhat for all three cell types but was generally higher in SVT2 material than in 3T3 or revertant materials; the ratio of C_1 to C_2 was similar for all three cell types.

Ubiquity of Substrate-Attached Proteins. Substrate-attached material was prepared from a number of normal and virus-transformed cell lines, including Swiss 3T3, SV40-transformed Swiss 3T3, murine sarcoma virus transformed BALB/c 3T3, hamster Nil-B, and SV40-transformed Nil-B cells, and analyzed after NaDodSO$_4$–PAGE. Leucine-radiolabeled material contained the same major and minor protein bands identified in Figures 1 and 2. Glucosamine-radiolabeled polysaccharide from the mouse cell lines consistently contained a major amount of GAG-1 with smaller and equivalent amounts of GAG-2 and GAG-3 as shown in Figure 2. The polysaccharides from the hamster cells contained a principle band of GAG-1 with a reduced proportion of GAG-3 to GAG-2.

The effects of cell removal from the substrate by two methods other than EGTA treatment were examined in terms of the substrate-attached protein distribution. EDTA-mediated removal resulted in deposition of the same major and minor proteins and polysaccharides reported above. Scraping of cells from the dish with a rubber policeman left the same major substrate-bound cell proteins (except C_3–C_5) but with a much higher proportion and larger number of minor proteins than removal with the chelating agents; however, substrate-attached material from *scraped* 3T3 or SVT2 cells contained undetectable levels of C_3–C_5.

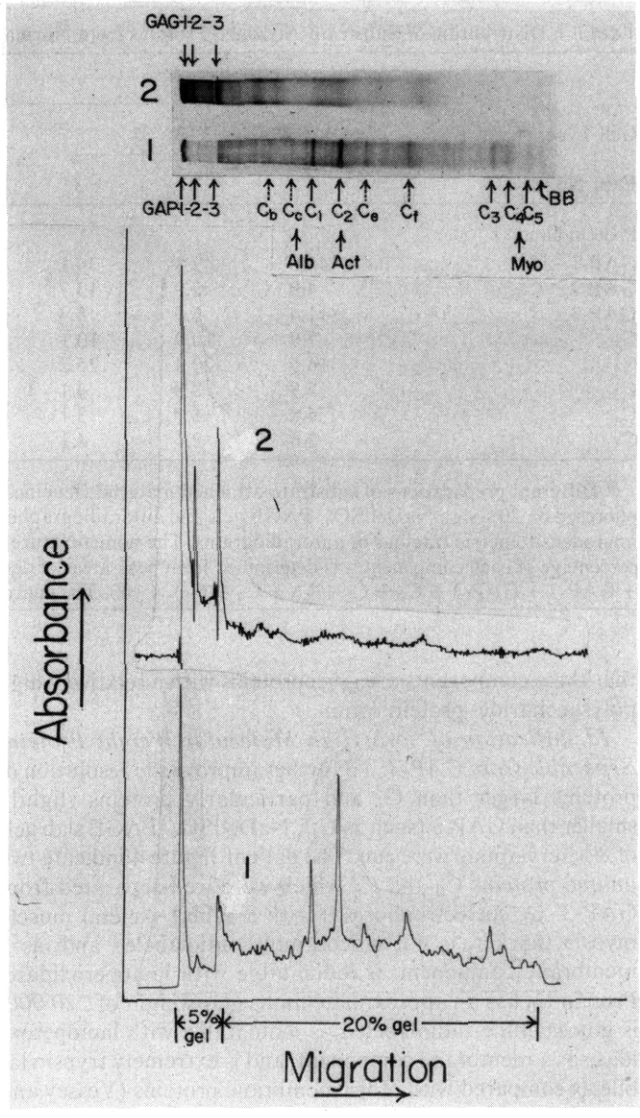

FIGURE 2: Glucosamine- or leucine-radiolabeled substrate-attached material. BALB/c 3T3 cells were grown in medium containing [^{14}C]-glucosamine or [^{14}C]leucine during exponential growth and the appropriate radioactive substrate-attached material was electrophoresed on a 20% slab NaDodSO$_4$–PAGE gel as described in Materials and Methods: well 1, [^{14}C]leucine-radiolabeled material; well 2, [^{14}C]glucosamine-radiolabeled material. Microdensitometric tracings of autoradiograms were made as described in the Materials and Methods; a photograph of the autoradiogram appears at the top of the figure. Major polysaccharide components are denoted as GAG (glycosaminoglycans), major protein components as GAP (glycosaminoglycan-associated protein) or C with an appropriate subscript number, and minor proteins with C and a subscript letter; BB indicates the bromphenol blue dye front of electropherograms. The following marker proteins were used: Alb, albumin; Act, actin; Myo, myoglobin.

Substrate-attached materials from cells grown on plastic or Brockway glass substrates are compared in Figure 3. Both 3T3 (Figures 3-1 and 3-2) and SVT2 (Figures 3-3 and 3-4) cells deposited similar proportions of GAP, C_2, C_3, C_4, and C_5 when grown on either substrate. However, both cell types when grown on glass (Figures 3-2 and 3-4) deposited a reduced amount of C_1 and a large amount of C_d', a slightly larger protein than the minor component C_d seen with plastic-grown cells (Figure 1). Also two high molecular weight components C_x and C_y appeared which have not been observed using plastic substrates; radiolabeling studies with glucosamine indicated

[2] GAP-1 and -3 bands correspond to the "S_1" and "S_2" proteins reported previously (Culp and Buniel, 1976).

TABLE I: Distribution of Substrate-Attached Proteins from Normal and Virus-Transformed Cells.[a]

Cell Type:	Fraction of Major Proteins[b] (%)								
	3T3			SVT2			Revertant		
Expt No.:	1	2	3	1	2	3	1	2	3
Protein band									
GAP-1	45.0	42.8	34.1	28.8	37.2	23.4	42.2	43.9	46.7
GAP-2	4.9	2.9	13.7	2.4	3.2	4.5	9.7	1.6	4.1
GAP-3	11.4	5.9	5.3	13.2	17.4	16.1	15.3	5.4	14.5
C_1	7.9	10.9	10.1	4.9	5.6	5.3	6.3	12.9	9.3
C_2	16.5	20.4	25.2	15.1	8.4	13.6	21.1	21.2	13.8
C_3	5.9	8.3	4.1	14.2	12.5	18.7	2.8	7.5	4.0
C_4	5.8	6.7	3.1	17.0	11.2	13.0	1.8	5.0	5.1
C_5	2.6	2.1	4.1	4.4	4.5	5.4	0.9	2.5	2.5

[a] Different preparations of substrate-attached material (leucine-radiolabeled) indicated by the various experiment numbers were electrophoresed on 20% slab $NaDodSO_4$–PAGE gels and autoradiographed as described in Materials and Methods. Peak areas were measured from microdensitometric tracings of autoradiograms. The nomenclature for identifying major protein bands is given in Figure 1 or the text. [b] The percentage of each component was determined from peak areas of densitometer tracings using the formula $\text{percentage}_x = \text{Area}_x/\Sigma\text{Areas}(\text{GAP-1} + \text{GAP-2} + \text{GAP-3} + C_1 + C_2 + C_3 + C_4 + C_5) \times 100$. The contribution of radioactive minor bands was not taken into consideration

that these components are glycoproteins with a relatively high polysaccharide–protein ratio.

Identification of Two High Molecular Weight Proteins Separable from GAP-3. To further improve the resolution of proteins larger than C_1 and particularly proteins slightly smaller than GAP-3 (such as C_a), $NaDodSO_4$–PAGE slab gels of 8% acrylamide were run. The data of Figure 4 indicate two unique proteins C_0 and C_a which were well-separated from GAP-3. C_a coelectrophoreses with a rabbit skeletal muscle myosin marker, is not glucosamine-radiolabeled and, as a membrane component, is iodinatable with lactoperoxidase. Protein C_0 has an approximate molecular weight of 220 000, is glucosamine-radiolabeled, is iodinatable with lactoperoxidase as a membrane component, and is extremely trypsin labile as compared with other membrane proteins (Vessey and Culp, manuscript in preparation); this component is therefore similar to the LETS (LETS = large, external, transformation sensitive; Hynes and Bye, 1974) or CSP glycoprotein (CSP = cell surface protein; Yamada and Weston, 1975) which has been implicated in adhesion mechanisms. The data of Table II indicate no consistent differences in the proportion of either C_0 or C_a to the major protein C_1 between normal and transformed cells, and both proteins were present in the substrate-attached material from all the cell lines examined.

Identity of Substrate-Attached Proteins. Several approaches have been used to further characterize the nature of the major substrate-attached proteins. The apparent molecular weights are given in Table III. Protein C_2 appears to be actin since (a) it coelectrophoresed with marker rabbit skeletal muscle actin in these one-dimensional $NaDodSO_4$–PAGE gels; (b) it coelectrophoresed with marker actin in a two-dimensional gel system described by O'Farrell (1975) (the material was isoelectric focused on a disc gel in the first dimension and slab $NaDodSO_4$–PAGE analyzed in the second dimension); (c) most of its tryptic peptides are identical with peptides from rabbit skeletal muscle actin as found by Gruenstein et al. (1975); and (d) it is readily radiolabeled by cell growth in radioactive leucine, methionine, or proline—amino acids which are present in actin in similar concentrations (Collins and Elzinga, 1975).

Although protein C_1 exhibited an apparent molecular weight of 56 000 (Table III) consistent with its being tubulin, several pieces of evidence indicate that it is indeed not tubulin. C_1 electrophoresed on $NaDodSO_4$–PAGE gels as a protein slightly smaller than pig brain tubulin. Secondly C_1 was not radiolabeled by incorporation of precursor [^{14}C]tryptophan, whereas actin (C_2) was readily labeled; actin and tubulin have a similar complement of tryptophan residues (4 per 45 000 mol wt for actin (Collins and Elzinga, 1975) and 4–5 per 55 000 mol wt for tubulin (Stephens, 1970)). Also [^{3}H]colchicine did not bind to substrate-attached material.

Proteins C_3, C_4, and C_5 appear to be histones. They consistently coelectrophoresed with three major bands of calf histone (in some gels, the center band of calf histone split into a doublet; in the same gels, C_4 also split into a doublet which comigrated with the calf histone central doublet). Also, C_3 and C_4 contained a high concentration of radioactive lysine relative to the GAP proteins, C_1, and C_2 and relative to the leucine-radiolabeling pattern, all of which are consistent with their being the lysine-rich histones (DeLange and Smith, 1975). Proteins C_3–C_5 were not radiolabeled with [^{14}C]tryptophan under conditions where the GAP proteins and C_2 were radiolabeled; histones are highly deficient in tryptophan (DeLange and Smith, 1975).

Some information has been obtained on the nature of the GAP proteins which coelectrophorese with the hyaluronate polysaccharide. Raising the sodium dodecyl sulfate concentration of samples prior to boiling and electrophoresis to 2% only slightly diminished the proportion of GAP-1 to C_1 or C_2 with no effects on GAP-2 or GAP-3. Cell growth for several generations in radioactive proline resulted in the same relative pattern of major peaks after $NaDodSO_4$-PAGE as leucine radiolabeling—an indication that a major portion of GAP protein is not proline-rich collagen.

Addition of nonradioactive calf histone to samples prior to boiling and electrophoresis resulted in extensive loss of GAP-1 protein (compare Figures 5-2 and 5-4 whose samples contained carrier histone with samples in Figures 5-1 and 5-3 which did not). Both 3T3 and SVT2 material was affected, using either autoradiography or fluorography. GAP-2 and GAP-3 proteins were not affected. After histone addition, the dissociated proteins from the GAP-1 region did not appear in the 20% separating gel as unique bands of protein, as increased pools of histones C_3–C_5, or as low molecular weight polypeptides at

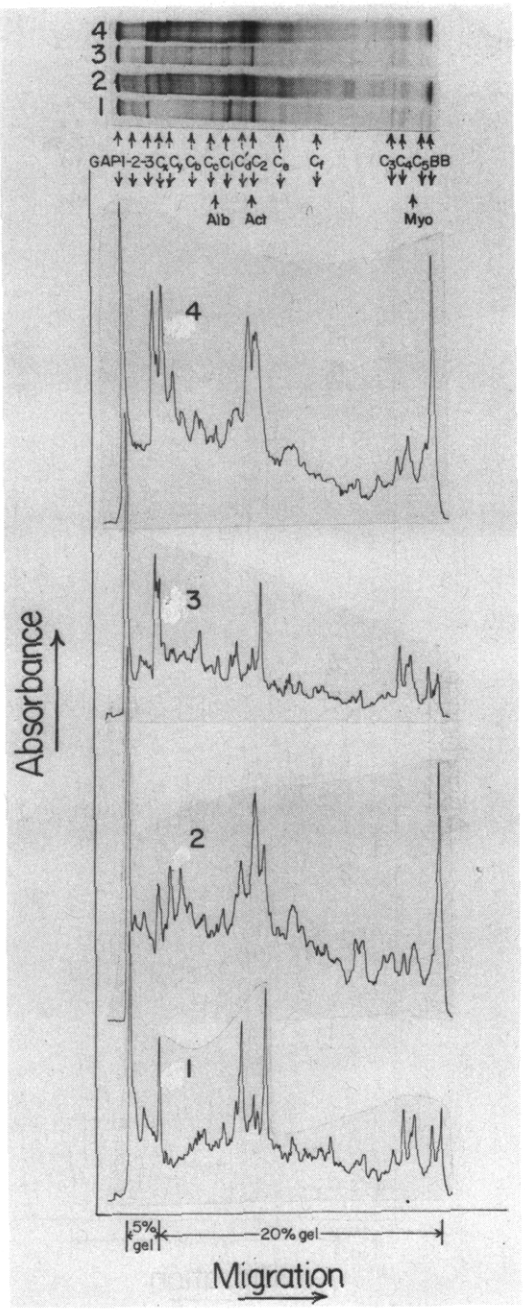

FIGURE 3: Comparison of substrate-attached material from plastic- or glass-grown cells. BALB/c 3T3 or SVT2 cells were grown on glass or plastic substrates in medium containing [^{14}C]leucine and substrate-attached material was electrophoresed on a 20% slab $NaDodSO_4$–PAGE gel as described in Materials and Methods. The following samples were electrophoresed: well 1, 3T3 on plastic; well 2, 3T3 on glass; well 3, SVT2 on plastic; well 4, SVT2 on glass. The methodology for analysis of autoradiograms and nomenclature for identifying major and minor cell proteins are given in Figures 1 and 2. All samples were run on the same gel. Marker proteins: Alb, albumin; Act, actin; Myo, myoglobin.

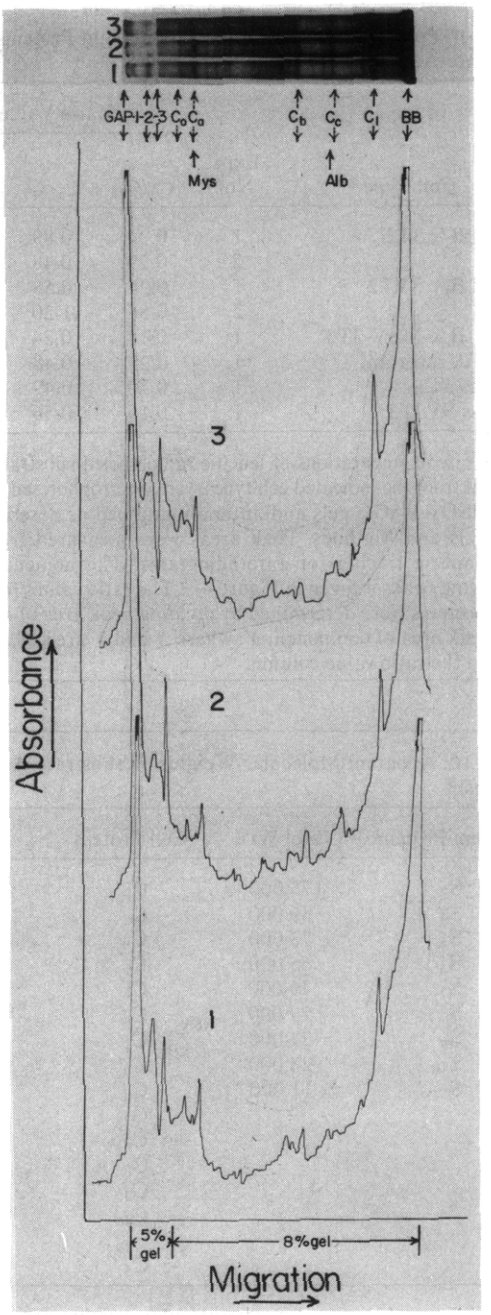

FIGURE 4: Resolution of high molecular weight substrate-attached proteins. BALB/c 3T3, SVT2, or Con A revertant cells were grown in medium containing [^{14}C]leucine and substrate-attached material was electrophoresed on a 8% slab $NaDodSO_4$–PAGE gel as described in Materials and Methods. All samples were run on the same gel. The following samples were electrophoresed: well 1, 3T3 material; well 2, SVT2 material; well 3, Con A revertant material. The methodology for analysis of autoradiograms and nomenclature for identifying major and minor cell proteins are given in Figures 1 and 2. Marker proteins: Alb, albumin; Mys, myosin.

the bromphenol blue front. Presumably they became highly dispersed throughout the gel because of size heterogeneity. There was no alteration of the distribution of GAG-1, -2, or -3 polysaccharides following histone treatment.

Effects similar to those reported in Figure 5 were also obtained by (a) removing cells with an EGTA solution which contained 0.1 mg/ml of nonradioactive calf histone or (b) incubating substrates coated with substrate-attached material, after EGTA-mediated removal of cells, with 0.1 mg/ml nonradioactive calf histone at 37 °C before $NaDodSO_4$ removal of the substrate-attached material. These experiments did not affect the relative distribution of GAP-2, GAP-3, C_1, C_2, C_3–C_5, C_0, or C_a.

Comparison of Substrate-Attached Proteins with EGTA-Solubilized Proteins. EGTA treatment of cells results in leakiness of cytosol proteins (Culp and Black, 1972a) as well as possible leaching of "peripheral" cell surface components (Singer, 1974). Therefore, substrate-attached proteins were coelectrophoresed with the EGTA-solubilized material (after

TABLE II: Proportion of High Molecular Weight Proteins in Substrate-Attached Material.[a]

Cell Type	Expt No.	Ratio Value[b] C_0/C_1	C_a/C_1	C_0/C_a
BALB/c 3T3	1	0.34	0.89	0.39
	2	0.29	0.48	0.62
BALB/c SVT2	1	0.24	0.58	0.41
	2	0.36	1.30	0.27
BALB/c MSV-3T3	1	0.14	0.24	0.59
Con A revertant	1	0.23	0.48	0.47
Swiss 3T3	1	0.70	0.49	1.42
Swiss SV3T3	1	0.16	0.56	0.29

[a] Different preparations of leucine-radiolabeled substrate-attached material from the indicated cell types were electrophoresed on 8% slab $NaDodSO_4$–PAGE gels and autoradiographed as described in the Materials and Methods. Peak areas were measured from microdensitometric tracings of autoradiograms. The nomenclature for identifying peaks is given in Figure 4. [b] The ratio values for the indicated proteins were determined by dividing peak area of component X by peak area of component Y, where X and Y are specifically denoted in the ratio value column.

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

Serum Protein	Mol Wt	Cell Protein	Mol Wt
S_3	175 000	C_0	220 000
S_4	85 000	C_a	200 000
S_5	73 000	C_x	175 000
S_6	65 000	C_y	145 000
S_7	49 000	C_b	85 000
S_8	29 000	C_c	67 000
S_9	27 000	C_1	56 000
S_{10}	23 000	C_d'	49 000
S_{11}	11 000	C_d	48 000
		C_2	45 000
		C_e	37 000
		C_f	27 000
		C_3	14 300
		C_{4a}	13 500
		C_{4b}	13 000
		C_5	11 000

[a] These are the approximate molecular weights determined for proteins of less than 100 000 mol wt on 20% $NaDodSO_4$–PAGE slab gels using bovine serum albumin, ovalbumin, actin, and myoglobin as molecular weight markers; the size of proteins greater than 100 000 was determined on 8% $NaDodSO_4$–PAGE slab gels using myosin, *E. coli* β-galactosidase, and bovine serum albumin as markers. Proteins S_1, S_2, GAP-1, GAP-2, and GAP-3 were too large to obtain reliable sizing information. Molecular weight values were obtained from plots of the log of the molecular weight vs. migration distance.

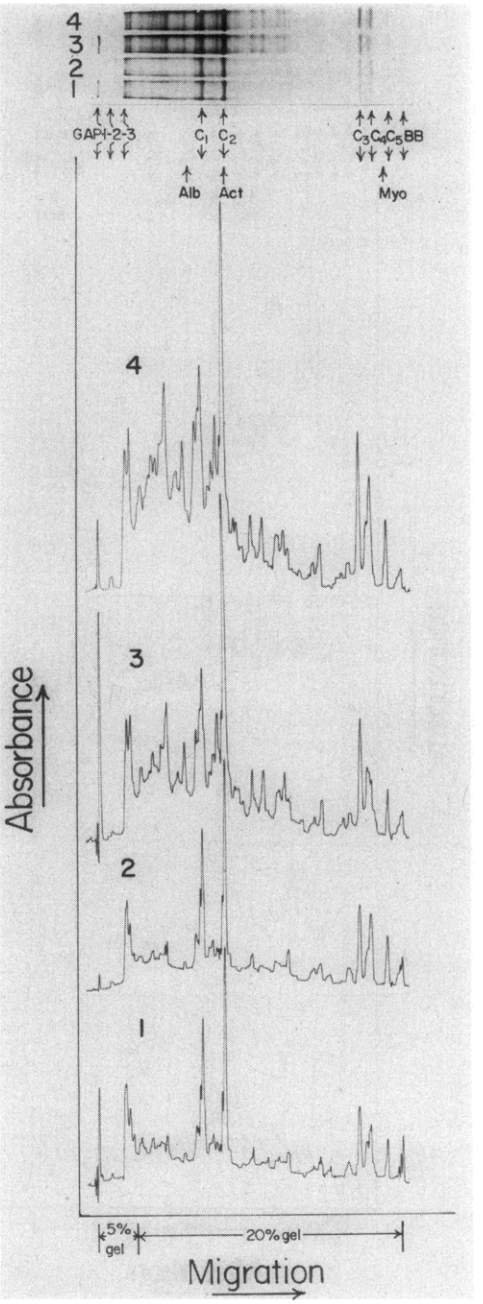

FIGURE 5: Histone dispersal of GAP-1 proteins. BALB/c 3T3 or SVT2 cells were grown in medium containing [^{14}C]leucine and substrate-attached material was electrophoresed on a 20% slab $NaDodSO_4$–PAGE gel as described in Materials and Methods. All samples were run on the same gel. However, the indicated samples received an aliquot of 1 mg/ml calf histone to make the final concentration in the sample 0.1 mg/ml just prior to boiling and electrophoresis. The following samples were electrophoresed: well 1, 3T3 material; well 2, 3T3 material plus histone; well 3, SVT2 material; well 4, SVT2 material plus histone. The methodology for analysis of this fluorogram and nomenclature for identifying major and minor cell proteins are given in Figures 1 and 2 or the Materials and Methods. Marker proteins: Alb, albumin; Act, actin; Myo, myoglobin.

separation from cells by centrifugation) to determine if substrate-bound proteins might also be solubilized. The $NaDodSO_4$–PAGE electropherograms indicated sizable pools of C_2 and C_3 in the solubilized material from 3T3 or SVT2 cells, as well as small amounts of the GAP proteins, C_0, C_a, C_b, and C_c. There were no detectable amounts of the other substrate-attached proteins in the solubilized fraction.

Deposition of Substrate-Attached Proteins during Initial Attachment of Cells. Mapstone and Culp (1976) have shown very similar kinetic relationships between the initial attachment of EGTA-subcultured cells to plastic substrate and deposition of substrate-attached proteins and polysaccharide with maximal binding within the first hour of attachment. The substrate-attached material deposited during the first hour was extracted as described in Materials and Methods and electrophoresed for comparison with material accumulated on the substrate during long-term growth of cells. The material from 3T3 or SVT2 cells (Figures 6-2 and 6-4) contained the same

major protein components identified on 20% gels as material from long-term growth of cells (Figures 6-1 and 6-3) in approximately the same relative proportions, except for a somewhat elevated amount of C_1. Similarly 8% NaDodSO$_4$–PAGE gels indicated that C_0 and C_a were also deposited during initial attachment in the same relative proportion to C_1 as observed for long-term-growth preparations (see Table II). Polysaccharide from initial attachment material of 3T3 or SVT2 cells contained a high proportion of GAG-3 compared with GAG-1 or GAG-2, whereas long-term-growth material contained a preponderance of GAG-1.

Identification of Turnover Proteins. Pulse-chase analyses with radioactive leucine have previously identified two different metabolic pools of substrate-attached protein (Culp et al., 1975): a major portion of the protein is stably bound to the substrate while a small pool turns over with a half-life of 2–4 h. The proteins deposited during a 2-h pulse of radioactive leucine are displayed in Figure 7-1 for 3T3 cells or Figure 7-3 for SVT2 cells with the familiar triplet of GAP proteins and C_1–C_5. C_d appeared as a major protein during these short pulses and coelectrophoresed with the minor protein identified as C_d during long-term radiolabeling experiments (see Figure 1B). After 24 h of chasing with nonradioactive leucine in the medium during which 70% of the radioactive protein is lost from the substrate (Culp et al., 1975), the profiles of Figure 7-2 for 3T3 cells and 7-4 for SVT2 cells indicate that (a) a small portion of the GAP proteins, C_1, C_2, C_3, C_4, and C_5, have disappeared and (b) C_d has been completely lost from the substrate. Turnover of these proteins did not result in the appearance of new substrate-attached proteins; presumably these proteins were "reingested" into cells or discarded into the medium. The small pool of substrate-attached sulfated GAG also turned over with a half-life of 2–4 h (Culp, unpublished data) and may be associated with the labile GAP proteins. Eight percent gels indicated that short pulses of radioactive leucine resulted in a much higher proportion of C_0 to C_a or C_1 compared with long-term labeling (Table II). Chasing after short pulses, on the other hand, demonstrated much more extensive turnover of C_0 than C_a, C_1, or the GAP proteins.

Deposition of Substrate-Attached Proteins by Cells Attaching to or Growing on Coated Substrate. When 3T3 cells were grown for several generations in medium containing radioactive leucine on substrates coated with *nonradioactive* 3T3 substrate-attached material (by prior growth of 3T3 cells to confluence and removal by EGTA treatment) (Culp, 1974), they deposited the same major radioactive protein components on the substrate (Figure 8-2) as cells grown on uncoated substrate (Figure 8-1) in similar relative proportions, except for a greater amount of GAP-3 and a reduced amount of GAP-1. Similar evidence was also obtained for SVT2 cells growing on 3T3 substrate-attached material. Eight percent gels indicated that C_0 and C_a were deposited equally well by 3T3 or SVT2 cells growing on coated substrate.

Examination of material deposited during initial attachment of leucine-radiolabeled 3T3 cells to substrates coated with *nonradioactive* substrate-attached material revealed (Figure 8-4) the same major GAP proteins and C_1–C_5 as deposited by cells attaching to uncoated substrate (Figure 8-3), as well as (a) an increased amount of C_d (this protein coelectrophoresed with the turnover protein C_d discussed previously), (b) an increased amount of GAP-1 and a decreased amount of GAP-3, and (c) increased amounts of C_3–C_5. SVT2 material displayed the same pattern. Eight percent gels indicated that C_0 and C_a were deposited in equivalent relative amounts by cells attaching to coated or uncoated substrates.

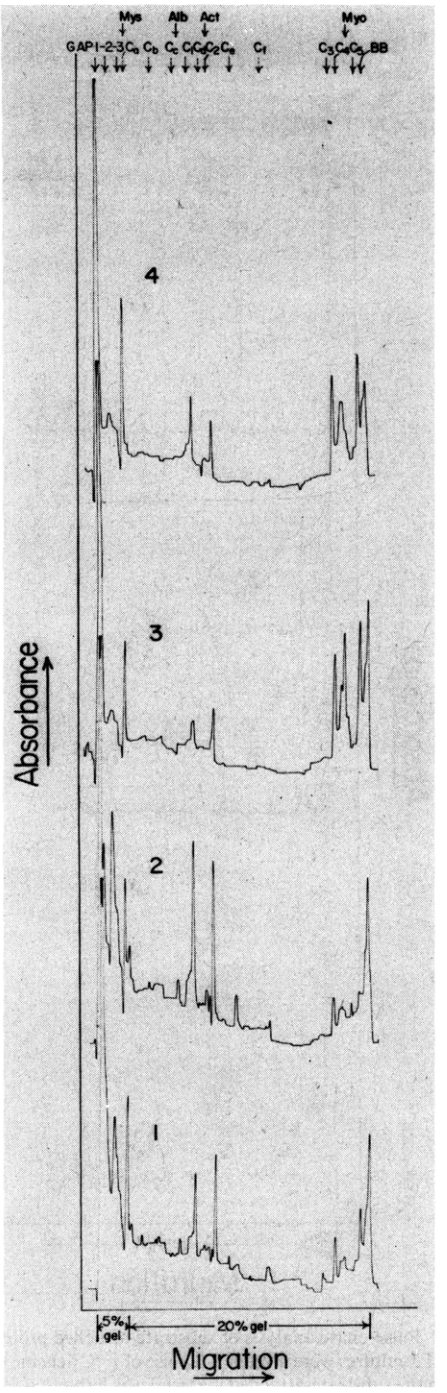

FIGURE 6: Comparison of substrate-attached proteins deposited during long-term growth or initial attachment of cells. BALB/c 3T3 or SVT2 cells were grown during exponential growth in medium containing [^{14}C]leucine, after which the cells were removed by EGTA treatment and the substrate-attached material was harvested as the *long-term-growth sample*. The EGTA-suspended cells were then allowed to attach to fresh plastic tissue culture dishes containing MEM X 4 (including serum) and [^{14}C]leucine. After 1 h of attachment, during which deposition of substrate-attached material was shown to be maximal (Mapstone and Culp, 1976), cells were removed by EGTA treatment and the freshly deposited substrate-attached material was harvested as the *initial attachment sample*. The following samples were electrophoresed on a 20% NaDodSO$_4$–PAGE gel as described in Materials and Methods: well 1, 3T3 long-term-growth material; well 2, 3T3 initial attachment material; well 3, SVT2 long-term-growth material; well 4, SVT2 initial attachment material. All samples were run on the same gel. The methodology for analysis of autoradiograms and nomenclature for identifying major and minor cell proteins are given in Figures 1 and 2. Marker proteins: Mys, myosin; Alb, albumin; Act, actin; Myo, myoglobin.

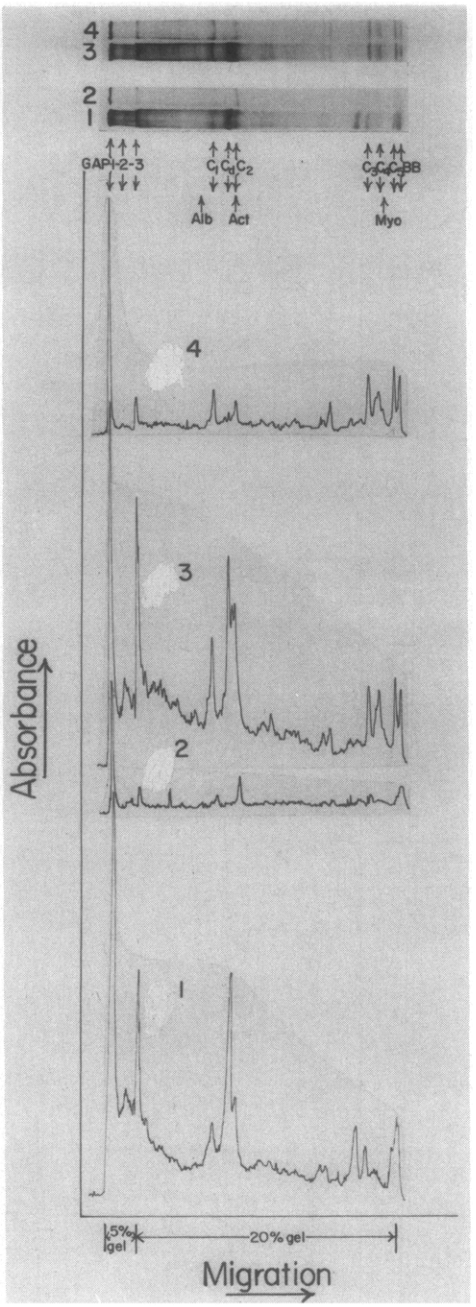

FIGURE 7: Pulse-chase analysis of substrate-attached proteins. BALB/c 3T3 or SVT2 cultures were given a 2-h pulse of [^{14}C]leucine (5.0 μCi/ml), after which the substrate-attached material was harvested from one batch of cells as described in Materials and Methods (PULSE samples). The second batch of cells was washed well with PBS and then chased for 24 h with MEM X 4 containing no radioactive leucine, after which substrate-attached material was harvested (CHASE samples). The following samples were electrophoresed on a 20% NaDodSO$_4$–PAGE gel as described in Materials and Methods: well 1, 10 000 cpm of 3T3 PULSE sample; well 2, 3000 cpm of 3T3 CHASE sample (this reduced amount of radioactivity affords direct comparison with the PULSE sample since it has previously been shown that 70% of the radioactivity was lost from the substrate-attached material during the 24-h CHASE period (Culp et al., 1975)); well 3, 10 000 cpm of SVT2 PULSE sample; well 4, 3000 cpm of SVT2 CHASE sample. All samples were run on the same gel. The methodology for analysis of autoradiograms and nomenclature for identifying major and minor cell proteins are given in Figures 1 and 2. Marker proteins: Alb, albumin; Act, actin; Myo, myoglobin.

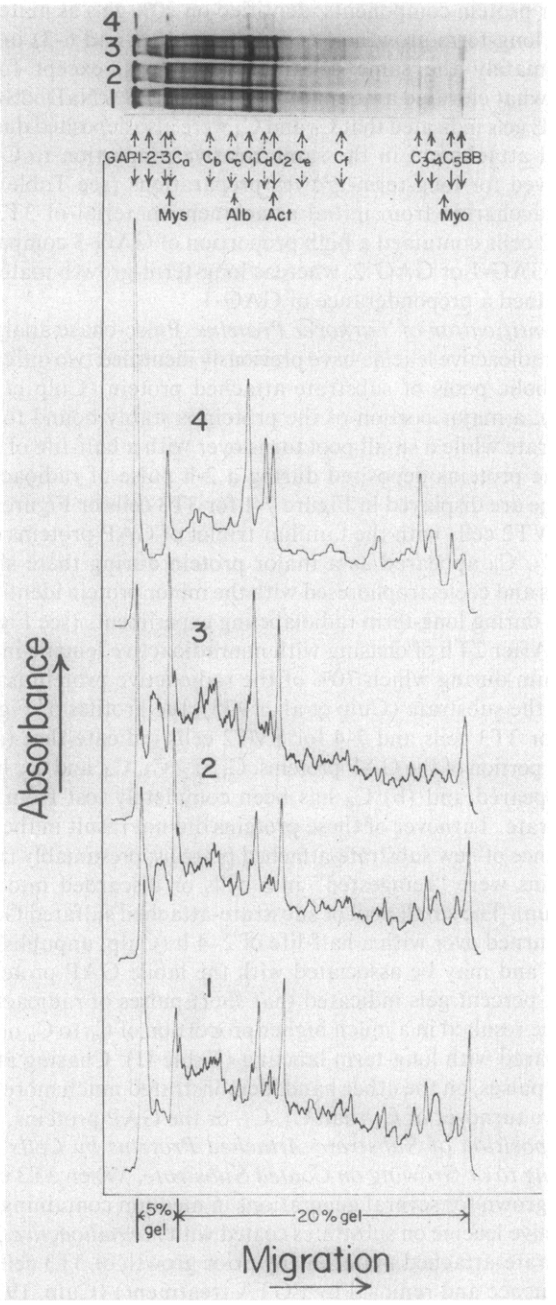

FIGURE 8: Substrate-attached proteins deposited by cells growing on or attaching to coated substrate. BALB/c 3T3 cells were grown in medium containing [^{14}C]leucine for 72 h on fresh substrate or substrate coated with nonradioactive 3T3 substrate-attached material (prepared by growth of 3T3 cells to confluence and removal by EGTA treatment) before harvesting the substrate-attached material (long-term-growth materials). 3T3 cells were also grown in medium containing [^{14}C]leucine, subcultured with EGTA, and permitted to attach to uncoated or coated substrate for 1 h before harvesting of the substrate-attached material as described in the legend to Figure 6 (initial attachment materials). The following samples were electrophoresed on a 20% NaDodSO$_4$–PAGE gel as described in Materials and Methods: well 1, 3T3 long-term-growth material; well 2, 3T3 material deposited during long-term growth on coated substrate; well 3, 3T3 initial attachment material; well 4, 3T3 material deposited during initial attachment to coated substrate (only 5000 cpm of this sample was used). All samples were run on the same gel. The methodology for analysis of autoradiograms and nomenclature for identifying major and minor cell proteins are given in Figures 1 and 2. Marker proteins: Mys, myosin; Alb, albumin; Act, actin; Myo, myoglobin.

Discussion

This study has characterized the substrate-attached proteins which remain adherent to the tissue culture substrate after normal or virus-transformed cells are removed by treatment with the Ca^{2+}-specific chelating agent EGTA and which may mediate substrate adhesion of cells (Culp and Black, 1972a;

Culp, 1974, 1975; Culp et al., 1975; Weiss et al., 1975; Mapstone and Culp, 1976). A limited number of serum and cell proteins have been separated from the hyaluronate proteoglycans originally identified in substrate-attached material by Terry and Culp (1974).

The presence of actin in substrate-attached material further implicates the subsurface membrane cytoskeletal microfilaments in substrate adhesion. Actin-containing microfilaments were originally identified by electron microscopic techniques as being closely associated with membrane sites involved in cell-to-cell and cell-to-substrate adhesion (McNutt et al., 1971, 1973; Perdue, 1973) and later identified by anti-actin immunofluorescence (Pollack et al., 1975). Little is known about the molecular interaction of the actin microfilaments with the surface membrane. Perhaps EGTA treatment weakens the subsurface cytoskeleton of the cell resulting in cell shape change and breakage of the main cell body away from the footpads by which the cell adheres to the substrate (Revel et al., 1974; Rajaraman et al., 1974), leaving a footpad vesicle containing a small pool of intramembranous microfilaments which may be linked to a "transmembrane" component (Jackson et al., 1973; Singer, 1974; Mueller and Morrison, 1974). Or the microfilaments may persist as substrate-adherent material as a result of the membrane being "torn" away from the substrate adhesion site by shearing forces generated during the EGTA treatment. A variety of experimental approaches will be required before the molecular relationship of the subsurface microfilaments with the bilayer membrane and other surface proteins in the substrate adhesion site can be firmly established. Special interest should be focused on possible differences in these relationships between normal and virus-transformed cells.

The presence of a major protein C_a which displayed several properties of myosin heavy chain[3] is interesting in light of the recent discovery of myosin in fibroblasts as a possible surface membrane component (Willingham et al., 1974; Painter et al., 1975). Much more evidence will be required before the exact nature of C_a is known.

Several properties of protein C_0 are consistent with its being the so-called LETS (Hynes and Bye, 1974) or CSP (Yamada and Weston, 1975) glycoprotein. In contrast to previous studies which have shown this component to be missing from surface membrane preparations of Rous sarcoma virus transformed chicken cells (Wickus et al., 1974; Yamada and Weston, 1975) and hamster sarcoma virus transformed hamster cells (Hynes and Humphreys, 1974), our studies have shown the prominence of C_0 in the substrate-attached material from both SV40-transformed and MSV-transformed mouse cells, implicating this surface membrane component in the substrate adhesion process. Yamada et al. (1975) have recently shown that the CSP protein agglutinates formalinized sheep erythrocytes, consistent with its potential importance in cell adhesion.

The presence of histones in substrate-attached material, particularly from virus-transformed cells, may be artifactual as a result of leaking from the EGTA-treated cells (Culp and Black, 1972a). Consistent with this idea was the absence of histones from substrate-attached material from scraped 3T3 or SVT2 cells and their absence from surface membrane preparations (Vessey and Culp, unpublished data). The histones may also be derived from a small proportion of cells which are dying and lysing during culture growth. Their presence may complicate interpretation of experiments dealing with attachment and growth of cells on substrate-attached material (Culp, 1974; Weiss et al., 1975).

The pools of C_0, C_a, and C_2 in the EGTA-solubilized fraction may indicate that the substrate adhesion site contains even higher levels of these components, some of which may have been leached off the substrate during EGTA treatment. On the other hand, solubilized components may result in artifactual binding to the substrate, although some evidence suggests that this may not be true. Autoradiographic evidence (Culp, 1975) indicates that substrate-attached material is only deposited at sites of cell adhesion to the substrate and in focal pools similar in density to the footpads by which the cell adheres to the substrate. Secondly, several methods of cell removal yield the same distributions of substrate-attached proteins, except for the histones which appear to be artifactually bound during the EGTA treatment. And finally, analysis of substrate-bound proteins after a wide variety of cellular growth and attachment experiments, particularly during reattachment of EGTA-subcultured cells, indicated similar proportions of these proteins (except for the histones) and argues for the importance of these components in the substrate adhesion process (Mapstone and Culp, 1976). Ultimately, the specificity by which these components become associated in the substrate-attached material will only be resolved by detailed analysis of the molecular topography and binding relationships of these various components.

The proteins associated with the three size classes of high molecular weight proteoglycans have been partially characterized as three size classes from normal, transformed, and revertant cell types. A major portion of GAP-1 was highly heterogeneous and competitively displaced from binding to GAG-1 by addition of positively charged histones without any effect on the distribution of glucosamine-radiolabeled polysaccharides in these gels. The minor portion of GAP-1 and all of GAP-2 and GAP-3 were not dissociated by a number of vigorous treatments. Much more information will be required, perhaps along the lines of study performed with cartilaginous hyaluronate proteoglycans (Heinegard and Hascall, 1974; Hardingham and Muir, 1974), before the exact molecular composition of these GAG proteoglycans and their prospective role in substrate adhesion are determined.

Pulse-chase experiments with radioactive leucine indicated that a small portion of all the major substrate-attached proteins from 3T3 or SVT2 cell turn over. Perhaps these components are found in a minor class of cell adhesion sites which are very transiently bound to the plastic. The kinetics of turnover of a portion of GAP proteins was similar to the turnover behavior of the small pool of sulfated GAG found in substrate-attached material (Terry and Culp, 1974; Roblin et al., 1975; Culp, unpublished data). C_d was the principle protein identified during short radiolabeling periods and completely turned over by loss from the substrate. A major portion of C_0 was also very labile during the chase period. The very different turnover patterns of these proteins should prove useful in a further study of the dynamics of substrate adhesion.

Culp and Mapstone (1976) have previously shown that deposition of substrate-attached material by newly attached SVT2 cells was inhibited by a coating of 3T3 substrate-attached material; deposition by 3T3 or Con A revertant cells was unaffected. $NaDodSO_4$-PAGE analyses have now revealed very little qualitative differences in these SVT2 preparations. Therefore the inhibition effect exerted by coated

[3] The myosin-like nature of C_a has now been confirmed by showing that this protein is selectively immunoprecipitated from Triton-solubilized membrane preparations with an antibody prepared against purified mouse L cell myosin and kindly provided by Dr. Ira Pastan.

substrate during SVT2 attachment may be a quantitative phenomenon applying to all the surface membrane components coming into contact with the substrate.

Slab NaDodSO$_4$–PAGE analysis provides an effective method for separating the limited number of serum (Culp and Buniel, 1976) and cell proteins from the high molecular weight proteoglycans tightly bound to the substrate. The presence of a limited number of proteins in the substrate-attached material from a variety of cell types offers considerable encouragement in the eventual resolution of the molecular topography of the substrate adhesion site and whether this site differs for normal and malignant cells.

Added In Proof

Data from scanning electron microscopic studies (J. Rosen and L. Culp, manuscript in preparation) indicate that substrate-attached materials are membranous vesicles whose size and density on the substrate are identical to "footpads" by which cells adhere to the substrate. This supports the biochemical evidence that EGTA treatment does *not* necessarily dissociate extra-surface materials directly involved in the cell–substrate adhesion site, but may weaken the subsurface cytoskeleton of the cell allowing pinching-off of footpads by shearing forces generated in the solution. These footpad vesicles probably contain all the macromolecules necessary for cell–substrate adhesion, including surface-associated microfilaments.

Acknowledgments

The author acknowledges the excellent technical assistance of Mrs. Josefina Buniel and Mr. Carl Martino with these experiments.

References

Bonner, W. M., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83.
Collins, J. H., and Elzinga, M. (1975), *J. Biol. Chem. 250,* 5915.
Culp, L. A. (1974), *J. Cell Biol. 63,* 71.
Culp, L. A. (1975), *Exp. Cell Res. 92,* 467.
Culp, L. A., and Black, P. H. (1972a), *Biochemistry 11,* 2161.
Culp, L. A., and Black, P. H. (1972b), *J. Virol. 9,* 611.
Culp, L. A., and Buniel, J. F. (1976), *J. Cell. Physiol. 88,* 89.
Culp, L. A., Terry, A. H., and Buniel, J. F. (1975), *Biochemistry 14,* 406.
Curtis, A. S. G. (1973), *Prog. Biophys. Mol. Biol. 27,* 317.
DeLange, R. J., and Smith, E. L. (1975), *CIBA Found. Symp. 28,* 59.
Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.
Grinnell, F. (1974), *Arch. Biochem. Biophys. 160,* 304.
Grinnell, F. (1975), *Arch. Biochem. Biophys. 169,* 474.
Gruenstein, E., Rich, A., and Weihing, R. R. (1975), *J. Cell Biol. 64,* 223.
Hardingham, T. E., and Muir, H. (1974), *Biochem. J. 139,* 565.
Heinegard, D., and Hascall, V. C. (1974), *J. Biol. Chem. 249,* 4250.
Hynes, R. O., and Bye, J. M. (1974), *Cell 3,* 113.
Hynes, R. O., and Humphreys, K. C. (1974), *J. Cell Biol. 62,* 438.
Jackson, R. L., Segrest, J. P., Kahane, I., and Marchesi, V. T. (1973), *Biochemistry 12,* 3131.
Kraemer, P. M. (1971), in Complex Carbohydrates of Animal Cells: Biochemistry and Physiology of the Cell Periphery in Biomembranes, Vol. I, Manson, L., Ed., New York, N.Y., Plenum Press.
Mapstone, T. B., and Culp, L. A. (1976), *J. Cell Sci. 20,* 479.
Maurer, H. R., and Allen, R. C. (1972), *Z. Klin. Chem. Klin. Biochem. 10,* 220.
McNutt, N. S., Culp, L. A., and Black, P. H. (1971), *J. Cell Biol. 50,* 691.
McNutt, N. S., Culp, L. A., and Black, P. H. (1973), *J. Cell Biol. 56,* 412.
Mueller, T. J., and Morrison, M. (1974), *J. Biol. Chem. 249,* 7568.
O'Farrell, P. H. (1975), *J. Biol. Chem. 250,* 4007.
Painter, R. G., Sheetz, M., and Singer, S. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1359.
Perdue, J. F. (1973), *J. Cell Biol. 58,* 265.
Pollack, R., Osborn, M., and Weber, K. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 994.
Poste, G., Greenham, L. W., Mallucci, L., Reeve, P., and Alexander, D. J. (1973), *Exp. Cell Res. 78,* 303.
Rajaraman, R., Rounds, D. E., Yen, S. P. S., and Rembaum, A. (1974), *Exp. Cell Res. 88,* 327.
Revel, J. P., Hoch, P., and Ho, D. (1974), *Exp. Cell Res. 84,* 207.
Revel, J. P., and Wolken, K. (1973), *Exp. Cell Res. 78,* 1.
Roblin, R., Albert, S. O., Gelb, N. A., and Black, P. H. (1975), *Biochemistry 14,* 347.
Rosenberg, M. (1960), *Biophys. J. 1,* 137.
Singer, S. J. (1974), *Annu. Rev. Biochem. 43,* 805.
Stephens, R. E. (1970), *J. Mol. Biol. 47,* 353.
Stephenson, J. R., and Aaronson, S. A. (1972), *J. Exp. Med. 135,* 503.
Takeichi, M. (1971), *Exp. Cell Res. 68,* 88.
Taylor, A. C. (1961), *Exp. Cell Res., Suppl. 8,* 154.
Terry, A. H., and Culp, L. A. (1974), *Biochemistry 13,* 414.
Weiss, L. (1961), *Exp. Cell Res. 25,* 504.
Weiss, L. (1962), *J. Theor. Biol. 2,* 236.
Weiss, L., Poste, G., MacKearnin, A., and Willett, K. (1975), *J. Cell Biol. 64,* 135.
Wickus, G. G., Branton, P. E., and Robbins, P. W. (1974), in Control of Proliferation in Animal Cells, Clarkson, B., and Baserga, R., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory, p 541.
Willingham, M. C., Ostlund, R. E., and Pastan, I. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4144.
Yamada, K. M., and Weston, J. A. (1975), *Cell 5,* 75.
Yamada, K. M., Yamada, S. S., and Pastan, I. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3158.
Yaoi, Y., and Kanaseki, T. (1972), *Nature (London) 237,* 283.