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Metabolic Properties of Substrate-Attached Glycoproteins from Normal and Virus-Transformed Cells†

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ABSTRACT: Balb/c 3T3, SV40-transformed 3T3 (SVT2), and Con A revertant variants of transformed cells leave a layer of glycoprotein on the culture substrate upon EGTA mediated removal of cells. The metabolic properties of this substrate-attached material (glycoprotein) have been examined. Pulse and cumulative radiolabeling experiments with glucosamine and leucine precursors established that this substrate-attached material accumulates on the substrate in growing cultures until cells have completely covered the substrate. The synthesis and/or deposition of the material diminished dramatically in cultures whose substrates had been completely covered with cells as observed microscopically, even though the contact-inhibited cell lines continued to make cell-associated and medium-secreted glycoproteins and transformed cells continued to divide and form multilayered cultures. Pulse-chase analysis using long periods of pulsing with radioactive leucine demonstrated that these glycoproteins are deposited directly on the substrate by cells and not subsequent to secretion into the medium. The substrate-attached material accumulated during long pulses was stably adherent to the substrate and displayed little appreciable turnover during 3 days of chasing of either sparse or dense cultures. Short-term pulse-chase analysis with leucine revealed two metabolically different pools of material—one which turns over very rapidly with a half-life of 2–3 hr (observed in both low-density and high-density cultures) and a second pool which is stably deposited on the substrate and whose proportion increased with the length of the radiolabeling period. No appreciable differences in the metabolic properties of substrate-attached material were observed in the three cell types studied during growth on a plastic substrate. These results are discussed with regard to the implicated roles of these glycoproteins in mediating adhesion of normal and virus-transformed cells to the substrate.

A class of glycoproteins has been found at the surface of normal and virus-transformed fibroblasts which appears to mediate adhesion of cells to the culture substrate (Culp, 1974)—so-called substrate-attached material which remains on the substrate subsequent to EGTA[1] mediated removal of cells. The more adherent Balb/c 3T3 and Con A revertant variants (Culp and Black, 1972a) of transformed 3T3 cells deposit more of this material than SV40-trans-

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[1] Abbreviations used are: Con A, concanavalin A; CPC, cetylpyridinium chloride; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; MEM X4, Eagle's minimal essential medium supplemented with four times the concentration of vitamins and amino acids; SV40, Simian virus 40.

formed cells (Culp and Black, 1972b). It has been speculated (Culp, 1974) that these glycoproteins may be a class of macromolecules whose primary function may be the control of *cell-to-substrate* adhesion and whose secondary function may be the control of the social behavior of normal and malignant cells *via cell-to-cell* adhesion.

Terry and Culp (1974) subsequently identified the major polysaccharide component of substrate-attached material as hyaluronic acid, which may be linked covalently to some of the protein of these preparations. Little is known about the protein components of these "supramolecules." In this study we identify some of the metabolic properties of these substrate-attached glycoproteins,[2] such as their accumulation on the substrate, their rate of synthesis, and their turnover as a function of growth of contact-inhibited and virus-transformed cells.

Materials and Methods

Cell Growth. Mouse fibroblast Balb/c 3T3 cells (clone A31), SV40-transformed A31 cells (clone SVT2), and the Con A-selected revertant variants (Culp and Black, 1972a) were grown as described previously (Terry and Culp, 1974). All cells were grown in Eagle's minimal essential medium (MEM X 4) supplemented with four times the concentration of amino acids and vitamins, 10% fetal calf serum, penicillin (250 units/ml), and streptomycin (0.25 mg/ml). Cells were incubated in an environment of 5% CO_2 in air which was humidified and maintained at 37°. They were routinely passaged in Brockway glass 32-oz. tissue culture bottles by using a trypsin–ethylenediaminetetraacetic acid solution; cultures for experimental purposes were grown in Lux plastic tissue culture dishes. All cells used for experimentation were *Mycoplasma* free according to the radiolabel assay of Culp and Black (1972b).

Radiolabeling Procedures. Trypsinized cells were inoculated into triplicate 60-mm plastic tissue culture dishes at the indicated densities for each datum point; 24 hr later, the medium was changed to medium containing radioactive precursor: to radiolabel glycoproteins, 2–3 μCi/ml of D-[1-^{3}H]glucosamine (specific activity 2.6–3.0 Ci/mmol) was included or, to radiolabel proteins, medium containing a reduced level of leucine (5 mg/l.) and supplemented with 10 μCi/ml of L-[4,5-^{3}H]leucine (specific activity 55 Ci/mmol) was used. Short-term pulse experiments were performed with leucine-free medium supplemented with 10 μCi/ml of [^{3}H]leucine. None of the radiolabeling conditions used in these studies was deleterious to cell growth or protein synthesis. Control dishes were used to determine cell number.

Isolation of Cell Fractions. The medium from 60-mm culture dishes was decanted and 50-μl aliquots were withdrawn for precipitation analysis. The cell layer was rinsed three times with phosphate-buffered saline; 2 ml of EGTA (0.5 mM in phosphate-buffered saline) was added and the culture was incubated on a rotary shaker at 37° for 30 min. The suspension of cells (called the cell fraction in these studies) was gently pipetted to remove any attached cells and 0.5-ml aliquots were analyzed by precipitation assays. Distilled water was used to rinse the substrate thoroughly. The substrate-attached fraction was removed from the surface of the substrate with either 0.1% sodium dodecyl sulfate (for direct determination of radioactive content in Bray's solution (Bray, 1960)) or 0.1 N NaOH (for precipitation with trichloroacetic acid or cetylpyridinium chloride) by incubation at 37° for 30 min on a rotary shaker. These concentrations of reagent were found to remove the substrate-attached material quantitatively. All measurements are made of the radioactive content of the entire fraction per 60-mm dish.

Precipitation Assays. Aliquots of cell (0.5 ml), medium (50 μl), and substrate-attached (0.2–0.5 ml) fractions were brought to a final volume of 0.5 ml with distilled water. To assay for cetylpyridinium chloride (CPC) precipitable radioactivity (Terry and Culp, 1974), sodium heparin (100 μg) was used as carrier glycosaminoglycan and CPC was added at three times the heparin concentration. The reaction mixture was allowed to incubate at 25° for 30 min, and the precipitates were collected on Millipore filters having a 0.45-μ pore size. After washing the Millipore filters were dried, and their radioactive content was determined using a Packard scintillation counter.

Precipitability with trichloroacetic acid was tested by making the samples 5% (w/v) in Cl_3CCOOH after the addition of 100 μg of carrier bovine serum albumin to precipitate proteins, some glycoproteins, and some glycosaminoglycans (Kraemer, 1971a,b). Precipitation occurred during incubation in the cold (0–4°) for 30 min. The precipitates were filtered through Whatman 2.4 cm GF/C glass fiber filters which were assayed for their radioactivity in the same manner as Millipore filters.

Materials were purchased from the following sources: D-[1-^{3}H]glucosamine (specific activity 2.6–3 Ci/mmol) and L-[4,5-^{3}H(N)]leucine (specific activity 55 Ci/mmol) from Amersham/Searle Corp.; EGTA from Eastman Organic Chemicals; cetylpyridinium chloride (hexadecylpyridinium chloride) and bovine albumin (fraction V) from Sigma Chemical Co.; MEM X 4 from Grand Island Biologicals Co.; fetal calf serum from GIBCO or Flow Laboratories; plastic tissue culture dishes from Lux Scientific Co.; 32-oz. Brockway glass prescription bottles from Brockway Glass Inc; Millipore membranes (25 mm, HAWP, 0.45-μ pore size) from Millipore Corp.; Whatman glass fiber filters (GF/C) from A. H. Thomas Co.; and sodium heparin (injectable) from Upjohn Co.

Results

Recent studies have shown that the major components left on the plastic substrate of cultured mouse fibroblasts subsequent to EGTA-mediated removal of cells are hyaluronic acid polysaccharide and protein components which have not been well-characterized (Terry and Culp, 1974). Pulse radiolabeling experiments were performed to measure the rate of synthesis of these substrate-attached glucoproteins relative to cell-containing and medium-secreted glycoproteins during growth of 3T3 cells, as measured by glucosamine incorporation into CPC-precipitable glycosaminoglycans (Terry and Culp, 1974). Cetylpyridinium chloride has been shown to be an effective precipitating agent of acidic glycosaminoglycans (Terry and Culp, 1974). As shown by Table I, the synthesis and/or deposition of substrate-attached material dramatically diminished after the 3T3 cells had covered the substrate (approximately 1×10^6 cells/60-mm dish) before they became growth inhibited (approximately 1.3×10^6 cells/dish). On a per-cell basis, production of substrate-attached material diminished 14-fold in contact-inhibited cells, while incorporation into acidic glycosaminoglycan-like materials of the cell fraction dimin-

[2] The term "glycoprotein" in these studies refers to a nonhomogeneous mixture of leucine- and glucosamine-radiolabeled macromolecules.

Table I: Incorporation of [^{3}H]Glucosamine into CPC Insoluble Macromolecules by 3T3 Cells.[a]

Time Period of Radiolabeling (Days after Inoculation)	Medium		Cell		Substrate Attached		Cell Number[c] ($\times 10^{-6}$)
	cpm/dish	cpm/cell[b]	cpm/dish	cpm/cell[b]	cpm/dish	cpm/cell[b]	
1 (2–3)	234,000	246	144,440	152	2560	2.70	0.95
2 (4–5)	166,750	137	64,870	53	280	0.23	1.22
3 (6–7)	110,250	79	20,160	14	300	0.21	1.39
4 (8–9)	107,500	87	25,300	21	200	0.16	1.23

[a] 3T3 cells (0.25×10^6) were inoculated into 60-mm culture dishes containing MEM X 4. At the beginning of the indicated periods after inoculation, the medium was removed in duplicate dishes and replaced with MEM X 4 containing 2.5 μCi/ml of [^{3}H]glucosamine; 48 hr later, radiolabeled cultures were fractioned into cell, medium, and substrate-attached fractions and analyzed by CPC precipitation for acidic glycosaminoglycans as described in the Materials and Methods section. Data are the averages of two dishes with an error of ±6% for the cell and medium measurements and no more than ±10% for measurements of the substrate-attached material. [b] Calculated by dividing the amount of radioactivity in the particular fraction by the number of cells at the end of the radiolabeling period. [c] At the time of fractionation of the cell cultures, an unlabeled culture of cells was used to determine the cell number in a hemocytometer after suspension with trypsin.

Table II: Incorporation of [^{3}H]Glucosamine into CPC Insoluble Macromolecules by SVT2 Cells.[a]

Time Period of Radiolabeling (Days after Inoculation)	Medium		Cell		Substrate Attached		Cell Number ($\times 10^{-6}$)
	cpm/dish	cpm/cell	cpm/dish	cpm/cell	cpm/dish	cpm/cell	
1 (2–3)	109,000	136	55,450	69	2370	2.95	0.80
2 (4–5)	367,750	120	430,650	141	8280	2.71	3.05
3 (6–7)	532,300	120	565,600	126	5040	1.12	4.48
4 (8–9)	503,000	94	629,980	118	3440	0.64	5.38

[a] 0.20×10^6 SVT2 cells were inoculated into 60-mm culture dishes and analyzed as described in Table I and the Materials and Methods section.

ished sevenfold and medium-secreted materials accumulated at a threefold lower rate. Thus, there appeared to be a preferential inhibition of formation and/or secretion of substrate-attached hyaluronic acid when 3T3 cells completely covered the substrate and became growth inhibited. Other experiments have shown that the availability of radioactive precursor did not become limiting.

This preferential inhibition of production of substrate-attached material was even more dramatic in SV40-transformed 3T3 cells (SVT2 cells) when they continued to grow as multilayered cultures (Table II). Synthesis and accumulation of cellular and medium-secreted acidic glycosaminoglycans continued unabated in dense cultures (cpm/cell in Table II), while synthesis and/or deposition of substrate-attached material (*i.e.*, accumulation) dropped fivefold when the cells covered the substrate (approximately 3×10^6 cells/60-mm dish).

Similar results were obtained using radioactive leucine pulses and Cl_3CCOOH precipitation (unpublished observations).

Cumulative radiolabeling of 3T3 culture fractions demonstrated the same phenomenon (Figure 1). Incorporation of radioactive glucosamine into substrate-attached material as measured by either CPC precipitability (quantitative for acidic glycosaminoglycans) or Cl_3CCOOH precipitability (quantitative for some types of glycoproteins and possibly some types of acidic glycosaminoglycans) continued until the cells covered the substrate (approximately 1×10^6 cells/dish) while incorporation into cellular and medium-secreted material continued after contact inhibition of growth, although at a diminishing rate.

Similar incorporation data were obtained for SVT2 cells (Figure 2), which completely covered the substrate at approximately $4–5 \times 10^6$ cells/dish[3] as observed in the microscope, and for revertant variants (Figure 3) of SVT2 cells, which are flatter, display contact inhibition of growth, and cover the substrate at $1.0–1.5 \times 10^6$ cells/dish. The limiting deposition of substrate-attached glycoprotein was not caused by limiting availability of radioactive glucosamine, since radioactivity increases at an expected rate in the cell and medium-secreted fractions and since a second addition of radioactive glucosamine at day 2 had no effect on the level of substrate-attached material. These data would suggest that cells no longer deposit these glycoproteins when they have covered the substrate and that they are a highly stable entity exhibiting very little turnover (or that turnover of this material by confluent cultures is balanced by continued synthesis and deposition). The pulse-labeling experiments of Tables I and II suggest, however that deposition of these glycoproteins onto the substrate does diminish in confluent cultures eliminating the balanced synthesis

[3] Transformed cells are smaller and exhibit no preference during growth for adhesion to the substrate or to neighboring cells. Therefore a much higher density of cells is required to confluently cover the culture substrate.

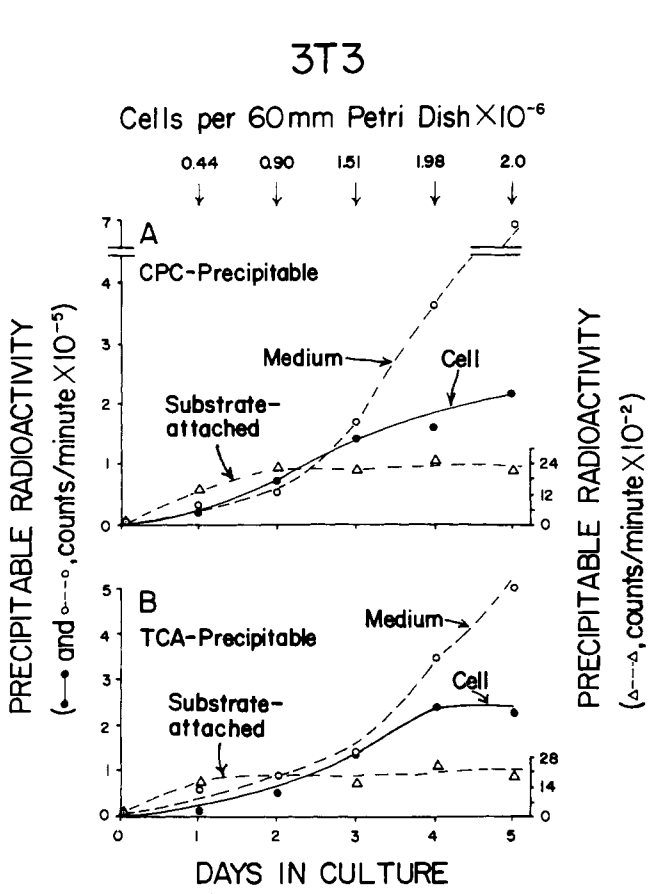

FIGURE 1: Glycoprotein accumulation in 3T3 cultures as a function of time; 0.3×10^6 3T3 cells were inoculated into 60-mm culture dishes containing MEM × 4; 24 hr later, the medium was changed to medium containing 2.0 μCi/ml of [^{3}H]glucosamine; fresh radioactive medium was substituted for old medium every other day. Duplicate dishes were fractioned every day into cell, medium, and substrate-attached fractions and assayed by CPC precipitation (A) or Cl_3CCOOH precipitation (B) as described in the Materials and Methods section. The measurements were averaged and calculated to give the total content of radioactivity per dish for each fraction. The cell number was determined at the time of fractionation by trypsinizing and counting cell suspensions in a hemocytometer.

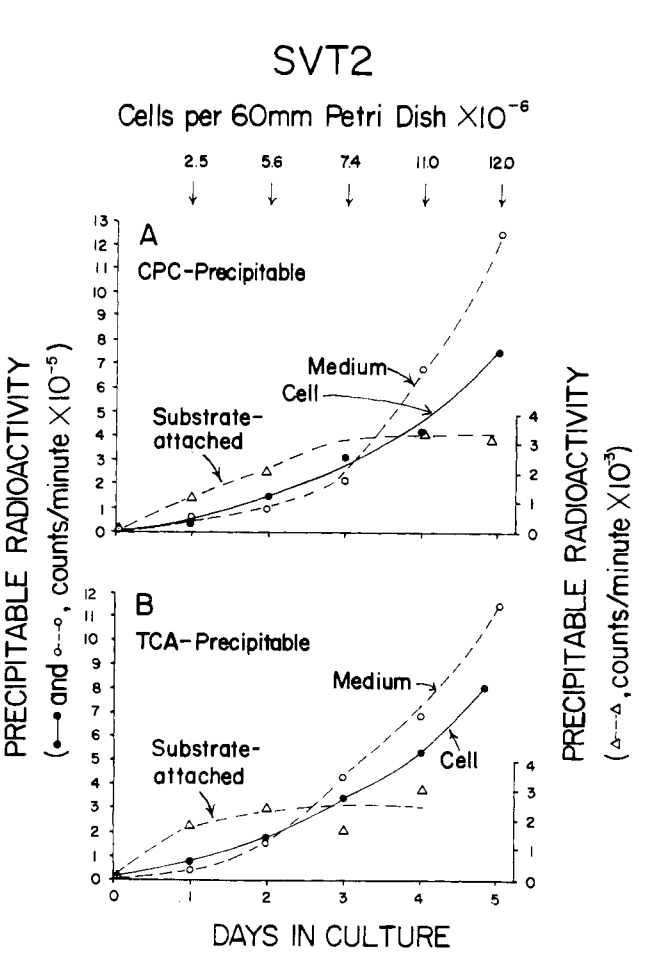

FIGURE 2: Glycoprotein accumulation in SVT2 cultures as a function of time. The same procedures were followed as described in the legend to Figure 1 and the Materials and Methods section, with the exception that 1.0×10^6 SVT2 cells were inoculated.

(with deposition) and turnover theory. The reduced accumulation of substrate-attached glycoproteins in confluent cultures was not due to reduced metabolic activity of the cells since transformed cells continued to divide in confluent cultures and the contact-inhibited cells continued to synthesize and secrete into the medium other classes of glycoprotein.

Similar results of cumulative radiolabeling with precursor leucine have been obtained (unpublished observations).

The experiments reported above do not define whether substrate-attached material turns over after deposition on the substrate or what the source of deposition of this material onto the substrate is (deposited directly by the cells or *via* secretion into the medium). We resorted to pulse-chase experiments using radioactive leucine to radiolabel substrated-attached glycoproteins, since radioactive leucine in proteins can be effectively chased. Unfortunately, it is not possible to chase radioactive glucosamine incorporation into glycoproteins by cells (Spear and Roizman, 1970; L. A. Culp, unpublished observations), so our results can only be definitive with regard to the protein portion of the substrate-attached material.

Two days of radiolabeling of 3T3 cells (Figure 4) demonstrated increasing levels of radioactivity in the medium,

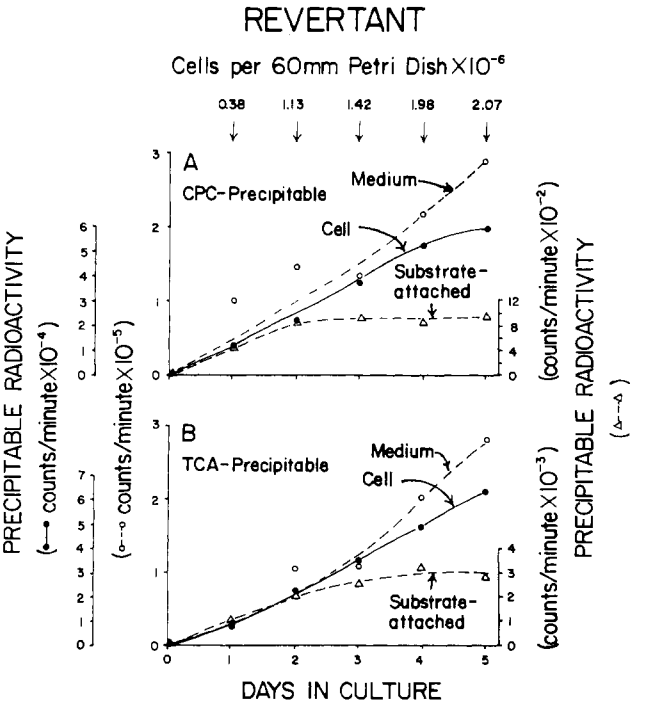

FIGURE 3: Glycoprotein accumulation in Con A revertant cultures as a function of time. The same procedures were followed as described in the legend to Figure 1 and the Materials and Methods section, with the exception that 0.2×10^6 revertant cells were inoculated.

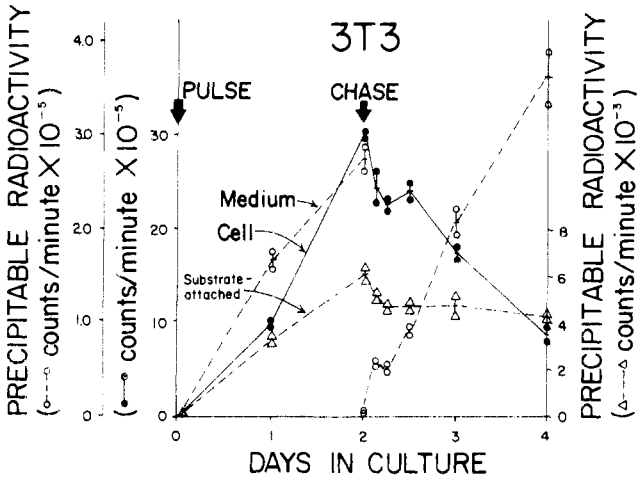

FIGURE 4: Long-term pulse-chase analysis of 3T3 cellular protein fractions. 0.25 × 10^6 3T3 cells were inoculated into 60-mm plastic tissue culture dishes. After 24 hr of growth, the PULSE medium was substituted, containing 10 μCi/ml of [^{3}H]leucine plus 5 mg/l. of carrier leucine. Nonradioactive CHASE medium, containing the normal concentration of leucine, was substituted for the PULSE medium after 48 additional hours of growth (day 2). Cells were approximately 90–100% confluent by day 4 (approximately 1.2 × 10^6 cells/dish). Aliquots of medium, cell, and substrate-attached fractions were precipitated with trichloroacetic acid and the total radioactive content of each fraction per 60-mm dish was determined as described in the Materials and Methods section.

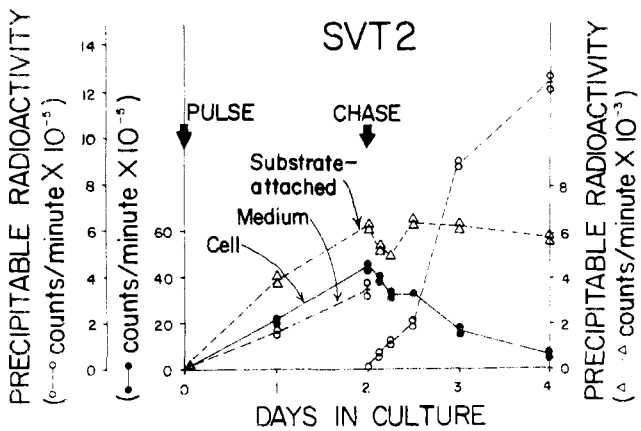

FIGURE 5: Long-term pulse-chase analysis of SVT2 cellular protein fractions. 0.5 × 10^6 SVT2 cells were inoculated into 60-mm dishes and analyzed as described in the legend to Figure 4 and the Materials and Methods section.

cell, and substrate-attached fractions. During a subsequent 48 hr of chasing with nonradioactive medium, radioactive protein was chased out of the cell fraction into the medium but not into the substrate-attached fraction—an indication that substrate-attached glycoproteins were not first secreted into the medium before deposition on the substrate. If such a phenomenon were occurring, we would expect a continued increase in the levels of radioactive substrate-attached material due to the persistent secretion of radioactive glycoproteins into the medium at an undiminishing rate.

The level of radioactive substrate-attached material (Figure 4) dropped slightly during the first few hours of chasing—possible indication of some turnover of this material. In general, the level of radioactive substrate-attached material remained quite stable for periods up to 72 hr during the chase—possible indication of very stable adherence of these glycoproteins to the substrate.

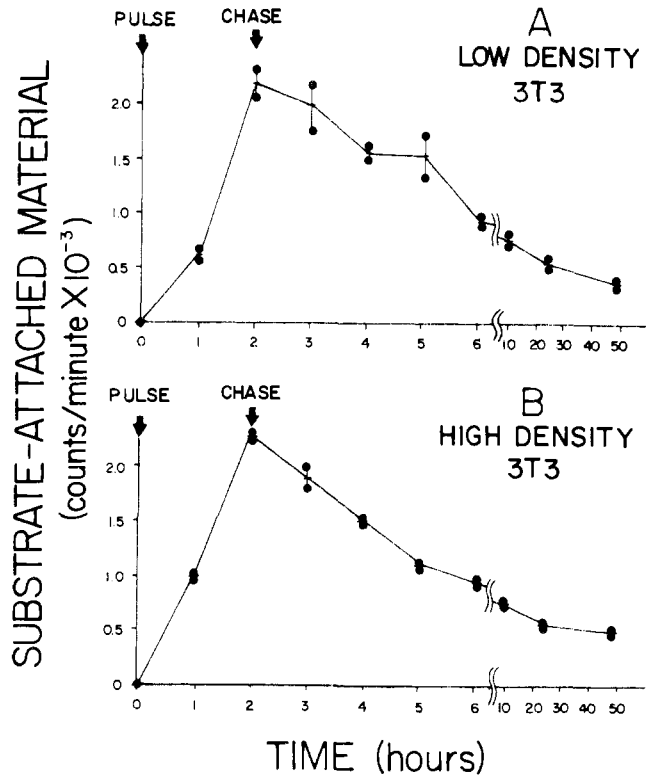

FIGURE 6: Short-term pulse-chase analysis of 3T3 substrate-attached glycoproteins. 3T3 cells were inoculated into 60-mm dishes and allowed to grow for 48 hr, when they were at a low density (A, 0.5 × 10^6 cells/dish) or high density (B, 2.0 × 10^6 cells/dish). At the beginning of the experiment, cells were washed with leucine-free MEM × 4 (at 37°) and the PULSE medium was added for 2 hr, containing no carrier leucine and 10 μCi/ml of [4,5-^{3}H]leucine. After 2 hr in the PULSE medium, the CHASE medium was substituted which contained the normal concentration of leucine. Duplicate dishes were treated as described in the Materials and Methods section with 0.1% SDS to obtain the substrate-attached fraction, aliquots of which were counted directly in Bray's scintillation fluid to determine the total radioactive substrate-attached content per dish.

Similar phenomena were observed for SVT2 cells (Figure 5) during long period pulse-chase experiments. Thus, substrate-attached protein behaves metabolically the same after deposition by normal or transformed cells.

Since long-term pulse-chase experiments would selectively establish the *accumulation* of material on the substrate, short pulses of radioactive precursor followed by chases were used to analyze for a pool of rapidly turning-over material. Short-term pulse-chase experiments did identify two different metabolic pools of substrate-attached material. The data of Figure 6A indicate that after a short 2-hr pulse approximately 75% of this material turned over during the first 10 hr of the chase (with a half-life of 2–3 hr), whether cells were at low density and growing (Figure 6A) or confluent and contact inhibited (Figure 6B). Approximately 25–30% of the substrate-attached material was quite stable to chasing. Identical phenomena were observed with low or high density SVT2 cells and by addition of conditioned pulse and/or chase media, rather than fresh medium. Turnover was therefore not an effect of the addition of fresh serum possibly resulting in turnover of membrane glycoproteins. Turnover of substrate-attached material was observed by determining the radioactive content directly in Bray's fluid or by analysis of Cl_3CCOOH precipitates. It thus appears that longer pulsing periods identified the accumulation and higher proportion of very stable protein compo-

nent(s) which do not turn over, while short pulses followed by a chase identify a pool of substrate-attached protein which turns over quite rapidly and whose formation may be independent of cell growth.

Discussion

Pulse radiolabeling experiments during different phases of growth of 3T3, SVT2, and Con A revertant cells have shown that synthesis and/or deposition of substrate-attached glycoproteins onto the culturing substrate diminishes greatly when the cells have completely covered the surface of the substrate (*i.e.*, become confluent). In the growth-inhibited 3T3 and revertant cells, synthesis of cellular and medium-secreted glycoproteins continued in the absence of further deposition of substrate-attached material—an indication that cells continued to be metabolically competent. These experiments do not distinguish, however, between the inhibition of synthesis of these glycoproteins or their continued synthesis in the absence of further deposition when cells have covered the substrate.

The persistent growth and piling of transformed cells after confluence suggest the possibility that substrate-attached glycoproteins continue to be synthesized and secreted into the intercellular space and no longer deposited onto the cell-saturated substrate, since these cells do not display density-dependent inhibition of growth (Stoker and Rubin, 1967; Green and Todaro, 1967), sugar transport (Schultz and Culp, 1973; Bradley and Culp, 1974), and other metabolic changes which may be related to growth control of normal cells (Yogeeswaran *et al.*, 1972; Sakiyama *et al.*, 1972; Fishman *et al.*, 1974; Cunningham, 1972).

Perhaps substrate-attached glycoproteins are intrinsic components of the cell surface which are deposited on the surface of the substrate during surface membrane and cell movement. Once these processes have been inhibited by contact between growth-regulated cells (Abercrombie *et al.*, 1970; Trinkaus *et al.*, 1971), new points of adhesion to the substrate may not be initiated. It has recently been shown (Culp, manuscript submitted for publication) that substrate-attached glycoproteins are deposited directly onto the substrate at the site of cell attachment as focal pools of material, in distributions which resemble the attachment points of cells observed in electron micrographs (Revel *et al.*, 1974). Other experiments (Mapstone and Culp, manuscript in preparation) suggest that there are sizable pools of these glycoproteins on the cell surface available for adhesion of the cell on the substrate. What controls the synthesis and topography of these materials, allowing them to adhere as focal pools?

Cumulative radiolabeling experiments also demonstrated the accumulation of radioactive substrate-attached material until cells confluently covered the substrate surface. The saturation level of substrate-attached material appeared to be very stable during further maintenance and growth of either contact-inhibited or transformed cells. These data are very similar to the kinetics of microexudate accumulation observed in a variety of normal and virus-transformed cell lines by Poste *et al.* (1973).

Autoradiography experiments (Culp, submitted for publication) have demonstrated that substrate-attached material is deposited at the site of cell attachment to the substrate, and not randomly on the substrate subsequent to secetion into the medium. This evidence has now been confirmed by the long-term pulse-chase analyses. Although cellular proteins continued to be secreted into the medium for at least 2 days during the chase, the level of radioactive substrate-attached material did not increase as a result of persistent accumulation or decrease as a result of turnover. Thus metabolic studies and autoradiographic evidence indicate that deposition of substrate-attached proteins is directly related to the extent of cell growth and coverage of the substrate and not the result of rapid secretion of these components into the medium followed by their random and rapid saturation of the substrate. Unfortunately, little is known about the accumulation kinetics or turnover of the hyaluronic acid portion of this material (Terry and Culp, 1974), because of the inability to perform pulse-chase experiments with precursor glucosamine and the lack of an effective specific inhibitor of polysaccharide synthesis in mammalian cells.

Short-term pulse-chase experiments, on the other hand, have revealed two metabolically different classes of the protein of substrate-attached material—one which turns over very rapidly with a half-life of 2–3 hr and a second which is stably deposited on the surface. Prior studies on the biochemical nature of substrate-attached material (Terry and Culp, 1974) were performed with cells radiolabeled during extended growth, which no doubt contain much larger proportions of the stably deposited protein components observed in longer pulses (Figures 4 and 5). It will be important to radiolabel cells for very short periods with or without subsequent chases to determine the biochemical nature of the rapidly turning-over material. Similarly, these experiments have dealt with substrate-attached material adherent to a plastic substrate, and it will be interesting to determine if there are metabolically different pools of this material deposited on glass or biopolymer-modified surfaces.

Is the turning-over material a second class of protein which may be linked to hyaluronic acid polysaccharide? Or is it a unique class of proteins (or glycoproteins) distinguishable from hyaluronic acid in substrate-attached material? Which class of components plays specific roles in affecting the kinetics of cell attachment to the substrate, the mobility of cells, and their effects on intercellular behavior (Culp, 1974)? Perhaps the *turning-over* material is biochemically identical with *accumulated* substrate-attached material but is labile because of deposition on the substrate at sites of new membrane synthesis and/or new adhesion foci which may be somewhat unstable. We might speculate that the turned over material is either reingested by cells, followed by catabolic breakdown, or labilized into the medium where much of the cellular glycoprotein appears to be secreted. Unfortunately, little is known about the composition of these medium-secreted glycoproteins.

The evidence in these studies has indicated remarkable similarity in the metabolic properties of substrate-attached material from cells with very different growth properties, for example, 3T3 cells, SV40-transformed 3T3 cells, and revertant variants of the transformed cells (Culp and Black, 1972a). Considerably more biochemical evidence on the nature of these components will be required before definitive roles can be made for them with the varying adhesiveness of normal and malignant cells.

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Synthesis of P^1-Dolichyl P^2-α-D-Mannopyranosyl Pyrophosphate. The Acid and Alkaline Hydrolysis of Polyisoprenyl α-D-Mannopyranosyl Mono- and Pyrophosphate Diesters†

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ABSTRACT: P^1-Dolichyl P^2-α-D-mannopyranosyl pyrophosphate (**9**) has been chemically synthesized by a method developed for the corresponding citronellyl derivative, which also contains a saturated α isoprene residue. In each case, the P^1-polyisoprenyl P^2-diphenyl pyrophosphate was treated with 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl phosphate to give a fully acetylated pyrophosphate diester, which was purified chromatographically and subsequently deacetylated. The citronellyl and dolichyl pyrophosphate diesters were compared with the previously synthesized citronellyl and dolichyl α-D-mannopyranosyl phosphate, respectively, by chromatography and by hydrolysis experiments. Good separations of the monophosphate from the corresponding pyrophosphate were achieved by silica gel tlc in a variety of solvent systems. Brief dilute acid hydrolysis of both the mono- and pyrophosphate diesters gave D-mannose and no α-D-mannosyl phosphate, the other products being polyprenyl phosphate and pyrophosphate, respectively. When the polyprenyl α-D-mannopyranosyl mono- and pyrophosphate diesters were treated with hot dilute alkali, the major products were polyprenyl phosphate and substances arising from the breakdown of D-mannose, indicating that the α-D-mannosyl phosphate bond was the most labile linkage in both compounds. However, the formation of a small proportion of free dolichol indicated that α-D-mannosyl phosphate was also formed to a minor extent. The interpretation of the results of the alkaline hydrolysis was complicated by the instability of D-mannose under basic conditions, it being almost completely degraded by even a brief treatment.

The recent demonstration that polyisoprenyl mannolipids are formed *in vitro* by enzymic systems obtained from mammalian and avian sources has prompted the study of the probable role of these compounds as intermediates in glycoprotein biosynthesis (Richards and Hemming, 1972; Baynes *et al.*, 1973; Waechter *et al.*, 1973). Partly on the basis of chromatographic and hydrolytic data, the compounds have been identified as phosphate diesters of D-mannose and of either dolichol or a very similar, long-chain isoprenol. In several instances, strong evidence for a monophosphate diester bridge was obtained from chromatographic comparison of the endogenous mannolipid phosphates with synthetic dolichyl α-D-mannopyranosyl phosphate (Warren and Jeanloz, 1973a,c). The compounds characterized in this way were formed by preparations from pig-liver endoplasmic reticulum (Evans and Hemming, 1973), calf pancreas (Tkacz *et al.*, 1974), human lymphocytes (Wedgwood *et al.*, 1974), and hen oviduct and bovine

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