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STUDIES OF SURFACE MEMBRANES OF TWO MOUSE FIBROBLAST CELL LINES

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SUMMARY

The proteins and glycoproteins of whole mouse fibroblast surface membranes were examined by polyacrylamide gel electrophoresis. Membranes derived from two mouse cell lines exhibiting different growth behavior and cells at different stages of growth were compared.

A new method for isolation of intact whole surface membranes from small numbers of cultured cells was utilized. The distribution of membrane proteins after polyacrylamide gel electrophoresis was determined by staining the gels or labeling the membranes with radioactive leucine and assaying sequential slices of the gel for radioactivity. No differences were seen between patterns derived from the membranes of the two cell lines, or between those of cells at different stages of growth.

Membrane glycoproteins were labeled with radioactive glucosamine and their distribution determined. The glycoprotein patterns of the two cell lines were similar, but differed from the protein patterns significantly.

INTRODUCTION

The cell surface membrane has been the subject of recent intensive investigations. Many of these studies have utilized enzymatic digestion[1–3], or chemical extraction[4] to examine small portions of these membranes. Other workers have studied whole cell surface membranes derived from large numbers of red blood cells[5,6], liver cells[7–9], or cultured L cells,[10] by solubilizing the membranes and separating the constituent molecules by polyacrylamide gel electrophoresis. Differences in cell surface membrane antigens[11], glycoproteins[1–3], and glycolipids[4] have been reported in cells exhibiting different growth characteristics. However, the relationship of the changes in surface membranes to differences in growth behavior and cell–cell interaction has not been well established.

Using a method developed in our laboratory[12], we have studied whole surface membranes from 3T3 and 3T6 mouse fibroblast cell lines, which exhibit markedly different properties of growth[13]. This method allows rapid isolation of highly purified surface membranes without enzymatic digestion from small numbers of cultured cells. The protein and glycoprotein components of these surface membranes were examined by solubilization and polyacrylamide gel electrophoresis.

MATERIALS

Acrylamide and bis-acrylamide were obtained from Eastman Kodak. The gel slicer was made by sandwiching double edged razor blades between 1 mm thick steel washers. [6-^{3}H]glucosamine, [1-^{14}C]glucosamine, [4,5-^{3}H]leucine and [^{14}C]leucine were purchased from New England Nuclear Corporation. Cells were grown in 75 cm^{2} polystyrene tissue culture flasks from Falcon Plastics.

METHODS

Cell cultures

The 3T3 and 3T6 mouse embryo fibroblast cell lines used in these studies were obtained from Dr. Howard Green. 3T3 cells grow as a monolayer to a density of $0.5 \cdot 10^{6}$–$1.5 \cdot 10^{6}$ cells per flask while the 3T6 cells grow over one another to a depth of 4–6 cells at a density of $2 \cdot 10^{6}$–$6 \cdot 10^{6}$ cells per flask[13]. These densities are maintained at a constant level over a period of 10 days. The cells were grown in 12 ml of Vogt's modification of Eagle's medium containing 10 % calf serum.

Membrane isolation

The cells were washed gently three times with 10-ml portions of 0.16M NaCl containing 0.01 % $CaCl_2$. A solution of 0.001 M $ZnCl_2$ and dimethyl sulfoxide (4:1, v/v) was used to cover the cells for 10 min. This solution was followed by 15 ml of saturated fluorescein mercuric acetate in 0.02 M Tris buffer, pH 8.1. The flask was then shaken on a rotating platform at 120 rev./min. After 15 min the fluorescein mercuric acetate was decanted and new fluorescein mercuric acetate solution was added and the cells shaken again for 15 minutes. The fluorescein mercuric acetate solutions containing a suspension of cellular surface membranes were pooled and isolated by centrifugation at 600 $\times$ g for 10 min. After washing the pellet four times with 10 ml of H_2O, the membranes were suspended in 1 ml of H_2O and stored at 5° until used.

Labeling of confluent cells with radioactive leucine and glucosamine

In order to obtain surface membranes labeled with radioactive leucine or glucosamine, cells were grown to confluence (7 days after transfer) and 12 ml of a special medium was added. This medium contained ^{3}H-labeled leucine or ^{14}C-labeled leucine, 0.1 μC/ml or 1.0 μC/ml respectively, and the non-radioactive leucine was reduced to 10 % of that used in the original medium. Leucine labeled surface membranes were harvested after a 48 h incubation. Medium containing ^{3}H-labeled glucosamine or ^{14}C-labeled glucosamine at a final concentration of 10 μC/ml and 2.5 μC/ml respectively, containing no glucose and using pyruvate 0.02 M as a source of carbohydrate, was used to label the cells with radioactive glucosamine. Membranes labeled with glucosamine were harvested after a 5 day incubation.

Labeling of nonconfluent cells with radioactive leucine or glucosamine

Cells were grown, labeled and their surface membranes harvested, before reaching confluence, by the addition of the radioactive label at an earlier stage of growth. Special medium containing either radioactive leucine or glucosamine was added to

cells 2 h after their transfer. Membranes were harvested after a 3 day incubation with leucine special medium and after a 5 day incubation with glucosamine medium, before the cells had reached confluence.

Solubilization of membranes and polyacrylamide gel electrophoresis

Membrane preparations containing approximately 100 μg of protein or 7000 counts/min were solubilized in 0.26 ml of 0.1 M phosphate buffer, pH 6.8, containing 2 % sodium dodecyl sulfate and 1 % mercaptoethanol, by heating in a boiling water bath for 1 min. To the solubilized membranes, 0.02 ml of 50 % sucrose and 0.02 ml of 0.001 % bromphenol blue were added, resulting in a solution with a volume of 0.3 ml which was loaded on a 5 % polyacrylamide gel (0.5 mm × 100 mm). Gels were polymerized and electrophoresis carried out according to the method of MAIZEL[14], using 0.1 M phosphate buffer, pH 6.8, containing 0.1 % sodium dodecyl sulfate. Electrophoresis was carried out at 10 V/cm (100 V across the gel) until the front of the bromphenol blue dye was at the bottom of the gel (2.5 h). Gels were frozen at —70° and then sliced into 75 fractions using the apparatus described previously. Each fraction was weighed, which allowed correction for the 10 % variation in fraction size when the gel was sliced in this fashion. After drying overnight in a plastic scintillation vial, the gels were dissolved in 0.1 ml of 30 % H_2O_2 at 60° for 2 h[15]. 1 ml of Nuclear Chicago solubilizer and 10 ml of toluene based scintillation fluid were added to the solubilized gel slices which were then counted in a Packard Tri Carb 3800 liquid scintillation spectrometer. When double label studies were carried out crossover from ^3H to ^{14}C was made less than 1 % and crossover from ^{14}C to ^3H was determined by adding [^{14}C]toluene to 5 vials and recounting. Data from double label experiments were normalized by making the total number of counts collected in each channel equal to one another before plotting. Unlabeled membrane gels were fixed overnight with 10 % trichloroacetic acid, stained with 0.25 % Coomassie blue for 2 h and destained with 7.5 % acetic acid.

Molecular weight determinations were calculated from the migration of known standards using the conditions of electrophoresis described[16].

Protein was determined by the method of Lowry[17].

RESULTS

Surface membranes

Phase microscopy of isolated surface membranes of 3T3 and 3T6 cells revealed a remarkably uniform appearance of large membrane fragments (Fig. 1). Electron microscopy of the final membrane pellets revealed large numbers of highly folded and tortuous membranes in which a unit membrane structure could be seen (Fig. 2). In none of the sections examined were nuclear or cytoplasmic organelles other than rare small vesicles seen.

Surface membrane proteins

3T3 and 3T6 surface membranes were completely solubilized by sodium dodecyl sulfate and mercaptoethanol treatment, as judged by the absence of a pellet after ultracentrifugation. When these solubilized membranes were electrophoresed on 5 % polyacrylamide gels in 0.1 % sodium dodecyl sulfate a large number of separate

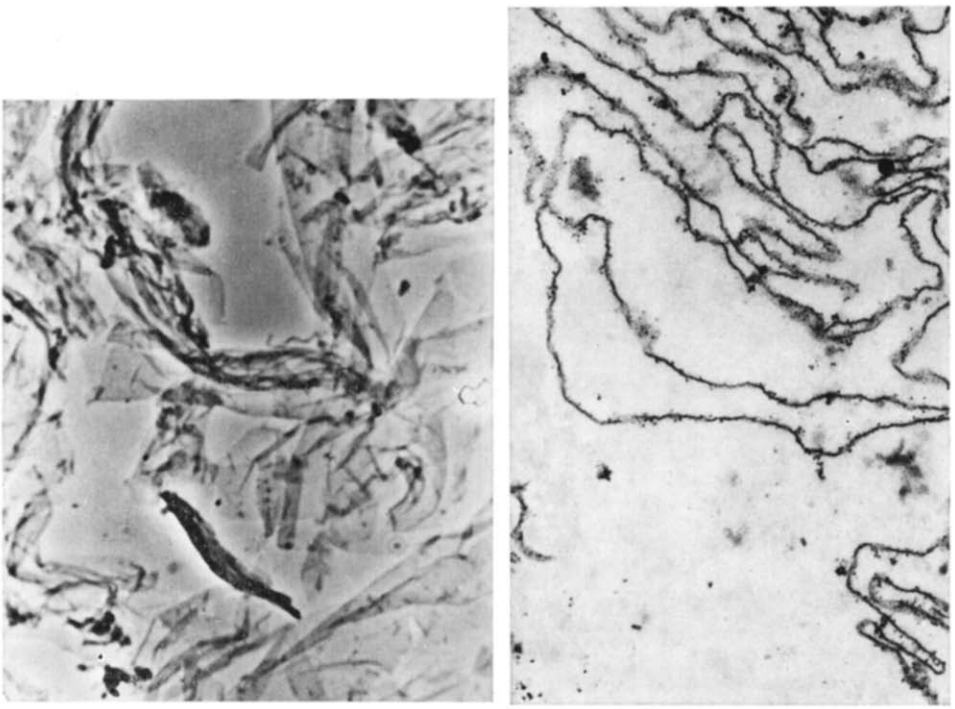

Fig. 1. Phase photomicrograph of isolated 3T6 surface membranes. Large membrane fractions of uniform appearance are present. × 640.

Fig. 2. Electron micrograph of 3T6 surface membranes. Long folded segments of membranes with occasional attached granules are seen. × 10500.

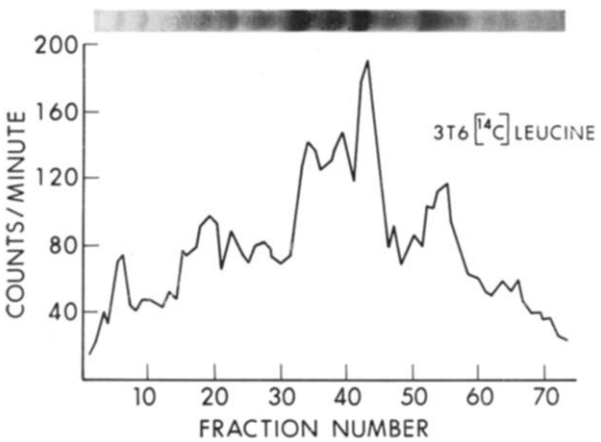

Fig. 3. A polyacrylamide gel stained with Coomassie blue after electrophoresis of 3T6 surface membranes is compared with another gel fractionated and assayed for radioactivity as described in the text after electrophoresis of [^{14}C]leucine labeled 3T6 membranes. Three major protein bands or peaks of approx. 80000, 66000 and 57000 daltons are seen in both techniques. The close similarity between the two methods of studying membrane protein is seen.

protein bands could be resolved. Coomassie blue staining revealed at least 14 distinct bands (Fig. 3). Addition of iodoacetate to a concentration of 0.03 M to the solubilized membranes did not alter their polyacrylamide gel electrophoretic staining pattern. The staining pattern of membranes from both cell types was similar with three major bands having molecular weights of approximately 80000, 66000 and 57000 daltons. Many minor bands with molecular weights ranging from 250000 to 30000 daltons were seen.

Because of the differences in protein staining and migration of membrane proteins from gel to gel, comparisons by this technique are difficult. In addition, Coomassie blue staining is nonstoichiometric and therefore quantitative comparisons of membrane proteins are not valid. By co-electrophoresing membranes labeled with ^{3}H-labeled leucine or ^{14}C-labeled leucine on the same gel, a direct quantitative comparison of two separate membrane preparations was possible.

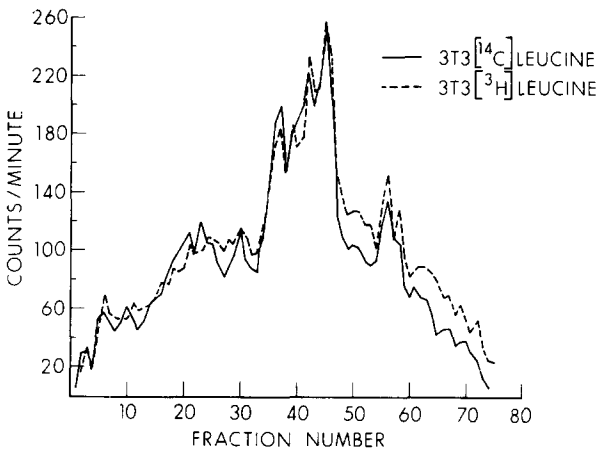

Fig. 4. 3T3 surface membranes labeled with [^{3}H]leucine and 3T3 surface membranes labeled with [^{14}C]leucine were co-electrophoresed on the same gel. The patterns of radioactively labeled proteins show no differences. This observation establishes the reproducibility of the membrane isolation technique.

Radioactively labeled surface membrane proteins were prepared by growing the cells in the presence of ^{3}H-labeled leucine or ^{14}C-labeled leucine for 2 days and the distribution of radioactivity determined in gel slices as described under METHODS. The distribution of radioactivity correlated quite closely with the staining pattern previously described (Fig. 3), although the resolution of proteins was not as discrete. The [^{3}H]leucine-labeled-3T6membranes and [^{14}C]leucine-labeled 3T6 membranes were mixed, then solubilized and co-electrophoresed. The close similarity of the ^{3}H and ^{14}C patterns is noted (Fig. 4). This established the reproducibility of the technique and served as a basis for considering differences in other studies. There were no differences seen when [^{3}H]leucine-labeled-3T3 membranes and [^{14}C]leucine-labeled-3T6 membranes were mixed and electrophoresed on the same gel (Fig. 5). Using this technique we compared surface membranes derived from actively dividing nonconfluent 3T3 cells to the same cells in a stationary confluent state. Again no differences were seen (Fig. 6).

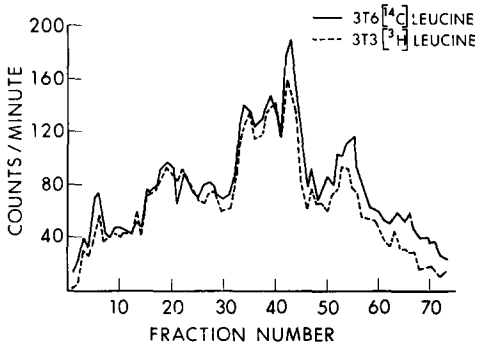

Fig. 5. Surface membranes from 3T3 cells labeled with [^{3}H]leucine and 3T6 cells labeled with [^{14}C]leucine were co-electrophoresed on the same gel. The close similarity of the labeled membrane proteins from these two cell lines is clearly demonstrated.

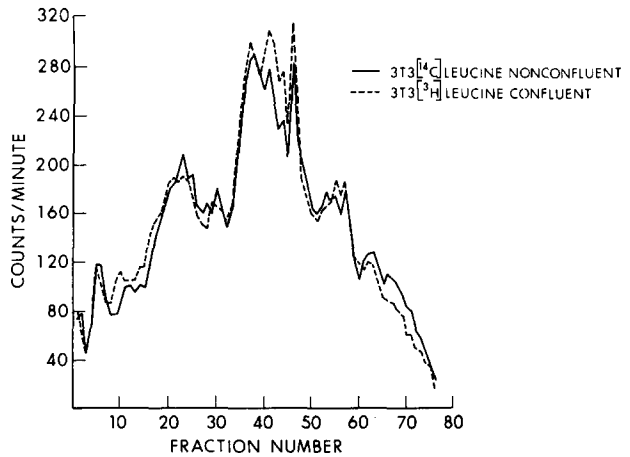

Fig. 6. The distribution of radioactively labeled proteins after co-electrophoresis of surface membranes from confluent 3T3 cells labeled with [^{3}H]leucine and nonconfluent actively growing 3T3 cells labeled with [^{14}C]leucine is depicted. No significant differences are seen.

Surface membrane glycoproteins

Surface membranes of 3T3 cells grown for 3 days in the presence of ^{3}H-labeled glucosamine to label glycoproteins and ^{14}C-labeled leucine to label proteins were electrophoresed. Differences in the distribution of glycoproteins and proteins were seen (Fig. 7). A large percentage of the glycoproteins (^{3}H counts) were always seen at the top of the gel where no corresponding protein band was present. The remaining glycoprotein peaks corresponded to the protein pattern (^{14}C counts). When membrane glycoproteins from 3T3 cells (^{14}C counts) were mixed with those from 3T6 cells (^{3}H counts) and electrophoresed similar patterns were obtained.

DISCUSSION

A new technique of surface membrane isolation developed in our laboratory allows the rapid isolation of purified membranes from small numbers of cultured

cells. Using material isolated by this method, we have shown that the proteins of cell surface membranes derived from cultured 3T3 and 3T6 mouse fibroblast cells are heterogeneous. Their molecular weights range from approximately 30000 to 300000 daltons. These data are in agreement with studies of whole surface membranes of cells

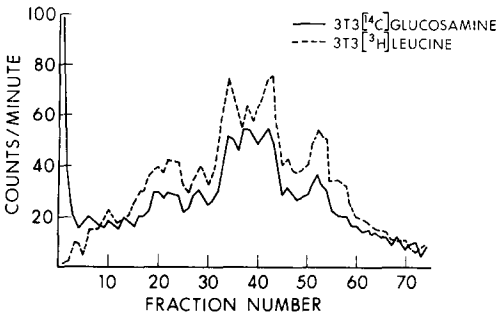

Fig. 7. The distribution of labeled proteins and glycoproteins after co-electrophoresis of surface membranes obtained from 3T3 cells labeled with [^{3}H] leucine and [^{14}C] glucosamine respectively is shown. A high molecular weight component labeled with glucosamine is present at the top of the gel, (fractions 1 and 2) where no leucine counts are seen.

isolated by other techniques[6–10]. The failure of iodoacetate to alter the electrophoretic pattern of solubilized membranes indicates that disulfide bonds broken by mercaptoethanol are not reformed in the presence of sodium dodecyl sulfate. More than 90 % of the radioactively labeled proteins or glycoproteins applied to the gels were recovered after electrophoresis. These data are in contrast with the study of LAICO *et al.*[18] who found a large percentage of membrane proteins in a fraction with a molecular weight of 5000 daltons. If a comparable small protein is present in the surface membranes of 3T3 and 3T6 cells it is not labeled by leucine after a 48 h incubation. The failure of iodoacetate to alter the distribution of radioactive proteins in the gels also indicates that these small proteins are not present as aggregates in our solubilized membrane preparations.

Comparison of surface membranes electrophoresed and then stained with Coomassie blue with surface membrane labeled with leucine and counted after electrophoresis gave similar patterns. This similarity suggests that there is active turnover of the major surface membrane proteins. Three major bands with molecular weights of 80000, 66000 and 57000 daltons constitute a large percentage of the total membrane proteins. This conclusion is supported by the heavy staining and the large number of counts present in these bands.

Patterns obtained from leucine labeled membranes remained constant, showing no variations between 3T3 and 3T6 or actively growing 3T3 and confluent 3T3.

Membrane glycoproteins were studied by labeling cells with glucosamine[19]. The patterns obtained from membranes labeled with glucosamine differed from those obtained with leucine labeled surface membranes. This difference consisted of a large molecular weight glucosamine labeled band where there was no corresponding leucine labeled band. The small amount of protein associated with this glucosamine-labeled macromolecule suggests that it may be a proteoglycan. 3T3 cells synthesize proteoglycans (hyaluronic acid) in culture[20]. BUCK *et al.*[3] have demonstrated a large

molecular weight glucosamine-labeled molecule present in cultured fibroblasts, which is susceptible to hyaluronidase digestion. Heparin sulfate has also been shown to be associated with membranes of cultured cells[21,22]. The nature and role of the macromolecule demonstrated in our mouse embryo fibroblasts is unclear, but its presence in large quantities on their surface membranes suggests that it may have an important function. The correlation between the leucine-labeled bands and the remaining glucosamine-labeled bands indicates that many of the proteins of the surface membrane are glycosylated. Previous studies of the whole surface membrane glycoproteins by polyacrylamide gel electrophoresis have been limited to liver[8,9] and red blood cells[23] in which glycoproteins were detected by the Schiff periodate staining procedure which demonstrated one or two major bands and many minor bands. Although the surface membrane glycoproteins from 3T3 and 3T6 cells had similar patterns after acrylamide gel electrophoresis, the degree of variation in the results did not permit any definite conclusions regarding minor differences between their surface membrane glycoprotein. It is interesting that the surface membrane sialic acid content of L cells have recently been reported to show inexplicable fluctuations[24].

We would conclude from these comparative studies that the differences in growth behavior between 3T3 and 3T6 cells, or confluent 3T3 and nonconfluent 3T3 cells, are neither mediated by nor reflected in major changes in the protein or glycoprotein composition of their surface membranes. With these techniques we cannot exclude significant alterations of a minor protein or glycoprotein nor differences in the configuration or position of proteins within the surface membranes which might account for the different growth behavior demonstrated by these cells. These observations cast a note of caution on the conclusions derived from studies of virus-transformed cells in which changes in contact inhibition and cell growth have been attributed to changes demonstrated in the surface membranes[2,4,25,26].

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