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Phospholipid Composition of Substrate Adhesion Sites of Normal, Virus-Transformed, and Revertant Murine Cells†

Martha K. Cathcart and Lloyd A. Culp*

ABSTRACT: The phospholipid composition of cell–substratum adhesion sites, obtained after EGTA-mediated detachment of cells from the tissue-culture substratum, was determined for [^{32}P]orthophosphate radiolabeled Balb/c 3T3, SV40-transformed (SVT2), and concanavalin A selected revertant variant cell lines. All of the major phospholipid classes were found in the substrate-attached material, but there was an enrichment for specific phospholipid species in this adhesive material as compared to whole-cell and surface-enriched membranes. The phospholipid composition was remarkably similar for the whole-cell and surface-enriched membrane fractions from the three cell lines. However, pronounced differences in the phospholipid composition of the adhesion sites were observed as a result of viral transformation—SVT2 sites were clearly enriched in phosphatidylethanolamine and depleted in phosphatidylcholine when compared to 3T3 sites. This alteration in adhesion site phospholipids of transformed cells reverted to 3T3-like values in the adhesive material of revertant cells. The composition of adhesive material of newly attaching cells was also examined to differentiate compositional differences between "footpad" adhesion sites and "footprints", adhesive material pinched off from the posterior of cells as they move across the substratum. Pulse and pulse-chase analyses of the [^{32}P]phospholipids revealed some differences in synthesis and turnover rates in the three cell lines; in addition, altered rates of deposition of newly synthesized material into adhesion sites of transformed cells were observed. These data afford further evidence that the cell–substratum adhesion sites are highly specialized areas of the cell surface enriched in components which are intricately involved in the adhesive process. The transformation-dependent changes in adhesion site phospholipids may help to determine the basis for the altered adhesive properties of transformed cells.

Normal cells and oncogenic virus-transformed cells exhibit considerably different growth, morphologic, and motility properties in vitro. One basic alteration in transformed cells is that they are less adherent to their tissue-culture substratum than normal cells (Gail & Boone, 1972; Sanford et al., 1967; Shields & Pollock, 1974; Weber et al., 1977), a characteristic which may mimic their behavior during metastasis in vivo. The adhesive interaction between a cell and its tissue-culture substratum has been determined to involve discrete attachment points on the undersurface of the cell (Brunk et al., 1971; Abercrombie et al., 1971; Harris, 1973; Revel et al., 1974; Culp, 1975; Rosen & Culp, 1977). The interaction is mediated by one or more serum components absorbed to the substrate (Revel & Wolken, 1973; Grinnell, 1974; Culp & Buniel, 1976; Stamatoglou, 1977), but the serum components as well as the cellular components which participate in this adhesive interaction at present remain undefined. The determination of the molecular mechanism of the interaction is of great interest not only for understanding growth and movement of cells in culture but also for understanding the alterations which accompany malignant conversion.

Treatment of normal or SV40[1]-transformed murine cells, attached to their serum-coated tissue-culture substrates, with the Ca^{2+}-specific chelator EGTA results in cell rounding and detachment (with the aid of rotary movement and gentle pipetting) (Culp & Black, 1972b). Scanning electron microscopic studies have revealed that, as the cell rounds up and detaches under these conditions, it pinches off and leaves behind firmly adherent footpads which had mediated the cell–substratum adhesion (Rosen & Culp, 1977). Pinched-off

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[1] Abbreviations used: CL, cardiolipin; DiPI, diphosphatidylinositol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; $NaDodSO_4$, sodium dodecyl sulfate; PA, PC, PE, PG, PI, and PS represent respectively phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylserine; SM, sphingomyelin; SV40, Simian virus 40; SAM, substrate-attached material; REV, revertant cells; PBS, phosphate-buffered saline; PBS II, phosphate-buffered saline containing 100 mg/L each of $MgCl_2$ and $CaCl_2$.

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adhesion sites are also left behind at the cell posterior as a result of normal movement of the cell on the substratum as indicated by biochemical evidence (Culp, 1976b) and by microscopic techniques (Chen, 1977). Substrate-attached material, comprised of adhesion sites which are derived from a small fraction of the cell's undersurface (Culp & Black, 1972b; Rosen & Culp, 1977), allows the study of the composition and variation of the adhesion sites from a variety of normal and transformed cells, perhaps providing a means for determining the basis for adhesive differences between normal and malignant cells.

Biochemical characterization of the substrate-attached material has revealed that it contains several protein, glycoprotein, and glycosaminoglycan components (Terry & Culp, 1974; Culp, 1976a,b; Rollins & Culp, 1979) including: the LETS glycoprotein [*l*arge *e*xternal *t*ransformation *s*ensitive glycoprotein (Hynes, 1976), also commonly referred to as fibronectin (Keski-Oja et al., 1976)]; the microfilament-associated proteins—actin, myosin, and α-actinin, a protein which is probably the 10-nm diameter filament subunit protein (Brown et al., 1976); and the major glycosaminoglycan components—hyaluronic acid, heparan sulfate, and the chondroitins. These components are present in amounts different from those in whole cells and membranes and therefore represent a specialized portion of the cell surface, enriched in adhesive material (Culp, 1978).

The presence of intracellular microfilament components in this adhesion site material in addition to the scanning electron microscopic observations of adhesion site formation strongly implies that this piece of the cell surface includes a portion of the plasma membrane. These observations made it imperative to investigate the lipid components, especially the major mammalian membrane lipid class, phospholipids, in this adhesive material. Using normal, SV40-transformed, and revertant murine cells, we have conducted a study of the phospholipid composition of cells, enriched surface membrane preparations, and substrate-attached material under various attachment and growth conditions. This paper provides evidence that the phospholipid composition of the substrate adhesion site is considerably different from that of the whole-cell and surface membrane preparations and is enriched in certain phospholipid species. Furthermore, evidence is presented which indicates that the phospholipid composition of the adhesion site is altered as a result of viral transformation.

Materials and Methods

Cell Growth. Balb/c mouse 3T3 (clone A31), SV40-transformed Balb/c 3T3 (clone SVT2), and concanavalin A selected revertant variant cells of the SVT2 line (clone 84) have been described previously (Culp & Black, 1972a). The cells were maintained between their 8th and 20th passages and were cultured in Eagle's minimal essential medium containing a fourfold concentration of vitamins and amino acids (MEM × 4), supplemented with streptomycin (0.25 mg/mL), penicillin (25 units/mL), and 10% donor calf serum for incubation at 37° C in a humidified atmosphere of 5% CO_2–95% air. For experimental purposes, cells were routinely cultured in Lux plastic tissue-culture dishes. The cells were free of mycoplasma according to the radiolabeling assay of Culp & Black (1972b).

Cell Fractionation. Cell-associated, substrate-attached, and enriched surface membrane fractions were prepared in the following manner.

(*A*) *Cell.* Cells were gently rinsed three times with PBS and released from their tissue-culture substrate by incubation for 30 min on a gyratory shaker at 37 °C with EGTA (0.5 mM in PBS). The cell suspension was gently pipetted over the tissue-culture substrate surface to ensure release of all cells. The cells were centrifuged out of the EGTA solution and washed once with PBS for subsequent analysis.

(*B*) *Substrate-Attached Material.* The tissue-culture substrates, after quantitative cell release by EGTA treatment, were subjected to a rinse with PBS and two subsequent rinses with distilled water. The substrate-attached material was then quantitatively extracted (Cathcart & Culp, unpublished experiments) during a 30-min treatment with 0.2% sodium dodecyl sulfate (w/v in distilled water) during gyratory shaking at 37 °C.

(*C*) *Surface Membrane Enriched Material.* Surface membrane enriched fractions were prepared by two methods: a modification of the aqueous two-phase dextran–poly(ethylene glycol) method of Brunette & Till (1971) and the polylysine-coated bead method of Cohen et al. (1977). The modified two-phase polymer method has been described previously (Vessey & Culp, 1978). Briefly, cells were removed from their tissue-culture substrate with EGTA and washed once in PBS and twice in NaCl (0.85%) plus KCl (0.02%). Cells were then suspended in 10^{-3} M $ZnCl_2$, incubated 10 min, cooled in an ice bath, and broken in a Dounce homogenizer with a tight-fitting pestle (approximately 150–250 strokes, depending on the cell type). When 90% of the cells was disrupted, as determined by phase-contrast microscopic monitoring, the homogenization was terminated. The membranous material, along with other insoluble materials, was pelleted out of the suspension by centrifugation (2400*g* for 15 min), resuspended in the two-phase mixture of dextran-500 and poly(ethylene glycol), and subsequently subjected to centrifugation to separate the mixture back into two phases (the surface membrane "sheet" aligns at the interface between the two phases). The latter centrifugation was performed at 7445*g* for a period of 10 min. The two phases and the interface material were twice decanted into a clean tube, mixed well, and subjected to further centrifugation to prevent trapping of nonmembranous components at the interface. The final interface of surface membrane enriched material was collected and diluted with distilled water to form a one-phase solution from which it was spun out of suspension by centrifugation at 2400*g* for 20 min.

The polylysine bead method of obtaining an enriched cell membrane preparation, briefly, included the following steps. Polylysine was covalently coupled to Bio-Gel P-2 polyacrylamide beads according to the method of Cohen et al. (1977). Cells were grown as indicated above and, after EGTA-mediated detachment from the tissue-culture dishes, were washed extensively (five to seven times) in PBS at 4 °C and then twice in sucrose acetate buffer, pH 5.0 [buffer 1 of Cohen et al. (1977)], at 25 °C. The beads were washed five times in 0.15 M Tris, pH 7.4, at 4 °C and finally twice in buffer 1 at 25 °C. Each was then suspended to 50% v/v in buffer 1. The beads were added dropwise to the cells with gentle agitation, incubated for 10 min with several gentle inversions, and allowed to settle, and the supernatent was aspirated off and discarded. The cell-bead pellet was washed three times in this manner to remove unbound cells. The tube was vigorously vortexed, filled with 10 mM Tris, pH 7.4, at 4 °C (buffer 2), and washed four times by letting the beads settle out of suspension. After the last wash few or no intact cells remained bound to the beads as determined by phase-contrast microscopy. The membrane-coated beads were then suspended in an equal volume of ice-cold buffer 2, sonicated with a microtip at 20 W for 5 s, and washed three times in buffer 2 until all particulate matter was removed from the beads. The

bead-bound membranes were then subjected to phospholipid extraction with organic solvents or other analyses.

Radiolabeling Procedures. (*A*) *Phospholipid Radiolabeling.* To uniformly radiolabel phospholipids (long term), trypsinized cells (2×10^6 SVT2, 1.5×10^6 3T3, or 1.0×10^6 revertant cells) were inoculated into 100-mm plastic tissue-culture dishes containing medium. Six hours later the medium was changed to medium containing 50–100 μCi/mL of carrier-free [^{32}P]orthophosphate. The cells were then incubated an additional 48 h (until approximately 80% of the dish surface was covered with cells). For *pulse* and *pulse-chase* analyses, cells were inoculated as indicated above, allowed to grow to approximately 50% confluence, and then exposed to medium containing 100 μCi/mL of carrier-free [^{32}P]orthophosphate for 2 h. Samples were obtained for further analyses (pulse) or the cells were rinsed with PBS before refeeding with nonradioactive medium for an additional 24 h (pulse chase). Phospholipid analyses of *reattaching* cells and substrate-attached material involved the preparation of uniformly ^{32}P-radiolabeled cells as indicated above. Cells were detached by use of EGTA (as indicated above), pelleted by centrifugation, resuspended in labeling medium, and inoculated into fresh dishes for 2 h with medium containing [^{32}P]orthophosphate (100 μCi/mL) to study the formation of newly organized footpad adhesion sites.

(*B*) *Protein Radiolabeling.* Cell-surface proteins were radiolabeled by lactoperoxidase catalyzed [^{131}I]iodination by use of glucose oxidase as the hydrogen peroxide generator as modified from Schenkein et al. (1972). Subconfluent cells (in situ) were rinsed three times with PBS containing 100 mg/L each of $MgCl_2$ and $CaCl_2$ (PBS II) and then exposed to radiolabeling medium for 10 min. The radiolabeling medium contained lactoperoxidase (0.2 mg/mL), KI (10^{-6} M), Na^{131}I (100 μCi/mL, carrier free), glucose oxidase (0.1 units/mL), and glucose (5 mg/mL) in PBS II. Subsequent to the 10-min radiolabeling period the medium was removed and the cells were rinsed with PBS containing 10^{-3} M KI. Cell, substrate-attached material, and surface membrane enriched fractions were then obtained as described above for further analysis.

Phospholipid Extraction and Analysis. Lipids were extracted from whole-cell and surface membrane enriched fractions by use of a standard organic solvent system (Bligh & Dyer, 1959). The cell or membrane pellet was suspended in a small volume of an aqueous medium and extracted with a one-phase methanol–chloroform–distilled H_2O (2:1:0.8) solvent system for 1 h with intermittent shaking. The insoluble material was pelleted by centrifugation and the supernatant fraction was set aside for lipid analysis. The pellet was then reextracted in the same manner. The two supernatant fractions were pooled, and one volume each of chloroform and distilled water was added. The mixture was centrifuged to aid in separation of the two phases. The lower chloroform phase was withdrawn and brought to dryness under a stream of nitrogen. The residue was immediately dissolved in chloroform and brought to a known small volume. Approximately 99% of the ^{32}P radioactivity in this phase was present in phospholipids as determined by chromatography on silica gel 60.

Extraction of substrate-attached material samples was carried out in a similar manner; however, these samples were extracted only once since the sample was completely soluble with no particulate matter for reextraction. Due to the large volume of the lower chloroform phase of some of these samples, evaporation was performed on a rotoevaporator, breaking the vacuum with nitrogen gas. The sample was then dissolved in 1–5 mL of chloroform, brought to dryness under a stream of nitrogen gas and then redissolved in a small aliquot of chloroform (0.1 mL or less). For compositional analysis of the specific phospholipids in the various fractions, aliquots were applied to EM brand precoated thin-layer plates with silica gel 60 and developed according to the two-dimensional chromatographic method of Broekhuyse (1969), in which the solvent system in the first dimension was chloroform–methanol–7 M ammonia (90:54:11 v/v) and in the second dimension was chloroform–methanol–acetic acid–distilled water (90:40:12:2 v/v). The plates with [^{32}P]phospholipid were autoradiographed by use of RP XOMAT film which was developed in an automatic processor in the Radiology Department of Case Western Reserve University. Radioactive spots were located on the plates, and the gel was scraped into scintillation vials in order of increasing radioactivity for determination of radioactive content. Phospholipid components were identified by cochromatography with standards (as detected by iodine staining) and by their reactivity to chemical detection spray reagents such as ninhydrin, Dragendorff, and Periodate–Schiff. $NaDodSO_4$ was shown to have minimal effects on lipid extraction procedures or phospholipid separation protocols.

Materials were purchased from the following sources: ^{131}I-labeled sodium iodide, E. R. Squibb and Sons, Inc.; [^{32}P]orthophosphate (carrier free), ICN Pharmaceuticals, Inc.; EGTA from Eastman Organic Chemicals; MEM × 4 from Grand Island Biologicals Co.; 32-oz Brockway glass prescription bottles, Brockway Glass, Inc.; plastic petri dishes, Lux Scientific Corp.; donor calf serum from Kansas City Biologicals Inc.; sodium dodecyl sulfate from Bio-Rad Laboratories; precoated thin-layer plates and silica gel 60 (EM brand) from Brinkmann Inc.; lactoperoxidase B grade lyophilized, Calbiochem Corp.; glucose oxidase from Boehringer Mannheim, Inc.; Bio-Gel P-2 (200–400 mesh) polyacrylamide beads from Bio-Rad Laboratories; poly-L-lysine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride from Sigma Chemical Co.; dextran 500, Pharmacia Fine Chemicals; Carbowax 6000 [poly(ethylene glycol)] from Union Carbide Corp.; Royal RP X-omat medical X-ray film from Eastman Kodak Co.; and phospholipid standards PS, PC, LPC, PE, and SM from Applied Sciences Laboratories and PS, PG, PI, LPE, PA, CL from Serdary Research Laboratories.

Results

Phosphate Incorporation into Cell and Substrate-Attached Fraction. Analyses of the cell fractions of the three cell lines indicated that approximately 30–40% (as determined by the chloroform solubility of phospholipid) of the total incorporated [^{32}P]orthophosphate appeared in phospholipid. On the other hand, in the substrate-attached fractions 70–80% of the ^{32}P-radiolabeled material was present in phospholipid. This variation in phospholipid content between the cell and substrate-attached fractions can be explained by the presence of large quantities of nucleic acid and phosphorylated proteins in the cell which of course are depleted in substrate-attached material (Culp & Black, 1972a; Culp, 1978). By the same criterion, it was determined that the phospholipid content of substrate-attached material accounts for 2.1% of total cellular phospholipid in 3T3 cells and 1.1% and 0.8% in SVT2 and revertant cell lines, respectively. These values are comparable to the protein content of this material.

It has previously been shown that the proteins and polysaccharides in the substrate-attached material are deposited as a result of direct cell–substrate contact and not artifactual binding of medium-secreted or EGTA-solubilized material to

the serum layer (Culp et al., 1975; Culp, 1975; Rosen & Culp, 1977; Culp, 1978). It was necessary to ensure that the phospholipids obtained in substrate-attached material are also actively deposited by the cells as adhesive material and not derived from absorption of phospholipids exuded by the cells into the medium or solubilization during EGTA treatment of cells with subsequent rebinding to the serum-coated substratum. To rule out these possibilities and to demonstrate that substrate-attached phospholipids are deposited directly by the cell, several experiments were performed. First, medium containing $^{32}PO_4^{3-}$ which had been used to radiolabel cells for 48 h was decanted into a fresh dish containing an absorbed serum layer with or without nonradiolabeled substrate-attached material. After a 1-h incubation period at 37 °C, the medium was withdrawn, and the substratum was treated with EGTA (as described under Materials and Methods) and $NaDodSO_4$ extraction to remove any material on the plastic tissue-culture dish. The amount of ^{32}P-radiolabeled phospholipid that bound to the substratum in these experiments was approximately 4% of the amount detected in the ^{32}P-radiolabeled substrate-attached material layer from the cultures used to obtain the conditioned medium. This 4% binding was observed on both the serum-absorbed and substrate-attached material-coated substrates.

Secondly, ^{32}P-radiolabeled cells were scraped from their original tissue-culture substrates with a rubber policeman and suspended in PBS in fresh dishes with nonradiolabeled substrate-attached material or serum-absorbed layers; the PBS suspension was then brought to 0.5 mM in EGTA and incubated on a rotary shaker for 30 min. The cells were gently pipetted and rinsed off the dish, and the dish-bound material was extracted with $NaDodSO_4$. The results of this experiment revealed that no more than 5–9% of the total [^{32}P]phospholipid in substrate-attached material could be due to rebinding of EGTA-soluble material, although some of the low level of radioactivity in this experiment resulted from transient adhesions formed between the cell and substratum, which were broken off and remained substrate bound (unpublished experiments).

In addition to the two control experiments just described, autoradiography experiments similar to those of Culp (1975) were performed to relate the location of substrate-attached material to the location of cells. Tissue-culture dishes with ^{32}P-radiolabeled cells or substrate-attached material were rinsed with PBS, dried at 60 °C, layered with stripping film, exposed for varying lengths of time, and then developed. The autoradiographs indicate that the [^{32}P]phosphorus incorporated into substrate-attached material is present on the substratum in a punctate pattern from which cell patterns and cell shapes can be discerned and is not present as a uniform layer which covers the whole substratum (data not shown). All of these studies indicate that 95% or more of the ^{32}P-radiolabeled phospholipids in substrate-attached material is actively deposited by the cells by a contact-mediated process and not as a result of rebinding of exuded phospholipids to the serum-absorbed or substrate-attached material layer.

Phospholipids in 3T3 and SVT2 Cell and Substrate-Attached Fractions. We examined the distribution of the various phospholipid species in the EGTA-released 3T3 and SVT2 cells. Inorganic [^{32}P]orthophosphate incorporation into cellular phospholipid achieves a steady-state level within 24 h of exponential growth; therefore compositional analyses were performed after a 48-h radiolabeling period to ensure uniform radiolabeling. The results obtained in the compositional analyses of cell fractions were very reproducible as indicated by the low standard deviations in Table I. The substrate-attached fractions displayed greater variability due to the smaller amounts of radiolabeled phospholipid in this fraction. The distribution among the different phospholipid species in cell fractions was found to be very similar for the 3T3 and SVT2 cell lines. The major phospholipid species in each of these cell fractions was PC, followed by PE and PI. Although some differences were observed, the general distribution was very similar.

Table I: Phospholipid Distribution in Cell and Substrate-Attached Fractions[a]

fraction	phospholipid component	percentage of total phospholipid: 3T3 cell line	SVT2 cell line	REV cell line
cell	origin[b]	0.2 (±0.1)[c]	0.1 (±0.1)	0.2 (±0.2)
	LPC	1.2 (±0.1)	0.5 (±0.3)	0.2 (±0.1)
	SM	3.7 (±0.1)	4.5 (±0.8)	5.8 (±1.4)
	PC	61.2 (±0.8)	54.6 (±2.2)	56.0 (±4.5)
	LPE	0.3 (±0.1)	0.3 (±0.2)	0.3 (±0.2)
	PI	8.0 (±0.2)	9.0 (±1.0)	11.8 (±0.6)
	PS	3.3 (±0.3)	6.0 (±0.6)	4.6 (±0.5)
	PA	0.5 (±0.2)	0.3 (±0.1)	0.4 (±0.1)
	PE	15.9 (±0.8)	20.4 (±1.2)	16.2 (±2.9)
	PG	0.6 (±0.2)	0[d]	0.9 (±0.4)
	CL	2.8 (±0.3)	3.4 (±0.1)	2.9 (±0.4)
substrate-attached	origin	0.4 (±0.2)	0.8 (±0.4)	0.3 (±0.1)
	LPC	0.6 (±0.3)	0.4 (±0.2)	1.8 (±0.7)
	SM	2.4 (±0.3)	3.2 (±0.2)	7.7 (±4.0)
	PC	37.4 (±0.2)	26.9 (±2.3)	38.4 (±1.3)
	LPE	2.4 (±0.5)	3.1 (±1.7)	2.4 (±0.3)
	PI	3.0 (±0.5)	6.5 (±1.5)	9.5 (±1.7)
	PS	17.9 (±1.8)	14.0 (±4.0)	7.9 (±2.4)
	PA	0.5 (±0.1)	1.1 (±0.3)	1.7 (±1.3)
	PE	28.6 (±5.6)	35.0 (±3.2)	28.8 (±0.9)
	PG	0.8 (±0.5)	0	0.8 (±0.5)
	CL	5.4 (±3.0)	8.8 (±1.9)	2.8 (±1.8)

[a] Cells were radiolabeled for 48 h in medium containing [^{32}P]-orthophosphate (100 μCi/mL). The phospholipids were extracted and analyzed from cell and substrate-attached fractions as described under Materials and Methods. Data are presented as percentage of total phospholipid. [b] Less than 1% and usually less than 0.3% of the total radiolabeled material remained at the origin. [c] The numbers in parentheses represent the standard deviation of the data from a mean derived from at least four experiments. [d] Zero values indicate that the component was not detected.

The phospholipid distribution of the substrate-attached fractions of these two cell lines was also investigated (Table I). The two predominant phospholipid species in substrate-attached material were PC and PE, as they were in the cell fractions. Both 3T3 and SVT2 substrate-attached fractions possessed a lower relative amount of PC and a higher percentage of PE than their corresponding cell fraction, and the third major component in these fractions was PS, not PI as it was in the cell phospholipids. In addition there was a marked difference between 3T3 and SVT2 substrate-attached material: the major phospholipid species in 3T3 substrate-attached material was PC (3T3 PC/PE ratio = 1.31), whereas the predominant phospholipid in SVT2 substrate-attached material was PE, not PC (SVT2 PC/PE ratio = 0.77). No PG was found in either the cell or substrate-attached fractions from the SVT2 cell line. The relative amounts of PE and PS were significantly increased and the relative amount of PC was decreased in the substrate-attached fractions as compared to the cell fractions.

Phospholipids in Revertant Cell and Substrate-Attached Fractions. Revertant variant cells have reattained several properties characteristic of normal cells, including greater adhesivity to the tissue-culture substratum (Culp, 1974; Culp & Buniel, 1976). It was necessary to investigate this cell line

Table II: Phospholipid Distribution in Enriched Surface Membrane[a]

phospholipid component	percentage of total phospholipid		
	3T3 cell line	SVT2 cell line	REV cell line
LPC	1.0	0.5 (2.6)[b]	0.8
SM	5.8	6.5 (8.6)	4.4
PC	56.7	48.0 (43.4)	57.8
LPE	0.4	0.4 (1.8)	0.4
PI	8.5	9.2 (7.5)	12.2
PS	4.1	6.4 (11.6)	4.4
PA	0.1	0.1 (0.1)	0.4
PE	20.2	24.0 (21.8)	14.5
PG	0.2	0.4 (0.4)	0.7
CL	2.6	4.2 (1.6)	3.2

[a] Cells were grown as described in Table I, and surface-enriched membrane fractions were prepared according to the modified method of Brunette & Till (1971) as presented under Materials and Methods. [b] Numbers in parentheses represent the percentage of total phospholipid in each phospholipid component from surface-enriched membranes prepared according to the method of Cohen et al. (1977) as outlined under Materials and Methods.

to determine if the observed alteration in phospholipid distribution between 3T3 and SVT2 substrate-attached fractions correlated with the transformed phenotype. Although the phospholipid composition of the revertant cell fraction was very similar to that of 3T3 or SVT2 cells (Table I), the phospholipid composition of the revertant substrate-attached material did not resemble SVT2 substrate-attached material but more closely resembled the substrate-attached fraction from the 3T3 cell line. The relative percentage composition of the major phospholipid components in this adhesion site enriched material has therefore "reverted" along with phenotypic reversion, although this reversion is not observed in every phospholipid species.

Comparison of Phospholipids in Surface Membrane and Substrate-Attached Fractions. It is clear from the data of Table I that the phospholipid distribution in the substrate-attached fractions is considerably different from that of the cell fractions. Comparatively, the substrate-attached material has a smaller relative amount of PC and PI and an increased proportion of PE, PS, and LPE. To examine the cellular origin of this enrichment for different proportions of phospholipids in substrate-attached fractions, we isolated surface membrane enriched fractions from the three cell lines to determine if the enrichment for different phospholipid species in the substrate-attached material was due to the possibility that substrate-attached material contains highly purified plasma membrane as opposed to whole-cell membranes (plasma membrane plus internal endoplasmic reticular and organelle membranes). Both surface membrane enrichment techniques described under the Materials and Methods section were used to isolate SVT2 surface membranes. The two-phase polymer gradient technique, when performed on these cell lines, has been shown to yield a membrane fraction significantly enriched in surface membranes (Vessey, 1975). Attempts to characterize the degree of surface enrichment in the membrane fraction prepared by the method of Cohen et al. (1977) were not entirely satisfactory; surface/total protein ratios (^{131}I/[^{3}H]leucine) indicated only a two- to threefold enrichment for surface protein in membranes prepared by this method as compared to a minimum fivefold enrichment observed for the Brunette and Till method. There also appeared to be binding of [^{3}H]uridine-radiolabeled RNA to the beads which is probably related to the binding of cellular DNA to the polylysine coating as observed by Cohen et al. (1977). The

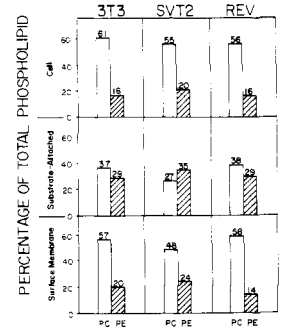

FIGURE 1: PC and PE distribution in various culture fractions. Phospholipid distributions in PC (plain bars) and PE (crosshatched bars) from cell, substrate-attached, and surface membrane enriched fractions of 3T3, SVT2, and revertant cell lines were calculated as percentage of total phospholipid and compared between the three cell lines and the three fractions (data from Tables I and II).

phospholipid class distribution in the two membrane preparations (Table II) was remarkably similar, yet, because of the previous extensive characterization of the Brunette and Till method for these cell lines (Vessey & Culp, 1978), this method was used to prepare surface membrane enriched fractions of the other two cell lines.

Upon examination of the phospholipid composition of the surface membrane enriched fractions in Table II, a closer resemblance to whole-cell than substrate-attached compositions is observed. This can be seen more readily in Figure 1 when comparison of the two major phospholipid components PC and PE is made between the various cell, substrate-attached, and surface membrane enriched fractions. This figure emphasizes the similarity of the cell and surface membrane enriched fractions of the three cell lines and in addition compares them to the compositionally different substrate-attached fractions. It also illustrates the pronounced PC/PE change with transformation in the substrate-attached fractions. These data suggest that substrate-attached material contains a distribution of phospholipid species that is distinctly different from the remainder of the surface membrane.

Substrate-Attached Fractions from Reattaching Cells. Subconfluent fibroblasts move around considerably on their tissue-culture substratum. In doing so they leave behind a trail of adhesion sites or "footprints" (Culp, 1976b; Chen, 1977). In addition to these abandoned adhesion sites, EGTA-mediated release of cells from their substratum causes the cell to pinch off "footpads" of active adhesion sites (Rosen & Culp, 1977). *Long-term* radiolabeled substrate-attached material then consists of a mixture of both footpad and footprint adhesive material (active and abandoned adhesion sites).

To take a direct look at adhesion sites which are newly formed and actively involved in adhesion at the time of EGTA-mediated cell release (footpads), the following experiment was performed. After EGTA-mediated detachment, uniformly ^{32}P-radiolabeled cells were reattached to fresh dishes in medium containing [^{32}P]orthophosphate for 2 h, and then cell and substrate-attached fractions were isolated. Phospholipid analysis of this material (Figure 2) revealed that the composition of the reattaching cell fraction was almost identical with that of long-term radiolabeled cells. This result would be expected because of the uniform radiolabeling of phospholipid in both of these experiments. The newly attaching footpad material, however, differed considerably from long-term radiolabeled substrate-attached material. Newly formed footpads (Table III) were relatively enriched in PC, SM, and LPC and exhibited a smaller relative amount of PE and PS. In comparing 3T3 to SVT2 *reattaching* (footpad)

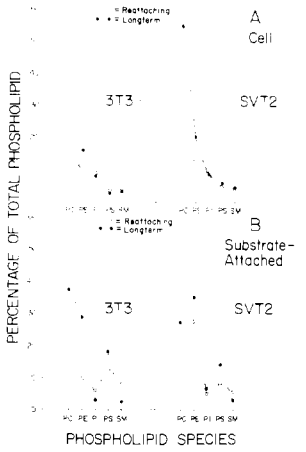

FIGURE 2: Reattaching vs. long-term radiolabeled cell and substrate-attached material. 3T3 or SVT2 cells were radiolabeled for 48 h with medium containing [^{32}P]orthophosphate (100 μCi/mL) (at this time approximately 80% of the tissue-culture surface was covered with cells). They were detached with EGTA, pelleted out of suspension by centrifugation, resuspended in radiolabeling medium (same as above), and replated in fresh dishes for 2 h in the same medium. After this 2-h attachment period, cell and substrate-attached fractions were prepared according to the procedures under Materials and Methods. The phospholipids were extracted and analyzed as described previously. Reattaching cell and substrate-attached fractions (○); long-term cell and substrate-attached fractions (●).

Table III: Phospholipid Distribution in Reattaching Cell and Substrate-Attached Fractions[a]

fraction	phospholipid components	percentage of total phospholipid: 3T3 cell line	percentage of total phospholipid: SVT2 cell line
cell	LPC	0.3	0.2
	SM	3.9	4.7
	PC	60.2	56.8
	LPE	0.1	0.2
	PI	8.8	8.9
	PS	3.7	5.2
	PA	0.1	0.3
	PE	18.7	19.0
	PG	0.2	0.4
	CL	2.4	3.4
substrate-attached	LPC	1.2	1.0
	SM	7.8	5.9
	PC	60.4	40.6
	LPE	0[b]	2.1
	PI	6.0	5.0
	PS	12.8	8.4
	PA	0.4	0.6
	PE	9.9	27.6
	PG	0.9	0.4
	CL	2.1	7.8

[a] 3T3 or SVT2 cells were grown for 48 h in medium containing [^{32}P] orthophosphate (100 μCi/mL), detached with EGTA, washed, and plated on fresh dishes in radiolabeling medium for 2 h. At this point cell and substrate-attached fractions were obtained, extracted, and analyzed for [^{32}P] phospholipid composition. These numbers represent the mean from four separate experiments, all of which yielded similar results. The SAM/cell ratio under these conditions was 0.033. [b] Zero indicates that the component was present in concentrations below the detectable level.

substrate-attached material (Table III), the 3T3 material had a much higher relative amount of PC and PS and a much lower amount of PE and CL than the SVT2 material and in this respect displays the differences observed in the long-term radiolabeled comparison of 3T3 and SVT2 substrate-attached fractions (Table I). Comparisons between the substrate-attached and cell fractions from *reattachment* experiments

Table IV: Phospholipid Composition of 3T3 and SVT2 Fractions with Pulse and Pulse-Chase Radiolabeling Conditions[a]

	pulse		pulse chase	
	3T3	SVT2	3T3	SVT2
cells				
DiPI	0.2	2.5	0[b]	0
LPC	0.2	0.1	0.3	0.2
SM	0.3	0.4	5.4	6.7
PC	32.2	24.6	58.1	54.9
LPE	0.4	0.4	0.1	0.1
PI	41.2	42.6	4.5	8.0
PS	0	0	7.8	7.2
PA	2.7	3.7	0.1	0.1
PE	18.8	20.8	19.9	18.6
PG	1.4	2.0	0.3	0.4
CL	1.8	3.7	2.6	4.2
substrate-attached				
DiPI	0	1.3	0	0
LPC	1.6	1.5	0.5	0.4
SM	1.1	4.4	3.1	3.1
PC	29.6	39.3	39.1	42.4
LPE	1.8	2.1	0.9	1.6
PI	30.0	23.9	7.6	5.3
PS	0	0	16.6	12.8
PA	3.4	2.8	1.0	1.7
PE	22.7	21.0	17.9	23.2
PG	3.2	0	1.8	2.5
CL	6.6	3.1	10.3	6.8

[a] 3T3 or SVT2 cells which covered approximately 50% of the tissue-culture substratum were exposed for 2 h to medium with [^{32}P] orthophosphate (100 μCi/mL), and the cell and substrate-attached fractions were prepared according to Materials and Methods (pulse) or the medium was changed to nonradioactive medium for 24 h and then the fractions were isolated (pulse chase). The phospholipids of all fractions were extracted and analyzed as described under Materials and Methods. The numbers represent the mean value from three or four separate experiments, all with similar results. The SAM/cell ratio of [^{32}P] phospholipids under pulse conditions was 0.003 and under pulse-chase conditions was 0.006. [b] Zero values indicate that the component was not detected.

revealed that, as in long-term radiolabeling conditions, a greater proportion of the cell phospholipid was in PI than PS, whereas the opposite was true in the substrate-attached fractions (Table III). The relative decrease in PC and increase in PE from cell to substrate-attached fractions, as seen in long-term radiolabeled fractions of all three cell lines, was observed only in the SVT2 cell line under reattaching radiolabeling conditions. But these changes were not as pronounced as in long-term radiolabeled studies. In comparing the phospholipid profiles in Tables I, II, and III it is interesting to note the similarity in composition between 3T3 and SVT2 footpad material with their corresponding long-term radiolabeled surface membrane and whole-cell preparations.

Phospholipid Metabolic Studies. In order to investigate the synthesis and turnover of phospholipids in this adhesive material, as well as to monitor the accumulation of phospholipid on the substratum, the following studies were performed.

(*A*) *Pulse and Pulse-Chase Analyses. Pulse* and *pulse-chase* analyses of the cell and substrate-attached fractions of the 3T3 and SVT2 cell lines were conducted to follow the rate of synthesis of the various phospholipid species in cells, the rate of deposition of the newly synthesized phospholipid species into the substrate-attached fraction, and the turnover characteristics of cell and substrate-attached phospholipids. Some of the results of these studies are given in Table IV. The cell phospholipid distribution after a 2-h pulse with [^{32}P]orthophosphate radiolabeling indicated that PI, PC, and PE (in this

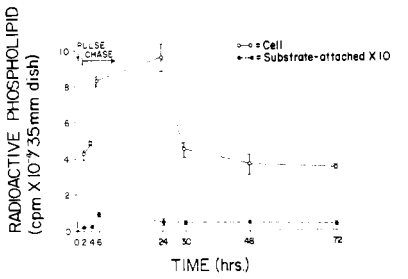

FIGURE 3: Turnover of cell and substrate-attached phospholipids. SVT2 cells were pulsed for 2 h in medium containing [^{32}P]orthophosphate (50 μCi/mL), the medium was then changed to nonradioactive medium, and the cells continued to grow up to 72 h. Three 35-mm dishes were treated with EGTA, and cell and substrate-attached fractions were isolated at various times after the beginning of the pulse. Phospholipids were extracted and the amount of radiolabeled material was determined for each fraction from each dish. Three values were averaged to give each point on the graph the indicated error bars. Cell (○); substrate-attached × 10 (●).

relative order) are the most rapidly synthesized components and together comprise around 90% of the newly synthesized radiolabeled phospholipid. It is also clear that PS has a very slow synthesis as it cannot be detected at 2 h. Pulse-chase analyses of the cell fraction revealed that the relative amounts of PE and PC become much greater than that of PI, perhaps indicating that PI has a short half-life relative to the other phospholipid classes.

Pulse analyses of substrate-attached phospholipids most likely provide information on the rate of deposition of newly synthesized phospholipid as well as rates of synthesis. After a 2-h pulse, the substrate-attached fractions of both 3T3 and SVT2 cell lines also contained primarily three radiolabeled phospholipid species—PI, PC, and PE—which differ in relative distribution from the cell fractions. Newly synthesized SM and PC appear to be deposited to a greater degree in SVT2 than 3T3 substrate adhesion sites. Of all the radiolabeling conditions studied diphosphatidylinositol was detectable only under pulse radiolabeling conditions and is most likely a short-lived intermediate. Diphosphatidylinositol, PC, and sphingomyelin were deposited to a greater degree and CL, PI, and PG to a lesser degree in SVT2 than in 3T3 substrate adhesion sites. Pulse-chase studies of the substrate-attached material reflect similar metabolic changes observed in the cell fraction, i.e., high turnover of PI and the relative stability of PS, SM, PC, and PE.

Studies were also performed to follow total phospholipid radioactivity with time after a 2-h pulse. The [^{32}P]phosphorus incorporated into cellular phospholipid after a 2-h pulse increased until 24 h (Figure 3), indicating fairly large precursor pools and, as indicated below, slow catabolic rates for the major components. The substrate-attached fraction showed accumulation of ^{32}P-radiolabeled phospholipid only up to 6 h and remained very stable after 24 h.

To examine the metabolism of PE and PC specifically during pulse-chase experiments on a per-cell basis, analyses of the radiolabeled phospholipid species were performed on cell and substrate-attached fractions of SVT2 cells after a 2-h pulse and at appropriate intervals thereafter. Figure 4A demonstrates their relative percentage distribution; the actual amounts of radioactive [^{32}P]phospholipid in a 35-mm plastic petri dish are given in Figure 4B. These data reveal that the relative percentage composition of PC and PE and their amounts of radioactivity per 35-mm dish in the substrate-attached fraction are fairly stable with time. In the cell fraction, although the PE is fairly stable in distribution by relative percentage and amount of radioactivity per dish, the

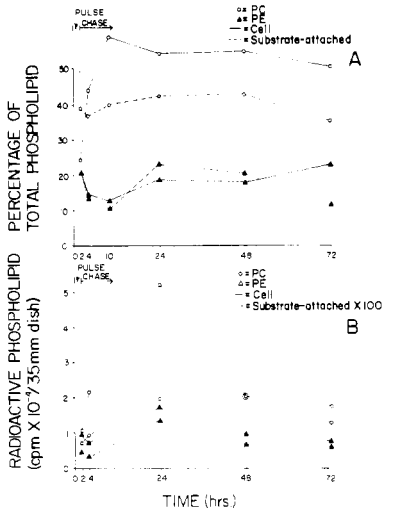

FIGURE 4: Turnover of PC and PE in cell and substrate-attached fractions. SVT2 cells were grown as described in Figure 3 but with 100 μCi/mL of [^{32}P]orthophosphate. Three 10-cm dishes were randomly selected for EGTA treatment and cell and substrate-attached fraction preparation at each time point. Phospholipids were extracted and analyzed according to the Materials and Methods section. The data points at 2, 24, and 48 h are an average of four separate experiments, whereas those at 4, 10 [(A) only], and 72 represent an average of two experiments. (○) PC; (▲) PE; (—) cell; (---) substrate-attached.

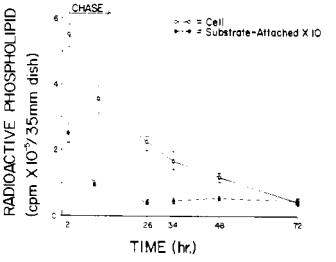

FIGURE 5: Turnover of newly attached cell and substrate-attached phospholipids. SVT2 cells were grown for 48 h in 35-mm plastic dishes in the presence of 50 μCi/mL of [^{32}P]orthophosphate, after which cells were detached with EGTA, suspended in medium containing [^{32}P]orthophosphate, and reattached to fresh dishes in the presence of medium containing [^{32}P]orthophosphate. After reattaching for 2 h the labeling medium was withdrawn, the plates were washed three times with PBS, and nonradioactive medium was added to the dishes. Cell or substrate-attached fractions were prepared from three 35-mm dishes (chosen at random) according to the Materials and Methods section at each time point. The error bars illustrate the variation in data among the three sample plates. (○) Cell; (●) substrate-attached.

PC varies markedly, climbing to almost threefold its relative percentage from 2 to 10 h and rising fivefold in actual counts per minute per dish from 2 to 24 h. It then drops back by 48 h to twice the amount of radioactivity incorporated at the end of the pulse. This demonstrates that the increasing relative percentage of PC as seen in the cell fraction in Figure 4A was due to an actual increase in the amount of PC and not merely due to a decrease in the actual amounts of the other components (data not shown for components other than PE and PC). After dropping down to the 48-h level the cellular PC achieves a steady level along with other components. In addition, these data, together with those of Table IV, establish that PI has a much higher turnover rate in both fractions than do the other classes of phospholipid.

Reattachment Chase. The fate of radiolabeled material after ^{32}P-radiolabeled cells were reattached in [^{32}P]orthophosphate-containing medium for 2 h was also studied. These experiments were modeled after the reattachment experiments reported above. Figure 5 follows the radiolabeled phospholipid

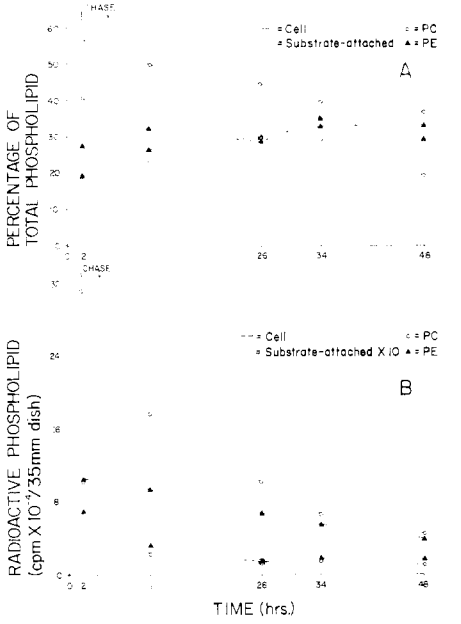

FIGURE 6: Turnover of PC and PE in reattached cell and substrate-attached fractions. SVT2 cells were grown as described in Figure 5 but with 100 μCi/mL of [^{32}P]orthophosphate. At various time points after replating, three 10-cm dishes were selected at random and treated with EGTA and then $NaDodSO_4$ for cell and substrate-attached fraction preparation. Phospholipids were extracted and analyzed according to the Materials and Methods section. The data at 2, 26, and 48 h represent an average of four separate experiments. The data points at 11 and 34 h are from two separate experiments. (○) PC; (▲) PE; (—) cell; (- - -) substrate-attached.

in cell and substrate-attached fractions during a nonradiolabeled medium chase which followed a 2-h reattachment of ^{32}P-radiolabeled cells in radiolabeling medium. These cells are synchronized to some extent in that they attach simultaneously 2 h preceding the chase, and the cells require a considerable adjustment period before dividing (Culp & Rosen, 1977). At 24 h after initiation of the chase, the total radioactive phospholipid content in the cell fraction was still decreasing, whereas the substrate-attached phospholipid had stabilized with minimal turnover. This may be related to cells walking out of their footpads and converting them to apparently nonlabile footprints. Figure 6 was constructed to allow the comparison of the fate of the two major phospholipid components during the chasing period. The cell fraction displayed a percentage decrease in radiolabeled PC and increase in PE while the substrate-attached fraction was comprised of a decreasing relative percentage of PC and a fairly stable amount of PE, verifying that PC catabolism does not generate PE. Figure 6B reveals that the decrease of PC and increase in PE in the percentage composition of the cell fraction were due to the more rapid turnover of PC relative to PE while both exhibited turnover to some extent. The substrate-attached fraction had a slightly higher turnover of PC than PE. Since PE was fairly stable in its contribution to the percentage composition of substrate-attached material, the rate of turnover of the other phospholipids as a whole must parallel it. The slightly greater PC turnover was enough to alter its percentage composition relative to the other phospholipids.

Discussion

It has been recognized for quite some time that phospholipids play some role in the process of cellular adhesion. Yet, it is difficult to distinguish between an active and passive participation in adhesion when the component under study comprises the fluid matrix mediating the organization and behavior of other surface components. It is quite possible that lipids serve merely a passive function by anchoring adhesive glycoproteins and proteoglycans, which could explain the temperature sensitivity of the adhesive process and stringent requirements for membrane fluidity (Mapstone & Culp, 1976; Pessac et al., 1977; Nath & Srere, 1977; Juliano & Gagalang, 1977) as well as the inhibitory effect of phospholipase A_2 on cellular adhesion (Curtis et al., 1975a,b). However, experiments such as those of Huang (1977), which demonstrate the agglutination of phospholipid particles in the presence of 10% serum and loss of the ability to agglutinate without serum, indicate that some adhesive interactions may involve cellular lipids alone (with serum components mediating the interaction) and that membrane interaction may operate by different mechanisms depending on their composition.

In this study, the phospholipid composition of the adhesion sites of normal and transformed cells was determined. The long-term radiolabeling studies (Table I) indicate that substrate-attached material—the material which mediates cell–substrate adhesion—possesses a very different phospholipid composition from that of the whole cell. Adhesion sites are greatly enriched in PE, PS, and LPE and depleted in PC and PI as determined by compositional analyses. Studies of the phospholipid composition of albeit impure but enriched fractions of surface membrane indicate that the unique composition of substrate-attached material does not appear to be due to the selection of surface-membrane phospholipid in substrate-attached material (Tables I and II and Figure 1). The results indicate that the cell–substratum adhesion site has a phospholipid composition different from the whole-cell or whole-surface membrane with selective enrichment for certain classes. There must then be specific interactions involving these phospholipids in the adhesion site either through direct involvement in the adhesion interactions or due to association with other components such as glycoproteins or proteoglycans which are essential for the adhesive interaction.

The radiolabeled phospholipids in substrate-attached fractions from long-term (48-h) cultures were found to be very different from those of newly attaching ones (2 h) although both studies involved cells which had uniformly radiolabeled phospholipids and the two cell fractions yielded identical phospholipid compositions (Tables I and III and Figure 2). The reasons for such differences could be the following: (1) that reattaching substrate-attached material is composed entirely of footpad material, whereas long-term substrate-attached material is made up of footpads plus footprints (see Results); or (2) that newly reattaching footpads are different from those of well-spread and established cells. These studies do not resolve these possibilities; however, our observations, along with others discussed below, appear to support the latter explanation.

The difference between reattaching substrate-attached material and well-established substrate-attached material does not seem to be due to differences in the phospholipid composition of footpad and footprint material. This conclusion is drawn as a result of the fact that during a 24-h period fibroblasts move more than one cell diameter away from their original locations (Gail & Boone, 1972). Analysis of the phospholipids in substrate adhesion sites during a 24-h chasing period revealed that most of the components, following an initial drop, were very stable (in comparison to cell fractions), both in actual counts per minute and percentage distribution (Figures 3–6). The stability of phospholipid components in this footprint material makes it difficult to believe that

footprints in well-established cultures account for the observed variation in relative PE and PC percentage from reattaching substrate-attached material.

The strong resemblance between the phospholipid distribution in reattaching substrate-attached material and long-term radiolabeled surface membranes is interesting when considered in light of unpublished data indicating that the composition of substrate-attached material after rubber policeman scraped detachment of cells is also similar to that of surface membrane. Therefore, EGTA treatment leaves a very select portion of surface membrane on the substrate, specifically that tightly associated with adhesive material (M. K. Cathcart and L. A. Culp, unpublished experiments). In addition, scraping cells from the substratum after long-term growth leaves approximately three times more substrate-attached material and EGTA-mediated removal of reattaching cells leaves four times more substrate-attached material behind than EGTA-mediated detachment of established cells. The greater amount of material left behind by reattaching cells or scraped cells seems to mask the difference between whole-cell surface membrane and adhesion site phospholipids. It appears then that reattaching cells have different adhesion site organization than well-spread established cells since they leave behind much more material upon EGTA-mediated cell detachment. Other investigators have reported that stabilization of adhesion follows the initial contact process (Grinnell, 1974; Pagano & Takeichi, 1977). Further evidence supporting an organizational maturation in the adhesion site is that newly attaching footpads are not as highly spread or bifurcated as footpads from established cells (Rosen & Culp, 1977). Also, newly attached cells are much more resistant to EGTA-mediated cell detachment than are cells which have been in culture for longer periods of time. Furthermore, cells are constantly moving about on the substratum, leaving a trail of pinched-off footprints (Chen, 1977; Culp, 1978); presumably there is some modification at the cell–footpad connection to permit cell dissociation from the adhesion site and concomitant footprint formation. Reattaching substrate-attached material has been shown to contain less actin per total protein than does well-established substrate-attached material (Culp, 1976b). Perhaps a lower level of microfilament organization in the adhesion site area allows for a larger part of the footpad to be pinched off from the cell body.

The enrichment for a specialized composition in a localized area is not unique to membrane lipids. Phagocytosis of latex beads is localized to topographically distinct areas which undergo internalization (Tsan & Berlin, 1971). The phospholipid composition analyses of phagosomes isolated from L cells indicate that the phospholipid distribution is similar to that of surface membranes, yet there are discernible differences (Brown & Klotz, personal communication). In addition, recent studies on erythrocyte membranes reveal a definite difference in phospholipid composition between the inner and outer layers of the lipid bilayer (Sandra & Pagano, 1978).

In addition to the maturation of the newly attaching cell footpads proposed herein and subsequent enrichment for a different phospholipid distribution in the adhesion site from whole cells or surface membrane, there are marked differences in the phospholipids between normal and transformed substrate-attached fractions (Table I). These differences are primarily increases in the relative percentages of PE and CL, decreases in PC, and loss of PG with viral transformation. All of these differences return to normal values in Con A selected revertant substrate-attached fractions. The most notable changes with transformation are those involving PC and PE in the substrate-attached fractions. The cell and surface membrane enriched fractions of all three cell lines—3T3, SVT2, and revertant—were very similar and verify previous reports of no observed differences in lipid composition with transformation (Cunningham, 1972; Kulas et al., 1972; Marggraf et al., 1972). The decrease in the relative amount of PC and increase in PE in the adhesion sites of the less adhesive transformed cell line may relate to other studies involving adhesion of cells. Hale et al. (1977) studied the effects of varying the phospholipid composition of normal and virus-transformed chick embryo fibroblasts, by supplementing delipidated serum with polar head groups (e.g., choline, ethanolamine, or their analogues), on the adhesion of the cells to their substratum. Their results imply that increased PC tends to promote adherence, whereas increased PE appears to decrease adherence. Another study of interest is one performed by Portoukalian et al. (1977) that involved a comparison of the phospholipid composition of the same cell type grown either in suspension or monolayer cultures. These investigators found that suspension-grown cells have relatively lower amounts of phosphatidylcholine and higher amounts of phosphatidylethanolamine than those grown on substrates. Both of these studies suggest that the increased relative amount of PE and decreased relative amount of PC as observed in the substrate-attached fractions could contribute to the diminished adhesive capacity of transformed cells.

Further differences between 3T3 and SVT2 phospholipids were observed in the cell fractions in the pulse experiments (Table IV). These indicate that the two cell lines have different rates of synthesis for a few of the phospholipid species. The similarity of the pulse-chase phospholipid distribution in the cell fractions reveals that the catabolic rates are similar. The differences observed between 3T3 and SVT2 substrate-attached fractions reveal different rates of deposition of newly synthesized material [SVT2 cells deposit much more newly synthesized PC than do 3T3 cells (Table IV)]. This altered deposition rate is also exhibited in studies of reattaching cells (Table III) in which uniformly radiolabeled phospholipids are deposited differently in 3T3 than in SVT2 substrate-attached fractions. In this instance, SVT2 cells put less PC and much more PE into their adhesive footpads. These observed differences may account for the differences observed in overall phospholipid composition of the adhesion sites isolated from the two cell types.

In summary the results of this study indicate that there is an enrichment for specific phospholipids in the cell–substratum adhesion site in comparison to whole-cell or surface membrane enriched fractions. Such specialization may reflect interaction of certain phospholipids with glycoproteins or proteoglycans which are enriched in this adhesive material. Furthermore, transformation-dependent differences were observed in the phospholipid composition of the substrate adhesion sites of normal, transformed, and revertant cells. This altered composition may help to determine the basis for the altered adhesive properties of transformed cells.

Acknowledgments

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Thermodynamics of Glucagon Aggregation†

Robert E. Johnson, Victor J. Hruby, and John A. Rupley*

ABSTRACT: Heats of dilution of concentrated glucagon solutions have been measured calorimetrically at 10 and 25 °C in 0.2 M potassium phosphate buffer of pH 10.6. Analysis of the data in terms of a monomer–trimer equilibrium gives the following thermodynamic parameters for the association reaction at 25 °C: $\Delta G°$ = −7.34 kcal/mol of trimer, $\Delta H°$ = −31.2 kcal/mol, $\Delta S°$ = −80 cal/(K mol), ΔC_p = −430 cal/(K mol). The sensitivity of heat of dilution data to the association constant and stoichiometry of the reaction is discussed.

There has been interest in recent years in the characterization of peptide-hormone receptor interactions, especially the determination of the relative importance of hydrophobic bonding in these interactions. Glucagon, a 29 amino acid peptide hormone, has received considerable attention. It has been implicated in the pathology of diabetes (Unger & Orci, 1975, 1977), its crystal structure has been determined at high resolution (Sasaki et al., 1975), its liver plasma membrane receptor has been characterized (Rodbell et al., 1975; Welton et al., 1977), and a wide variety of structure/function studies have appeared (Hruby et al., 1976; Epand et al., 1976). In these studies, the hydrophobic properties of glucagon have been implicated as important to the biophysical and biological properties of the hormone.

Edelhoch and his collaborators published recently a series of papers on the trimerization of glucagon, a process known to involve hydrophobic interactions in the crystal (Sasaki et al., 1975). Formisano et al. (1977) have presented a van't Hoff analysis of the effect of temperature on the concentration-dependent circular dichroism of glucagon in solution, from which they conclude that the formation of trimers involves a large decrease in enthalpy and a large decrease in entropy. For model compounds, in contrast, hydrophobic associations are typically characterized by small changes in enthalpy and large

† From the Department of Biochemistry and the Department of Chemistry (V.J.H.), University of Arizona, Tucson, Arizona 85721. *Received October 11, 1978; revised manuscript received December 14, 1978.* This work was supported by grants from the U.S. Public Health Service and the National Science Foundation.

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