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BIOSYNTHESIS OF PLASMA MEMBRANE COMPONENTS BY SV40-VIRUS-TRANSFORMED 3T3 MOUSE CELLS TEMPERATURE SENSITIVE FOR EXPRESSION OF SOME TRANSFORMED CELL PROPERTIES

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SUMMARY

We have studied the plasma membranes of an SV40-transformed 3T3 cell line temperature sensitive for the transformed growth phenotype (*ts* H6-15 cells), and have found that they vary little as a function of temperature of cultivation. Analysis by polyacrylamide gel electrophoresis was performed on plasma membranes prepared from *ts* H6-15 cells cultured at the permissive (32 °C) and non-permissive (39 °C) temperatures and radioactively-labelled in several ways. No significant differences were seen when the electrophoretic patterns of polypeptides of the plasma membranes of *ts* H6-15 cells, grown through 3–4 generations in medium containing radioactive leucine (32 °C and 39 °C temperatures) were compared. Plasma membranes derived from cells similarly grown in medium with radioactive glucosamine indicated that extensive alterations in the intrinsic glycopeptides occurred in association with alteration in growth phenotype. A shift towards decreased synthesis of large molecular weight ($\simeq$ 100 000–160 000) glycopeptides occurred in cells grown at the temperature of non-transformed growth (39 °C). A decrease in amount of a 120 000 molecular weight glycopeptide at 39 °C was the most prominent of these alterations.

We have studied the surface exposure of polypeptides and glycopeptides of intact cells grown at 32 and 39 °C, using lactoperoxidase-catalyzed iodination, $NaBH_4$ reduction of galactose oxidase-treated cells, and metabolic-labelling with glucosamine of trypsin-sensitive molecules. We found no major qualitative differences between whole cell extracts or between plasma membrane preparations of cells cultivated at the permissive and non-permissive temperatures. Of special interest was the observation that the formation and surface exposure of a trypsin-sensitive, 240 000 molecular weight polypeptide appeared not to be *ts* in *ts* H6-15 cells. The significance of these observations will be discussed.

INTRODUCTION

Previous studies in several laboratories have indicated that the transformation of cells in culture by viruses leads to major changes in the peptide, glycopeptide and

glycolipid components of the plasma membrane [1]. The significance of these surface alterations in respect to neoplasia is not clear since in many of these studies the normal and transformed cells being compared have differed considerably in their cell culture history. It is questionable, therefore whether these lines can be considered to be isogenic, except for the transformation event, a necessary condition if the comparisons are to be meaningful.

An opportunity to study the plasma membranes of normal and transformed cells in otherwise genetically identical cells has been provided recently by the isolation of a cell line, called *ts* H6-15 [2], which is temperature sensitive for the transformed phenotype.

When grown at 32 °C, *ts* H6-15 cells exhibit the following properties of virus-transformed cells [2–4]: they grow to high saturation density; they do not exhibit density-dependent inhibition of DNA synthesis; they have a lowered requirement for serum; they produce colonies on confluent sheets of normal 3T3 mouse cells and they are agglutinated by low concentrations of concanavalin A and wheat germ agglutinin. When grown at 39 °C, *ts* H6-15 cells behave like normal cells with respect to all these properties. We have confirmed the original finding [2] that *ts* H6-15 cells make no infectious SV40 virus and produce significant amounts of SV40-T antigen, whether grown at 32 or 39 °C. Because of this, and because only normal SV40 virus is rescued from *ts* H6-15 cells [2, 4], it had been concluded that the *ts* gene must be of cellular origin [2].

The genetic information stored in oncogenic viruses is not sufficient to code for the many cell surface alterations that appear to be correlated with transformation. It therefore seems likely that some of these alterations must result from the interaction of the virus genome (or its product(s)) with the normal cellular reactions of synthesis and turnover of the plasma membrane. The isolation of *ts* H6-15 cells has provided a tool for investigating this interaction. In this study we have used this cell line to address ourselves to one major question. Are the biochemical components of the plasma membrane, previously shown to be different in virus-transformed cells as compared with control cells, altered in *ts* H6-15 cells in association with modification of the growth phenotype by temperature shift?

MATERIALS AND METHODS

Cells

ts H6-15 cells were a gift from Dr. Claudio Basilico. Cells were maintained by continuous subculture [5] at low inoculum on a solid surface in medium 1066 [6], supplemented with 5% (v/v) foetal calf serum. Cultures were carried at both 32 and 39 °C in incubators flushed with 5 % CO_2/95 % air and maintained at 95 % relative humidity. Where noted, experiments were performed with cells grown in Dulbecco's modified Eagle's medium [7], supplemented with 10 % foetal calf serum. Cultures were found to be free of mycoplasma in a variety of tests [8], including scanning electron microscope observations performed by Professor Frances Doane.

Plasma membrane isolation

Plasma membrane fractions from approximately 2×10^8 cells were prepared by two methods. In the first [9, 10] cells grown to confluence in 32 oz. Brockway bottles

(Saniglas Co.) were removed from the growth surface by gentle treatment with trypsin [5]. Such treatment results in removal of a number of exposed surface glycopeptides and peptides, but leaves the cells fully viable and the plasma membrane otherwise intact. The cells were collected, washed in isotonic saline, and swollen in the presence of hypotonic zinc chloride solution [10]. The plasma membrane, released by Dounce homogenization, was collected and purified by centrifugation in a Dextran-polyethylene glycol aqueous two phase system [11].

In the second procedure [12], membranes were isolated without prior enzyme treatment of cells. Cells grown as above were washed twice with isotonic saline containing 0.01 % (w/v) $CaCl_2$ and incubated at room temperature with 15 ml of a solution of 1 mM $ZnCl_2$ and dimethylsulphoxide (4 : 1 mixture) for about 20 min (or that interval required to produce rounding of cells on the glass surface). The $ZnCl_2$/dimethylsulphoxide was replaced with an equal volume of a saturated solution of fluorescein mercuric acetate in 0.02 M Tris · HCl, pH 8.1. The cultures were agitated at room temperature on a gyrotory shaker platform (New Brunswick Scientific Co. Model G-10) at 105 rev./min for 20 min to release plasma membrane from cell residue. (This rotational speed had to be varied with different cell types, to obtain optimal yields of plasma membrane.) The fluid was collected and centrifuged at $4000 \times g$ for 10 min. The resulting pellet contained large sheets of plasma membrane (readily visible by phase contrast microscopy), nuclei and intact cells. The plasma membrane was purified by centrifugation in the two phase system as noted above [11]. Successful isolation of plasma membrane by this combined Scher and Barland [12] and Brunette and Till [11] procedure has already been described [13].

Purified plasma membrane preparations were finally collected by centrifugation at $6000 \times g$ for 20 min at 2 °C. If not analyzed immediately, they were stored at −20 °C.

Analysis of plasma membrane preparations by polyacrylamide gel electrophoresis

Plasma membrane fractions were dissolved in either 0.1 M sodium phosphate buffer (pH 7.5) or in 62.5 mM Tris · HCl (pH 6.8), each containing 2 % (w/v) sodium dodecyl sulphate and 1 % (v/v) 2-mercaptoethanol, to give an approximate protein concentration of 1.3 mg/ml. To obtain full solubilization these suspensions were heated in a boiling water bath for 2 min [14]. The solubilized membrane peptides and glycopeptides were analyzed by electrophoresis in polyacrylamide gels. Approximate molecular weights of membrane polypeptides were calculated by comparing their electrophoretic mobilities with those of the following proteins of known molecular weight: bovine serum albumin monomer, 67 000, dimer, 134 000; lactoperoxidase, 80 000; ovalbumin, 43 000; and the actin and myosin in a rabbit muscle homogenate, 45 000 and 200 000 respectively. The position of these markers are designated in figures by arrows and numbers (eg. 67 K, where K = 1000 daltons).

(a) *Cylindrical gels.* Aliquots of 100–200 μl (containing 100–400 μg of protein) were applied to polyacrylamide gels formed in 0.6-cm tubes to a length of 10–15 cm. Gels run in the sodium phosphate-buffered [15] system consisted of resolving gels of 5 % w/v polyacrylamide and stacking gels of 3 % w/v polyacrylamide. For the discontinuous Tris-buffered system of Laemmli [16], 6 % polyacrylamide resolving gels and 4 % polyacrylamide stacking gels were employed. Electrophoresis of samples was carried out at 8 mamps per gel, until the tracking dye reached 1 cm from the

bottom of the gel. In general, comparative duplicate gels were processed together in a 12-place electrophoresis apparatus (Buchler Instruments).

Coomassie Brilliant Blue [14] was used to stain peptides in gels. For analysis of radioactively-labelled material, the gels were frozen and cut into 1.5–2 mm segments with a set of fixed parallel razor blades. These slices were incubated overnight at 37 °C. in scintillation vials, with 1 ml 90 % (v/v) NCS Solubilizer. After the addition of 10 ml of scintillation fluid, the radioactivity was measured in a Nuclear Chicago Scintillation counter. The data was analysed as previously described [9] by a computer program which automatically plotted the results as % distribution of cpm of ^{3}H and ^{14}C within each gel. The program also plotted the % difference between distributions. The y-ordinates here give the actual data in cpm. The recovery of radioactive material within gels was 80–100 %.

(b) *Slab gels.* Gel slabs 17×15×0.15 cm with a 4 % w/v polyacrylamide stacking gel and a 7 % w/v polyacrylamide resolving gel were cast in an apparatus constructed by CPF Plastics (Port Credit, Ontario). The buffers used are those described by Laemmli [16]. Aliquots of 25–100 μl of material containing 50–200 μg of protein were applied to the top of the gels. Electrophoresis was at 8 V per cm until the Bromphenol Blue tracking dye reached 1 cm from the bottom edge of the gel. These gels were dried under vacuum and the location of ^{125}I-labelled material was detected by autoradiography on Kodak RP Royal X-Omat X-ray film.

Preparation of radioactively-labelled plasma membrane

(*a*) *By metabolic labelling.* To obtain plasma membrane generally labelled in protein and carbohydrate moieties, cells were grown at 32 or 39 °C for 3–4 generations as previously described [9] in medium containing radioactive leucine and glucosamine, respectively. Where noted, these media contained 0.1 μCi/ml of [6-^{3}H]Glc-NH_2HCl ($4 \cdot 10^{-3}$ μM; 25 Ci/mmol), 0.1 μCi/ml of [1-^{14}C]GlcNH_2HCl (0.3 μM; 348 Ci/mmol) or 1/5 the usual leucine concentration supplemented with L-[U-^{14}C]leucine (0.07 μCi/ml; 312 Ci/mmol) or L-[4,5-^{3}H]leucine (0.07 μCi/ml; 51 Ci/mmol).

A procedure for specific, metabolic labelling of glycoproteins at the extreme periphery of cells has been fully described [5]. Briefly, cells were grown to confluence and removed from the growing surface with trypsin under conditions which remove these glycoproteins but leave the underlying plasma membrane intact. The cells are then plated at 4×10^4 cells/cm^2 in medium containing 1/4 the normal glucose concentration. After 12 or 17 h for cells grown at 39 °C or 32 °C, respectively, this medium was decanted and replaced with 10 ml glucose- and serum-free medium containing 1 μCi/ml of [^{14}C]- or [^{3}H]GlcNH_2. The cells were incubated 1 h at the appropriate temperature prior to the harvest of plasma membrane.

(*b*) *By enzymatic labelling from without.* Externally-exposed peptides at the surface of cells were labelled with ^{125}I essentially as described by Hynes [17]. *ts* H6-15 cells, grown to confluence in 35 mm Petri dishes, were washed 3 times with phosphate-buffered saline [18] and then incubated for 10 min at room temperature in phosphate-buffered saline containing 400 μCi/ml of carrier-free Na^{125}I, 20 μg/ml lactoperoxidase, 5 mM glucose and 1.0 unit/ml glucose oxidase. This reaction mixture was decanted, the cells were washed three times with phosphate-buffered iodide (phosphate buffered saline in which the NaCl is replaced by NaI), and then scraped into this same solution containing 2 mM phenylmethylsulphonyl fluoride [19] to

inhibit proteases. Some cultures were treated with 10 μg per ml of trypsin for 10 min at room temperature before the cells were scraped. The cells were harvested by centrifugation at $800 \times g$ for 10 min at 4 °C and dissolved in the sample-dissolving buffer of Laemmli [16], supplemented with 2 mM protease inhibitor.

Molecules at the periphery of cells with exposed galactose-containing residues were labelled from without using galactose oxidase and [^{3}H]$NaBH_4$ as described by Gahmberg and Hakomori [20]. Cells were washed three times with phosphate-buffered saline and incubated for 15 min at 37 °C with 5 ml of this solution containing 10 units of *Vibrio cholerae* neuraminidase to remove sialic acid residues. The cultures were washed and then incubated for 15 min at 37 °C with 5 ml buffered saline containing 10 units of galactose oxidase. The cells were treated for 5 min with 5 ml cold phosphate-buffered saline, to which was then added 1 mCi [^{3}H]$NaBH_4$ (0.1 μmol, 10000 Ci/M). After 5 min the cultures were washed twice with saline solution containing 1 % (w/v) bovine serum albumin. Plasma membrane fractions were isolated by the second procedure described above.

Chemicals and reagents

All radioactive isotopes and NCS solubilizer were purchased from Amersham Searle. Foetal calf serum and trypsin were purchased from Reheis and Grand Island Biological Company, respectively. The fluors and phosphors for liquid scintillation counting came from Packard. Fluorescein mercuric acetate was purchased from Nutritional Biochemicals. Glucose oxidase and galactose oxidase were obtained from Worthington Biochemicals, lactoperoxidase from Calbiochem, and neuraminidase from Behringwerke. All chemicals were of analytical grade.

RESULTS

Previous studies [1, 5, 9, 21–23] in our laboratory had indicated that the plasma membranes isolated from normal 3T3 mouse cells differ chemically from those obtained from clonal isolates of 3T3 cells transformed by polyoma and SV40 viruses. Differences were detected in peptide, glycopeptide and glycolipid components. The availability of a *ts* mutant makes it possible to use genetically identical cells to assess the relationship of these alterations to the transformed growth phenotype.

Since the basic assumption inherent in our comparisons of *ts* H6-15 cells cultured at 32 and 39 °C was that these cells behaved as transformed and as normal cells respectively, we first confirmed the original observations of Renger and Basilico [2] and showed that *ts* H6-15 cells are *ts* for the following features generally considered characteristic of transformed cells: high saturation density, lack of density dependent inhibition of DNA synthesis, lowered requirement for serum growth factors and increased Concanavalin A agglutinability. We then compared the glycopeptide and polypeptide components of the plasma membranes of *ts* H6-15 cells cultured at 32 °C as transformed cells, with those of cells grown at 39 °C, in the normal growth pattern.

A. Comparison of leucine-labelled plasma membrane of ts H6-15 cells grown at 32 and 39 °C

Since comparative studies of the plasma membranes of control 3T3 mouse fibroblasts and cells transformed by polyoma and SV40 viruses had revealed differences in some of the minor polypeptide components, we examined these components

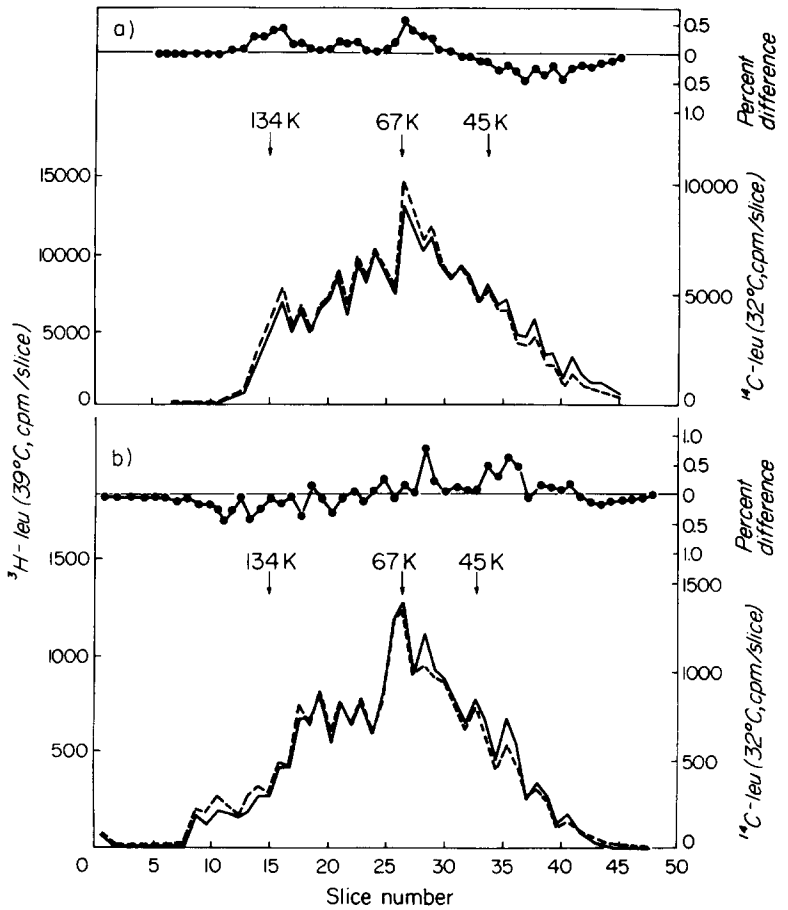

Fig. 1. Electrophoretic analysis of plasma membrane polypeptides of *ts* H6-15 cells grown at 32 and 39 °C. *ts* H6-15 cells were grown to confluence at 32 °C and at 39 °C, through 3–4 generations in medium containing labelled leu. Plasma membrane preparations, obtained as noted below, were analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods, along with the molecular weight markers shown. In this and all other such analyses comparative data have been aligned with respect to these molecular weight standards. Anode position in this and all other cylindrical gels is at the right. (a) Plasma membrane obtained from trypsin-released cells grown with [^{14}C]leucine at 32 °C (– – –) and [^{3}H]leucine at 39 °C (—). Recovery of [^{3}H] and [^{14}C]leucine was 92 and 86 % respectively. (b) Plasma membrane obtained from cells grown with [^{14}C]leucine at 32 °C (– – –) and [^{3}H]leucine at 39 °C (—) and then fixed with fluorescein mercuric acetate. Recovery of isotopes was 84 and 91 % respectively. ●–●, % difference between distribution of ^{14}C- and ^{3}H-labelled macromolecules separated within the gel.

in *ts* H6-15 cells at 32 and 39 °C. The cells were generally labelled with [^{14}C] or [^{3}H]leucine during long-term growth at 32 and 39 °C, respectively. Differentially-labelled plasma membrane preparations were obtained, combined, and then analyzed by polyacrylamide gel electrophoresis. The preparative procedures described in Fig. 1 were designed to permit comparison of plasma membrane from intact and trypsin-treated cells.

TABLE I

COMPARISON OF ELECTROPHORETIC ANALYSES OF LABELLED MACROMOLECULES IN PLASMA MEMBRANE PREPARATIONS DERIVED FROM *ts* H6-15 CELLS GROWN AT 32 AND 39 °C AND COMBINED

Where indicated cells were released by trypsin or fixed with fluorescein mercuric acetate prior to isolation of plasma membrane. % difference area is calculated from the % distribution of ^3H and ^{14}C label throughout the gels (see Materials and Methods). It is expressed as total % difference/2 ± S.E. ( ) indicates number of experiments.

Labelling procedure	Cells analysed	% difference area	Average variance
3–4 generations [^{3}H]-+[^{14}C]leucine	confluent, fixed	7.0±1.3 (3)	0.15
3–4 generations [^{3}H]-+[^{14}C]leucine	confluent, trypsinized	7.6±3.4 (2)	0.15
3–4 generations [^{3}H]-+[^{14}C]$GlcNH_2$	confluent, fixed	13.6±1.1 (4)	0.63
3–4 generations [^{3}H]-+[^{14}C]$GlcNH_2$	confluent, trypsinized	12.1±2.0 (3)	0.36
2–3 generations [^{3}H]-+[^{14}C]$GlcNH_2$	logarithmically growing cells	7.5±0.4 (2)	0.23
1 h [^{3}H]-+[^{14}C]$GlcNH_2$ pulse labelling of trypsin-sensitive material	non-confluent, fixed	13.0±3.4 (3)	0.77

The data shown in Fig. 1A and B suggest that there is very little qualitative difference between the leu-containing macromolecules of the plasma membranes isolated from *ts* H6-15 cells grown at 32 and 39 °C. A small quantitative difference was observed as is indicated in Table I. A comparison of Fig. 1a with Fig. 1b indicates that the two methods of plasma membrane preparation yield material with generally similar electrophoretic patterns, although the plasma membranes prepared from cells fixed with fluorescein mercuric acetate have components of molecular weights greater than 134 000 which are lacking in those prepared from trypsinized cells.

B. Comparison of plasma membranes of ts H6-15 cells generally labelled with $GlcNH_2$ at 32 and 39 °C

Our own studies [1, 5, 9, 23], as well as those of a number of other investigators [24–28], have revealed considerable differences between carbohydrate-containing proteins of normal and virus-transformed cells. In studying the glycoproteins of *ts* H6-15 cells grown at 32 and 39 °C, we first performed comparative analyses of $GlcNH_2$-containing glycopeptides, generally labelled during several generations of growth.

Plasma membrane preparations were obtained from confluent *ts* H6-15 cells differentially-labelled with [^{14}C]- and [^{3}H]$GlcNH_2$ during growth at 32 and 39 °C over extended time periods. These were solubilized and analyzed as described in the legend of Fig. 2. The electrophoretic profiles of the $GlcNH_2$-labelled macromolecules isolated from trypsin-released cells (Fig. 2a) and those fixed with fluorescein mercuric acetate (Fig. 2b) revealed marked differences. We consistently observed more extensive labelling of large molecular weight (100 000–160 000) glycopeptides in the plasma membranes of *ts* H6-15 cells grown at 32 °C. A glycopeptide (peak 4) of approximately 120 000 molecular weight was present in plasma membranes from cells grown at 32 °C, but was almost absent from cells grown at 39 °C. In cells grown at 39 °C,

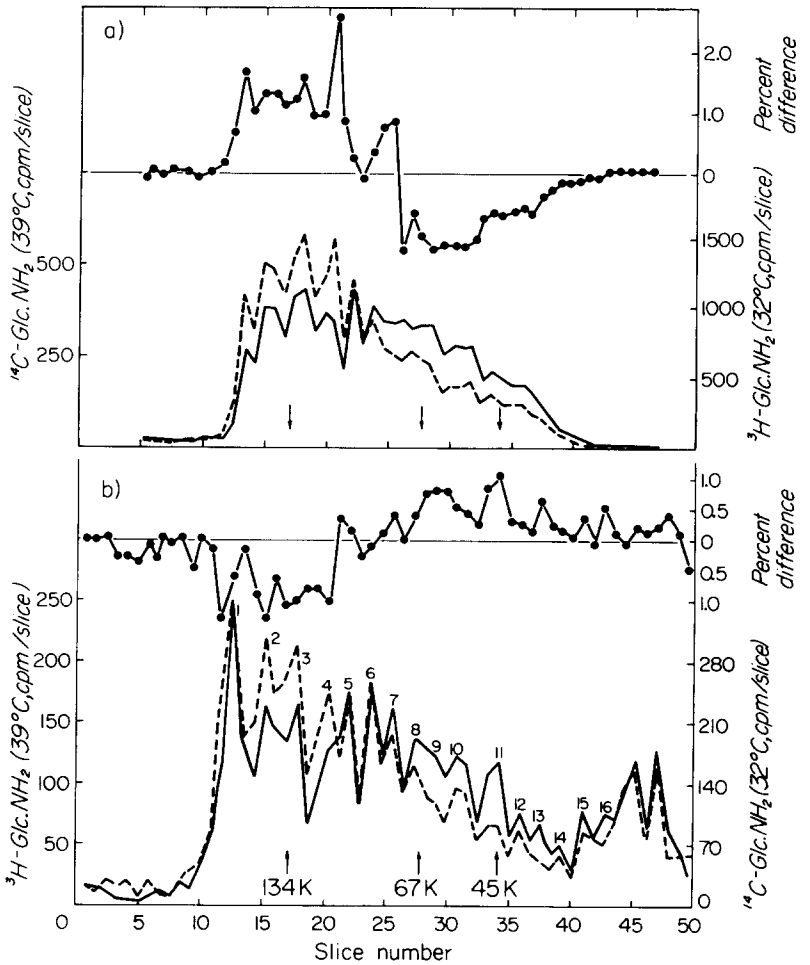

Fig. 2. Electrophoretic analysis of plasma membrane preparations from *ts* H6-15 cells grown at 32 and 39 °C with radioactive glucosamine. *ts* H6-15 cells were grown to confluence at 32 and at 39 °C through 3–4 generations in medium containing labelled Glc NH_2. Plasma membranes were isolated and analyzed as noted in the legend of Fig. 1. (a) Plasma membrane from trypsin-released cells grown with [^{3}H]Glc NH_2 at 32 °C (– – –) and with [^{14}C]Glc NH_2 at 39 °C (—). Recovery of isotopes from gel was 86 % and 82 %. (b) Plasma membrane from cells grown with [^{14}C]Glc NH_2 at 32 °C (– – –) and with [^{3}H]Glc NH_2 at 39 °C (—) and fixed with fluorescein mercuric acetate. Recovery of isotopes from gel was 82 and 91 %, respectively. ●–●, % difference etc. as in Fig. 1.

the smaller molecular weight components were more heavily-labelled. Additional variations were observed, as seen in Fig. 2b, peaks 13 and 16. GlcNH_2-labelled material of about 30 000 and 15 000 daltons respectively were present in plasma membranes from *ts* H6-15 cells cultured at 39 °C but absent from similar material from these cells cultured at 32 °C.

A comparison between Fig. 2a and 2b indicates that the electrophoretic profiles of plasma membranes of GlcNH_2-labelled cells fixed with fluorescein mercuric acetate contains material of large molecular weight (> 230 000) and of small molecu-

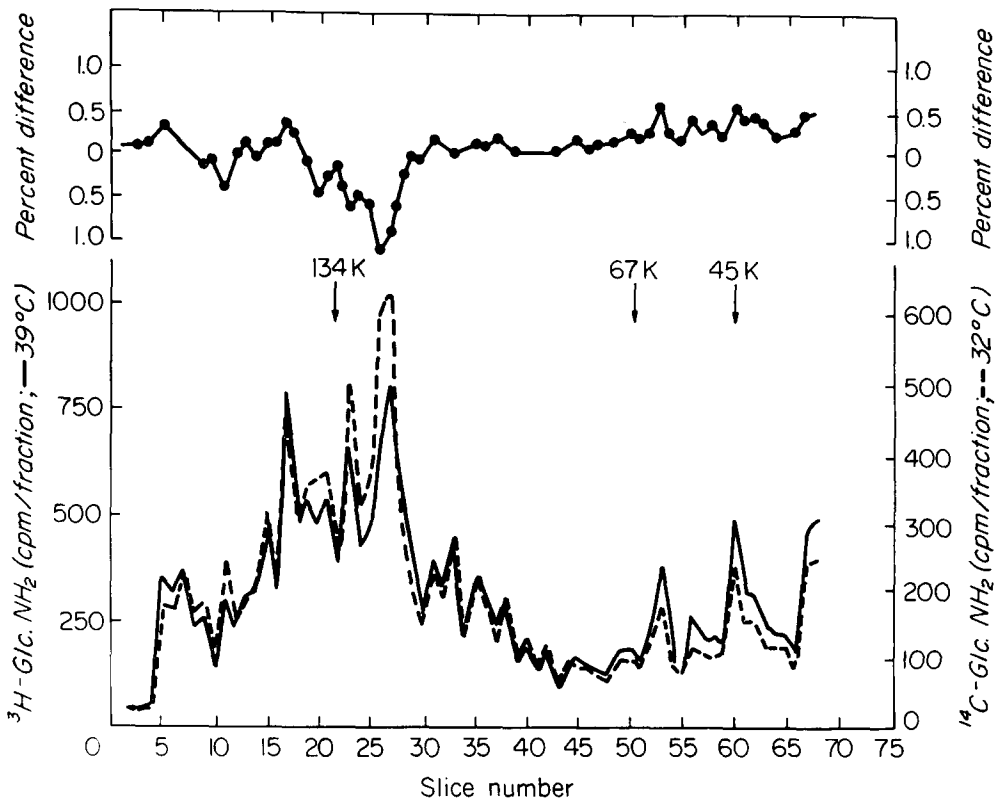

Fig. 3. Electrophoretic analysis of plasma membrane preparations from *ts* H6-15 cells growing logarithmically at 32 and 39 °C with radioactive glucosamine. *ts* H6-15 cells were grown through 2–3 generations in medium containing labelled $GlcNH_2$ to approximately mid-logarithmic phase. The cells were released from the growing surface with trypsin and plasma membranes were isolated and analyzed as noted in the legend of Fig. 1. Cells grown with [^{14}C]$GlcNH_2$ at 32 °C (- - -) and with [^{3}H]$GlcNH_2$ at 39 °C (—). Recovery of isotopes from gels was 88 and 84 % respectively. ●–●, as in Fig. 1.

lar weight (< 18 000) which are not detectable in the profiles of similar material obtained from trypsinized cells. These findings are in accord with earlier studies [5, 23] which indicated that glycoproteins of very large size are released from cells subjected to gentle trypsin treatment.

Confluent *ts* H6-15 cells cultured at 39 °C are in a state of quiesence as a result of topoinhibition of growth, while confluent cells at 32 °C are actively growing. The differences we observed between the plasma membrane glycopeptides of confluent *ts* H6-15 cells grown at the two temperatures could be a result of this difference in growth state. We have therefore examined the plasma membrane glycopeptides of *ts* H6-15 cells during logarithmic growth at the two temperatures. Fewer temperature-dependent differences were observed (Fig. 3). However the growth-temperature-dependent alteration in the 120 000 dalton component (Fig. 2, peak 4), observed with confluent cells was also detected in the plasma membranes of exponentially growing cells. This suggested that the presence of increased amounts of this material at 32 °C could be closely linked to the *ts* phenotype of this cell line.

C. Comparison of plasma membranes of ts H6-15 cells grown at 32 and 39 °C and specifically-labelled in peripheral surface glycoproteins

It had been demonstrated earlier that the surface glycopeptide fraction of 3T3 cells transformed by SV40 virus is chemically different from that of normal cells [5, 23]. It was therefore of special interest to compare this cell surface fraction in *ts* H6-15 cells grown at 32 and 39 °C. To specifically label the glycopeptides exposed at the cell periphery we adapted a procedure developed in previous studies with normal and virus-transformed mouse fibroblasts [5, 23]. *ts* H6-15 cells, grown to confluence at 32 and at 39 °C, were subcultured as described under Fig. 4. At hourly intervals thereafter cells were pulse labelled for 1 h with [^{3}H]GlcNH_2 and then analyzed for incorporation of GlcNH_2-labelled macromolecules at the cell periphery.

The data in Fig. 4 indicated that *ts* H6-15 cells begin to resynthesize surface glycopeptides immediately upon subculture by trypsin treatment. The maximum rate of incorporation of GlcNH_2 into acid-insoluble, trypsin-labile, cell-associated material was observed about 12–13 and 17–18 h post-plating of cells at 39 and 32 °C, respectively.

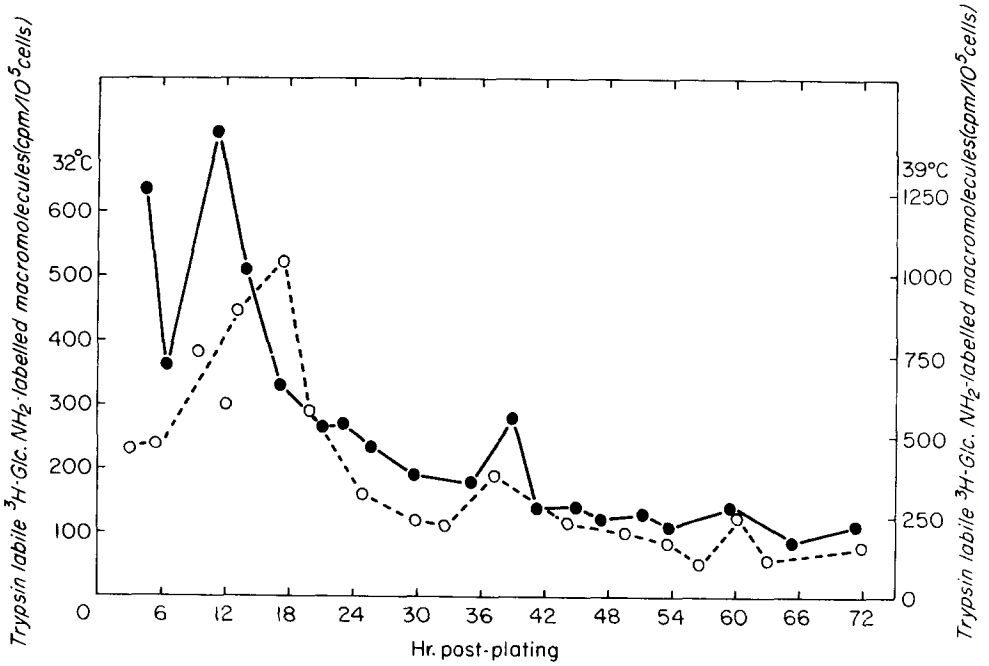

Fig. 4. Incorporation of [^{3}H]GlcNH_2 by *ts* H6-15 cells into trypsin-labile, acid-precipitable material, after subculture from confluence. *ts* H6-15 cells were grown to confluence at 32 °C (– – –) and 39 °C (—) and then subcultured with trypsin. At various intervals thereafter triplicate cultures were pulse labelled for one hour with [^{3}H]GlcNH_2 as described under "metabolic-labelling" in Materials and Methods. The cells were then subjected to the trypsin treatment used for subculturing to remove cell surface molecules while leaving the remaining cells fully viable. The resulting cell suspensions were centrifuged at $800 \times g$. The supernatant containing the trypsin labile cell surface components was treated with an equal volume of 10% trichloroacetic acid. The insoluble [^{3}H]GlcNH_2-labelled material was collected on glass fiber filters and counted.

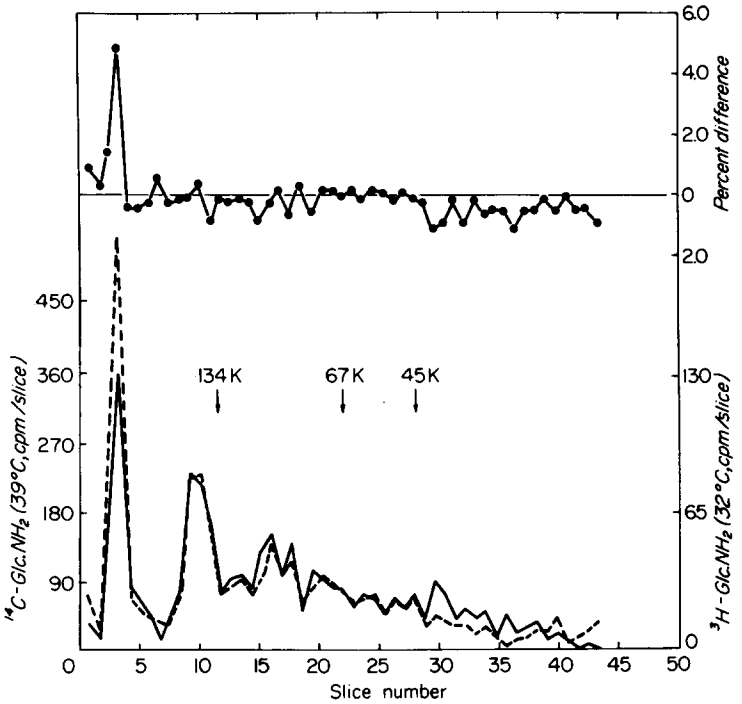

Fig. 5. Electrophoretic analysis of plasma membranes of *ts* H6-15 cells grown at 32 or 39 °C and specificially-labelled in surface glycoproteins. *ts* H6-15 cells were grown at 32 or 39 °C, subcultured with trypsin and then pulse-labelled in peripheral glycoproteins with radioactive $GlcNH_2$, as described under Materials and Methods (metabolic labelling). Plasma membrane preparations, made from cells fixed with fluorescein mercuric acetate were combined and analyzed by polyacrylamide gel electrophoresis as noted in the legend of Fig. 1. Cells were labelled at 32 °C (– – –) with [^{3}H]$GlcNH_2$ and at 39 °C (—) with [^{14}C]$GlcNH_2$. Recovery of ^{3}H- and ^{14}C-labelled macromolecules from the gels was about 88 and 81 % respectively. ●–●, as in Fig. 1.

Having established the conditions for maximum specific labelling of surface glycopeptides for *ts* H6-15 cells, at 32 and 39 °C, we then proceeded to a comparative analysis of these plasma membrane constituents as described in the legend of Fig. 5. The electrophoretic profiles obtained with plasma membrane preparations carrying surface glycopeptides specifically-labelled with [^{14}C]- and [^{3}H]$GlcNH_2$ at the low and high temperatures respectively, did not differ much qualitatively. However, quantitative differences between the extent of labelling of specific macromolecules at the two temperatures, was observed, particularly in the case of a very large molecular weight component which was recovered in the stacking gel (eg. Fig. 5, slices 3 and 4). More extensive labelling of this surface glycopeptide was observed in *ts* H6-15 cells grown at 32 °C than in cells grown at 39 °C.

D. Surface exposure of galactose-containing molecules of the plasma membranes of ts H6-15 cells grown at 32 and 39 °C

It had been reported that virus-transformed hamster fibroblasts are deficient, when compared with normal cells, in a large molecular weight (> 200 000), surface-

exposed, galactose-containing protein [24, 25]. This was indicated by the different susceptibility of neuraminidase-treated cells to oxidation by galactose oxidase. To examine the possible relevance of such a phenomenon to the transformed phenotype the following experiment was performed.

ts H6-15 cells were grown at 32 and 39 °C. These cells were treated with neuraminidase, galactose oxidase and NaB^3H_4 as described in the legend of Fig. 6. Plasma membrane preparations obtained from cells fixed with fluorescein mercuric acetate were analyzed, on a 6 % w/v polyacrylamide gel containing sodium dodecyl sulphate, using the buffer system of Laemmli to resolve surface material of high-molecular weight. As seen in Fig. 6, no significant differences in plasma membrane material of molecular weight > 70 000 were detected between the labelled galactose-containing molecules at the surface of *ts* H6-15 cells grown at 32 or at 39 °C.

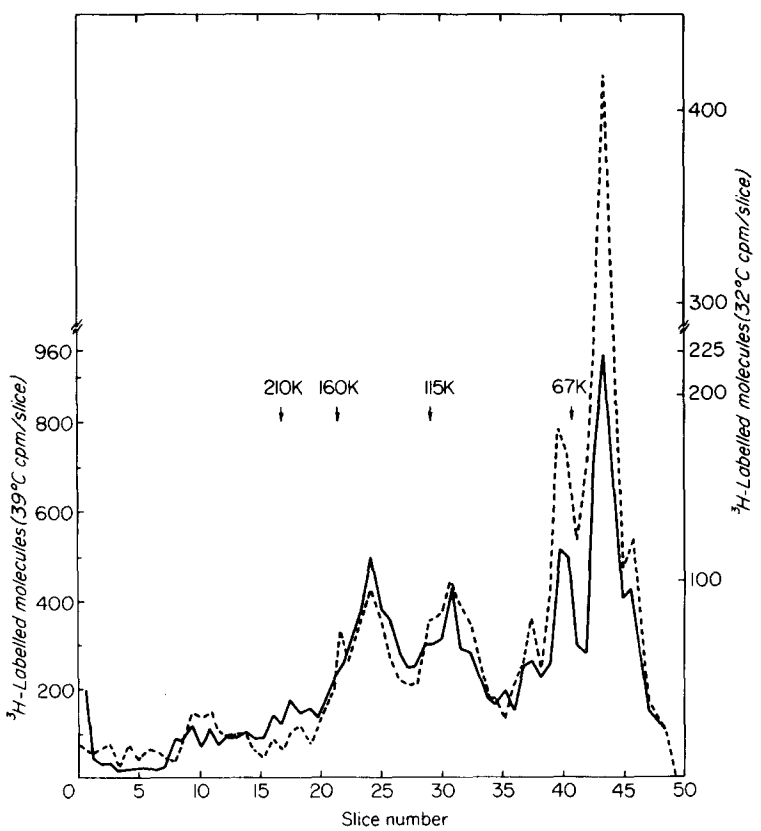

Fig. 6. Electrophoretic analysis of plasma membranes of *ts* H6-15 cells grown at 32 and 39 °C and labelled in surface-exposed galactose residues. *ts* H6-15 cells were grown at 32 (---) and 39 °C (—) and labelled with NaB^3H_4 and galactose oxidase as described in Materials and Methods. The plasma membranes were isolated from cells fixed with fluorescein mercuric acetate and analyzed by polyacrylamide gel electrophoresis using the procedure of Laemmli [16].

E. Surface exposure of polypeptides of the plasma membranes of ts H6-15 cells grown at 32 and 39 °C

A number of investigators have reported that virus-transformed fibroblasts are deficient in a large molecular weight, trypsin-labile polypeptide or glycopeptide, which in normal cells is detected by in situ lactoperoxidase-catalyzed iodination [17, 28–30].

ts H6-15 cells have been examined for their pattern of surface iodination as follows: cells grown at 32 and 39 °C were treated with lactoperoxidase and ^{125}I as described in the legend of Fig. 7. The cells were dissolved and the labelled polypeptides were analyzed by electrophoresis on slab gels. Fig. 7 presents an autoradiogram of one such analysis.

There are about ten ^{125}I-labelled bands visible in this autoradiogram. Some of them may be serum proteins that adhere strongly to cell surfaces or to the culture dishes [29, 30]. One of them comigrates with lactoperoxidase (80 000 daltons). About 100 polypeptide bands are seen when these gels of whole cell extracts are stained for protein with Coomassie blue. Gels of plasma membrane preparations generally labelled with leucine (Fig. 1), or stained with Coomassie blue [9], also have many more bands than the autoradiogram of ^{125}I labelled cells. These results, taken

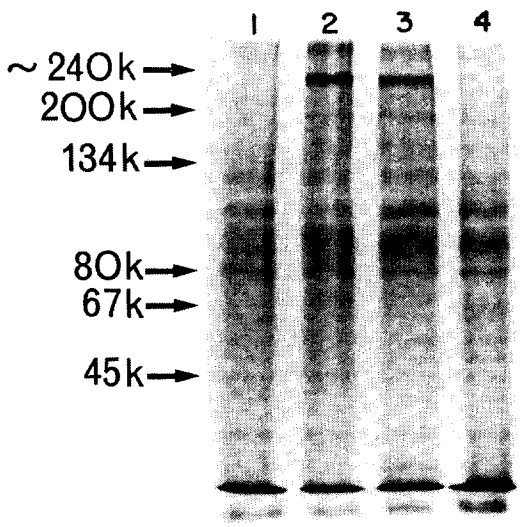

Fig. 7. Autoradiogram of an electrophoretic analysis of ^{125}I-labelled, surface-exposed proteins of *ts* H6-15 cells grown at 32 and 39 °C. Replicate cultures of *ts* H6-15 cells were grown to confluence at 32 or at 39 °C in Dulbecco's Modified Eagle's Medium supplemented with 10 % FCS. They were then treated in situ to label surface-exposed polypeptides, with lactoperoxidase (mol. wt. ≃ 80 000), glucose, glucose oxidase and ^{125}I, as described in Materials and Methods. Some cultures were treated with trypsin under conditions which remove such surface molecules but leave the underlying plasma membrane functionally intact. The cells were solubilized and equal amounts of trichloroacetic acid-insoluble, ^{125}I-labelled macromolecules were analyzed on polyacrylamide slab gels containing sodium dodecyl sulphate, using the Laemmli [16] buffer system. The position of molecular weight markers are designated by arrows. In lanes 1 and 2 material is extracted from cells grown at 32 °C; lanes 3 and 4 from cells grown at 39 °C. The cells in lanes 1 and 4 were treated with trypsin before extraction.

in conjunction with the trypsin lability of some of the ^{125}I labelled bands seen in Fig. 7, indicate that unexposed cellular polypeptides are not iodinated.

One polypeptide band with an apparent molecular weight of 240 000 is heavily labelled with ^{125}I and is quite sensitive to low levels of trypsin. Presumably, this is the *ts* H6-15 equivalent of the "transformation-sensitive" cell-surface component referred to above. The amount of this component accessible to lactoperoxidase catalyzed iodination is somewhat greater in *ts* H6-15 cells cultured at 32 °C than at 39 °C. Therefore its loss is not a prerequisite of transformed growth in these cells.

DISCUSSION

Many of the physiological properties that distinguish neoplastically-transformed cells from their normal counterparts have been attributed to one or more alterations in their plasma membrane structure [31, 32]. As a result, the surfaces of normal and transformed cells have been compared extensively. A wide variety of cell surface changes, including alteration of glycolipid, protein, and glycoprotein composition, have been found to accompany neoplastic transformation [5, 9, 21, 22, 33]. To determine which if any of these cell surface alterations is directly related to transformation we have studied the plasma membranes of a cell line temperature-sensitive for the transformed phenotype (*ts* H6-15).

Earlier studies of ours [9] and of others [28, 34, 35] have reported differences between the plasma membrane polypeptides of normal cells and their virally transformed counterparts, and between various clones of cells transformed by the same virus. It was, therefore, of interest to compare the plasma membrane polypeptide composition of *ts* H6-15 cells at the temperatures of normal and transformed growth. Our experiments with these cells failed to reveal any significant alteration in the polypeptides of the plasma membrane when material from cells cultured at 32 and 39 °C were compared. Thus it seems that such alterations are not determining features of the transformed phenotype.

Much recent work has focussed on the significance for growth regulation, of a large molecular weight, trypsin-labile protein which is present in normal cells, but is absent or depleted from virus-transformed cells. The loss of this 200 000–250 000 dalton, surface-exposed, plasma membrane constituent has been reported in chick embryo fibroblasts transformed by Rous sarcoma virus [28], in NIL hamster cells transformed by hamster sarcoma virus [17] and in 3T3 mouse fibroblasts transformed by SV40, polyoma and murine sarcoma viruses [29]. In chick embryo fibroblasts infected with the *ts* 68 mutant of RSV, this protein could be readily detected only in cells cultured at the non-permissive temperature [30]. These experiments suggested that the loss of the large molecular weight, surface-exposed protein may be a determinant of the growth of transformed cells.

We have shown here that a surface-exposed, trypsin-labile protein of about 240 000 molecular weight, detected by lactoperoxidase-catalyzed iodination, is present in roughly equal amounts on *ts* H6-15 cells grown either at 32 or 39 °C (Fig. 7). A similar observation with *ts* H6-15 cells was briefly reported by Hogg [29], who also found that LX cells, a cell line derived from mouse L-cells, which are chemically transformed, have such a large molecular weight protein on their surfaces.

These results suggest that the presence or absence of this protein cannot be an absolute determinant for regulation of cell growth.

A recent report by Hynes and Wyke [36] supports the suggestion that the absence of this high molecular weight protein is not a primary feature of cell transformation. These workers studied chick embryo fibroblasts infected with Rous sarcoma viruses temperature sensitive for the maintenance of transformed growth. They found that on a shift from non-permissive to permissive conditions, the reduction in amount of this surface protein occurred after the morphological and sugar transport rate changes characteristic of transformation. In studies of the effects of proteases on resting chick embryo fibroblasts, Teng and Chen [37] could find no correlation between the growth properties of these cells and the presence or absence of the 250 000-dalton, cell-surface protein.

Another cell surface change frequently associated with a shift to transformed growth is that of alterations in glycolipid composition. Specifically, a number of reports had indicated that transformed cells have simplified or incomplete glycolipids relative to their normal counterparts [22, 38–41]. However, it was demonstrated subsequently that some clones of virally-transformed cells do not exhibit this change [21, 42]. Indeed clonal variation with respect to glycosphingolipid biosynthetic pattern was found to be extensive. Since then a number of experimental approaches have confirmed the conclusion that an alteration in the pattern of glycosphingolipid biosynthesis is not a primary expression of the transformed cell growth phenotype.

Hammarström and Bjürsell [43], and Gahmberg et al. [44], studied the glycolipids of cells transformed with polyoma *ts* 3. These cells are temperature sensitive for the maintenance of transformed growth. The levels of some of the glycolipids of these cells varied with their temperature of cultivation, but the glycolipid compositions observed were different from both uninfected normal cells and BHK cells transformed with wild type polyoma virus. Den et al. [45] showed that revertants of several transformed cell lines, with apparently normal growth behaviour, retained the simplified pattern of ganglioside composition characteristic of their transformed parents. Sheinin and Murray (1974, unpublished data) found that *ts* H6-15 cells have a glycolipid composition resembling that of normal 3T3 cells whether cultured at 32 or 39 °C. Similar findings were reported by Mora [46]. Taken together, these various experiments indicate that a change in glycosphingolipid metabolism is not essential to the altered growth pattern of transformed cells.

The data discussed above indicates that alterations in the major polypeptides and glycolipids of the cell surface are not involved in the conversion to the transformed pattern of growth in *ts* H6-15 cells. Cell surface glycoproteins have been implicated in cell : cell interactions in a number of experimental systems and are, therefore, possible sites of expression of the *ts* gene of *ts* H6-15 cells. Previous work in our laboratory [5, 23] had indicated that surface glycoproteins sensitive to trypsin treatment were altered in virally-transformed cells. However, our studies on the resynthesis of these surface components in trypsin-treated *ts* H6-15 cells (Fig. 4) indicates that they do not vary qualitatively as a function of the temperature of cultivation of these cells.

Another probe for alteration in cell surface glycopeptides is the three-step neuraminidase, galactose oxidase, NaB^3H_4 treatment of intact cells developed by Gahmberg and Hakomori [20]. Application of this labelling procedure has suggested

that there may be differences in large galactopeptides at the surface of normal cells and their virus-transformed derivatives [24, 25, 44]. But as Fig. 6 demonstrates this technique did not reveal any temperature-sensitive differences in the surfaces of *ts* H6-15 cells. Thus our studies of the externally exposed glycopeptides of *ts* H6-15 cells suggest that these macromolecules are not sites of expression of this *ts* gene.

The only plasma membrane components of *ts* H6-15 cells which did exhibit temperature sensitivity were the intrinsic glycoproteins labelled by culturing the cells in the presence of radioactive $GlcNH_2$. The most prominent and consistent difference observed was a decrease in the amount of a 120 000 dalton, trypsin-insensitive glycopeptide in the plasma membranes of *ts* H6-15 cells cultured at 39 °C.

The techniques used in these experiments are not capable of resolving all cell surface polypeptides, and there may be differences between the surfaces of *ts* H6-15 cells cultured at the two temperatures that we cannot detect. However, the major impact of our studies lies in their demonstration that in *ts* H6-15 cells, the growth phenotype is not correlated with a number of the gross biochemical modifications previously implicated in the mechanism of virus transformation. As designed, they can have little to say about the role of these biochemical modifications in progression to, and expression of malignancy. One plasma membrane glycopeptide component (120 000 dalton) does exhibit temperature sensitivity which correlates with the growth behaviour of *ts* H6-15 cells. We plan to analyze it further with respect to structure and function, and examine other biochemical parameters of growth in these cells with the hope of elucidating the biochemical basis of the *ts* behaviour of *ts* H6-15 cells.

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REFERENCES

1 Sheinin, R. (1974) in Viruses, Evolution and Cancer (Kurstak, E. and Maramorosch, K., eds.), pp. 371–400, Academic Press, New York
2 Renger, H. C. and Basilico, C. (1972) Proc. Natl. Acad. Sci. U.S. 69, 109–114
3 Renger, H. C. and Basilico, C. (1973) J. Virol. 11, 702–708
4 Basilico, C., Renger, H. C., Burstin, S. J. and Toniolo, D. (1974) in Control of Proliferation in Animal Cells (Clarkson, B. and Baserga, R., eds.), pp. 167–176, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
5 Onodera, K. and Sheinin, R. (1970) J. Cell Sci. 7, 337–355
6 Parker, R. C., Castor, L. N. and McCulloch, E. A. (1957) in Cellular Biology, Nucleic Acids and Viruses, Vol. 5, pp. 303–313, N.Y. Acad. Sci. Spec. Publ., New York
7 Smith, J. D., Freeman, G., Vogt, M. and Dulbecco, R. (1960) Virology 12, 185–196
8 Hayflick L. (1973) in Tissue Culture: Methods and Applications (Kruse, Jr., P. F. and Patterson, Jr., M. K., eds.), pp. 722–728, Academic Press, New York
9 Sheinin, R. and Onodera, K. (1972) Biochim. Biophys. Acta 274, 49–63
10 Warren, L., Glick, M. C., and Nass, M. K. (1966) J. Cell. Physiol. 68, 269–281
11 Brunette, D. M. and Till, J. E. (1971) J. Membr. Biol. 5, 215–224
12 Scher, I. and Barland, P. (1972) Biochim. Biophys. Acta 255, 580–588
13 Carlsen, S. and Till, J. E. (1975) Can. J. Biochem. 53, 106–108

14 Maizel, J. V. (1971) in Methods in Virology (Maramorosch, K. and Kaprowski, H., eds), Vol. 5, pp. 179–246, Academic Press, New York
15 Baenziger, N. L., Brodie, G. N. and Marjerus, P. W. (1971) Proc. Natl. Acad. Sci. U.S. 68, 240–242
16 Laemmli, U. K. (1970) Nature 227, 680–685
17 Hynes, R. O. (1973) Proc. Natl. Acad. Sci. U.S. 70, 3170–3174
18 Dulbecco, R. and Vogt, M. (1954) J. Exp. Med. 99, 167–182
19 Fahrney, D. E. and Gold, A. M. (1963) J. Am. Chem. Soc. 85, 997–1000
20 Gahmberg, C. G. and Hakomori, S. (1973) J. Biol. Chem. 248, 4311–4317
21 Yogeeswaran, G., Sheinin, R., Wherrett, J. R. and Murray, R. K. (1972) J. Biol. Chem. 247, 5146–5158
22 Sheinin, R., Onodera, K., Yogeeswaran, G. and Murray, R. K. (1971) in The Biology of Oncogenic Viruses (Silvestri, L. G., ed.), pp. 274–285, North-Holland Publishing Co., Amsterdam
23 Sheinin, R. and Onodera, K. (1970) Can. J. Biochem. 48, 851–857
24 Gahmberg, C. G. and Hakomori, S. (1973) Proc. Natl. Acad. Sci. U.S. 70, 3329–3333
25 Critchley, D. R. (1974) Cell 3, 121–125
26 Vaheri, A. and Ruoslahti, E. (1974) Int. J. Cancer 13, 579–586
27 Chiarugi, V. P. and Urbano, P. (1972) J. Gen. Virol. 14, 133–140
28 Stone, K. R., Smith, R. E. and Joklik, W. K. (1974) Virology 58, 86–100
29 Hogg, N. (1974) Proc. Natl. Acad. Sci. U.S. 71, 489–492
30 Wickus, G. G., Branton, P. E. and Robbins, P. W. (1974) in Control of Proliferation in Animal Cells (Clarkson, E. and Baserga, R., eds.), pp. 541–546, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
31 Holley, R. W. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2840–2841
32 Wallach, D. F. (1969) Curr. Top. Microbiol. Immunol. 47, 152–176
33 Hynes, R. O. (1974) Cell 1, 147–156
34 Bussell, R. H. and Robinson, W. S. (1973) J. Virol. 12, 320–327
35 Wickus, G. G. and Robbins, P. W. (1973) Nat. New Biol. 245, 65–67
36 Hynes, R. O. and Wyke, J. A. (1975) Virology 64, 492–504
37 Teng, N. N. H. and Chen, L. B. (1975) Proc. Natl. Acad. Sci. U.S. 72, 413–417
38 Brady, R. O. and Mora, P. T. (1970) Biochim. Biophys. Acta 218, 308–320
39 Hakomori, S. and Murakami, W. T. (1968) Proc. Natl. Acad. Sci. U.S. 59, 254–261
40 Mora, P. T., Brady, R. O., Bradley, R. M. and McFarland, V. W. (1969) Proc. Natl. Acad. Sci. U.S. 63, 1290–1296
41 Hakomori, S., Saito, T. and Vogt, P. K. (1971) Virology 44, 609–621
42 Sakiyama, H. and Robbins, P. W. (1972) Fed. Proc. 32, 86–90
43 Hammarström, S. and Björsell, G. (1973) FEBS Lett. 32, 69–72
44 Gahmberg, C. G., Kiehn, D. and Hakomori, S. (1974) Nature 248, 413–415
45 Den, H., Sela, B.-A., Roseman, S. and Sachs, L. (1974) J. Biol. Chem. 249, 659–661
46 Mora, P. T. (1973) in Membrane-Mediated Information (Kent, P. W., ed.), pp. 64–84, Medical Technical Publishing Co. Ltd., Hackensack, New Jersey