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Membrane Proteins of Uninfected and Rous Sarcoma Virus-Transformed Avian Cells

ROBERT H. BUSSELL[1] AND WILLIAM S. ROBINSON
Department of Medicine, Stanford University, Stanford, California 94305

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A method for preparing large membrane fragments and cell ghosts was developed for uninfected and Rous sarcoma virus-transformed chicken embryo fibroblasts in culture. Membrane proteins were analyzed by electrophoresis in acrylamide gels containing sodium dodecyl sulfate. A major amino-acid-containing component of uninfected cell membranes was greatly diminished in amount or absent in membranes of virus-transformed cells. This component, called MP-1, had an electrophoretic mobility in sodium dodecyl sulfate-containing gels similar to that of a protein of a mol wt of 1.42×10^5. MP-1 was not altered by changes in cell growth rate or in cells infected with the nontransforming virus RAV-1.

Several properties of tissue culture cells transformed by Rous sarcoma virus (RSV) and other tumor viruses suggest that cell membrane changes are associated with viral transformation. Among these properties are loss of contact inhibition of cell movement (1, 30), changes in cell surface charge (1, 12, 13), changes in sugar transport (15, 16, 22), and changes in the agglutination of cells by wheat germ agglutinin and concanavalin A (7, 8, 20).

Recently, chemical differences between uninfected and virus-transformed cells have been reported. Alterations in membrane components such as carbohydrates (25, 26), glycolipids (14, 24), and glycopeptides (2) have been found in virus-transformed cells. Buck et al. (4, 5) showed that, when material labeled with radioactive fucose or glucosamine was removed from the surface of RSV-transformed cells by trypsin and digested with Pronase, it appeared to have a higher average molecular weight by Sephadex G-50 chromatography than did labeled material from uninfected cells. Similar changes were also observed in purified plasma membranes (4). These results suggested that the carbohydrate moiety of cell surface glycoproteins was altered by virus transformation. Chiarugi and Urbano (9) have also recently presented evidence for changes in the glycosylated portion of large membrane glycoproteins of polyoma virus-transformed hamster cells.

In this study, we isolated large membrane fragments rich in cell ghosts from uninfected and RSV-transformed chicken embryo cells and separated the membrane proteins by electrophoresis in sodium dodecyl sulfate (SDS)-containing gels. A major protein component of uninfected cell membranes was greatly diminished in amount or absent in membranes from virus-transformed cells.

[1] Present address: Department of Microbiology, University of Kansas, Lawrence, Kan. 66044.

MATERIALS AND METHODS

Chemicals. A mixture of 15 ^{3}H-amino acids (1 to 10 Ci/mmol), ^{14}C-amino acid mixture (~0.10 Ci/mmol), L-fucose-6-^3H (10 Ci/mmol), and thymidine-*methyl*-^3H (20 Ci/mmol) were purchased from New England Nuclear Corp. Ovalbumin was purchased from Pharmacia Fine Chemicals, Inc., crystallized bovine serum albumin was from Miles Laboratories, Inc., 2× crystallized rabbit muscle phosphorylase A was from Worthington Biochemical Corp., Triton X-100 was from Emulsion Engineering Corp., and toluene was from J. T. Baker Chemical Co.

Virus. The Bryan high-titer strains of RSV and Rous-associated virus (RAV-1) were grown in chicken embryo fibroblast (CEF) cultures to produce virus stocks with 10^6 to 10^7 focus-forming units per ml. The L-Kansas strain of Newcastle disease virus (NDV) was grown in embryonated eggs to produce virus stocks with titers of 10^8 to 5×10^8 plaque-forming units (PFU) per ml (3).

Cell culture. CEF cultures completely transformed by RSV(RAV-1) and parallel cultures of uninfected cells were prepared as previously described (27) and maintained in growth medium 1 (17) in 100-mm plastic tissue culture dishes. Confluent monolayers of CEF in 100-mm dishes were infected with NDV at a multiplicity of 5 to 10 PFU per cell as previously described (3).

Incubation of cells with ^{3}H-amino acids, ^{14}C-amino acids, ^{3}H-fucose, or ^{3}H-thymidine. Medium was removed from RSV(RAV-1)-transformed and uninfected cultures in 100-mm tissue culture dishes, the cells were washed one time with Tris-saline buffer, and 3 ml of amino acid-free medium (95 parts of medium 199 without amino acids, except for glutamine, and 5 parts of dialyzed calf serum) was placed on each culture for incubation at 41 C in a CO_2 incubator for 30 min. The medium on each culture was then replaced with 3 ml of amino acid-free medium containing 5 to 10 μCi of ^{3}H-amino acid mixture or 1 to 2 μCi of ^{14}C-amino acid mixture per ml, and incubation at 41 C was continued for the times specified for each experiment.

In some experiments, uninfected or RSV(RAV-1)-transformed CEF were incubated with ^{3}H-fucose (5 μCi per ml) in glucose-free medium (Earle balanced salt solution with amino acids and vitamins as in Eagle minimal essential medium) or with ^{3}H-thymidine (1 μCi per ml) in growth medium 1 (17), with 3 ml per dish for 2 h in a CO_2 incubator at 41 C.

Cell fractionation and membrane preparation. A method similar to that described by Atkinson and Summers (2) was used to prepare a membrane fraction rich in cell ghosts. In a usual experiment, 8 to 12 100-mm monolayer cultures of RSV(RAV-1)-transformed and uninfected CEF were washed three to five times with isotonic buffer (NaCl, 0.15 M; Tris-hydrochloride 0.01 M, pH 7.4) and the cells were then incubated for 30 min at 37 C with isotonic buffer containing 0.01 M EDTA. Cells were removed from the dishes by vigorous pipetting, collected by centrifugation at 230 $\times$ *g* for 6 min, suspended in 10 ml of hypotonic buffer (Tris-hydrochloride, pH 8.0, 0.01 M; sodium azide, 0.01 M) and again centrifuged at 230 $\times$ *g* for 6 min. Cells were then resuspended in 20 ml of hypotonic buffer containing sodium iodoacetate, 0.015 M, and held at room temperature for 5 to 10 min. Cells were then broken in a loose-fitting Dounce homogenizer by using 6 to 8 strokes for RSV(RAV-1)-transformed cells and 8 to 10 strokes for uninfected cells. Cell breakage was monitored by microscopy examination in all experiments. The homogenate was then centrifuged at 230 $\times$ *g* for 40 s, and the supernatant fluid was removed and recentrifuged in the same way to remove nuclei (11). The combined pellets are referred to as the 230 $\times$ *g* pellet. The supernatant fluid was layered over a discontinuous sucrose density gradient consisting of 6 ml of 45% (wt/wt) sucrose overlaid with 14 ml of 30% sucrose, both containing Tris-azide buffer, and centrifuged at 7,000 rpm and 2 C for 20 min in a Spinco SW27 rotor. The membrane band at the interface between the 45 and 30% sucrose layers was collected, diluted fivefold with hypotonic buffer, and centrifuged at 10,000 rpm and 2 C for 10 min in a Sorvall SS34 rotor. The pellet was resuspended in hypotonic buffer by pipetting and was rebanded in a discontinuous 30 over 45% sucrose gradient in a Spinco SW40 rotor, the membrane fraction was again collected, and membranes were recovered from the sucrose solution by centrifugation in a Sorvall SS34 rotor. The material in the pellet is referred to as the membrane fraction from the 30 over 45% sucrose gradient. In some experiments, the material remaining above the 30% sucrose layer after the first centrifugation in the SW27 rotor was recovered and centrifuged at 100,000 $\times$ *g* in a Spinco 50 Ti rotor for 2 h, which resulted in a 100,000 $\times$ *g* pellet and a 100,000 $\times$ *g* supernatant fraction.

Gel electrophoresis.. Electrophoresis in 5% polyacrylamide gels containing SDS was carried out as previously described (19). Membrane fractions were prepared for electrophoresis by dissolving in Tris-hydrochloride (pH 7.4, 0.01 M), 2% SDS, 2% 2-mercaptoethanol, and 6 M urea and by heating in a boiling water bath for 2 to 3 min.

Radioactive counting. Radioactive samples were assayed in a scintillation spectrometer after trichloroacetic acid precipitation as previously described (3) or by making aqueous samples (or polyacrylamide gel slices) to a volume of 0.75 ml by adding 1% Triton X-100 in water and then added 10 ml of toluene-Triton X-100 (3 vol plus 1 vol) scintillation fluid.

RESULTS

Membrane preparation. The conditions for cell breakage by Dounce homogenization were critical and slightly different for uninfected and RSV(RAV-1)-transformed cells. Cells transformed by RSV(RAV-1) were larger in size and more fragile than were uninfected cells, and fewer homogenizing strokes were needed to break the transformed cells.

By using microscopy criteria, almost all unbroken cells and most nuclei were removed by two centrifugations at 230 $\times$ *g* for 40 s. Microscopy examination of the final membrane preparation, from RSV(RAV-2)-transformed or uninfected CEF banded two times in discontinuous 30 over 45% sucrose gradients, demonstrated the presence of many ghosts and large membrane fragments and few, if any, recognizable whole cells or nuclei. The nuclei remaining in the supernatant fluid after centrifugation at 230 $\times$ *g* were found to pass through the 45% sucrose layer to the bottom of the tube, unlike the cell ghosts and large membrane fragments which floated on the 45% sucrose layer during centrifugation at 7,000 rpm for 20 min in the 30 over 45% discontinuous sucrose gradient. During the latter step, only large membrane fragments sedimented down the gradient, and small particles and soluble proteins remained at the top of the gradient.

An experiment was done to determine the distribution of the DNA of an unfractionated cell homogenate after centrifugation in a 30 over 45% discontinuous sucrose gradient. Uninfected cell cultures were incubated with ^{3}H-thymidine to label cell DNA or with ^{3}H-fucose, and cell homogenates of each were prepared by Dounce

homogenization. Both homogenates, without preliminary low-speed centrifugation at 230 × *g*, were layered over discontinuous 30 over 45% sucrose density gradients and centrifuged at 7,000 rpm and 2 C for 20 min. Fractions were collected from the bottom of each tube, and the pellets on the bottom of the tubes were recovered for determination of trichloroacetic acid-precipitable radioactivity. The results for the two sucrose gradients are shown in Fig. 1. More than 98% of the tritium-labeled DNA was in the pellet on the bottom of the centrifuge tube, and less than 2% was floating on the 45% sucrose layer. This result is consistant with the microscopy finding that almost all nuclei pass through the 45% sucrose layer.

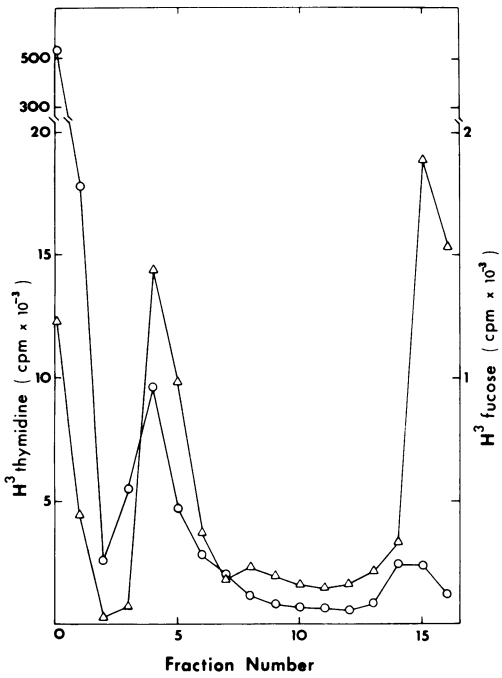

FIG. 1. *Distribution of radioactivity in discontinuous sucrose density gradients after centrifugation of homogenates of cells that were incubated with* ^{3}*H-thymidine* (○) *or* ^{3}*H-fucose* (△). *Eight 100-mm cultures of uninfected chicken embryo fibroblasts were incubated with* ^{3}*H-fucose and eight were incubated with* ^{3}*H-thymidine, and the cells from each set were then removed from the dishes and disrupted by homogenization as described in Materials and Methods. Both whole unfractionated cell homogenates were layered over separate discontinuous 30 over 45% sucrose density gradients and centrifuged at 7,000 rpm and 2 C for 20 min in a Spinco SW27 rotor. Fractions were collected from the bottom of each tube, and samples were removed for trichloroacetic acid precipitation and counting.*

The trichloroacetic acid-precipitable ^{3}H-fucose-labeled material had a different distribution in the sucrose gradient. Almost one third was at the interface between 30 and 45% sucrose, and a significant proportion remained at the top of the centrifuge tube.

In a typical experiment, when uninfected or RSV(RAV-1)-transformed cells were incubated with ^{3}H-thymidine for 1 to 2 h, the total trichloroacetic acid-precipitable ^{3}H-thymidine in the final membrane preparation, prepared by the standard procedure described in Materials and Methods, was less than 0.5% of that in the unfractionated cell homogenate. In similar experiments, the recovery of trichloroacetic acid-precipitable ^{3}H-fucose, ^{3}H-glucosamine, or ^{3}H-amino acids in the final membrane preparation was around 5% of that in the starting homogenates.

When the final membrane preparations from ^{3}H-amino acid-labeled uninfected cells and ^{14}C-amino acid-labeled RSV(RAV-1)-transformed cells were centrifuged together in a linear sucrose density gradient, both radioactive membrane preparations were fairly homogeneous with respect to buoyant density. In several experiments, the radioactive RSV(RAV-1) cell membranes had a slightly lower mean buoyant density and were more heterodisperse than were the membranes from uninfected cells (Fig. 2).

Proteins in membrane preparations from uninfected, RSV(RAV-1)-transformed and NDV-infected cells. Uninfected CEF cultures, RSV(RAV-1)-transformed cells, and cells 10 h after infection with NDV were incubated with a ^{3}H-amino acid mixture for 1 h, and cell membrane preparations were then made from the cells of each type. Fig. 3 shows the distribution of ^{3}H-amino acid-labeled proteins present after electrophoresis in SDS-gels. Many labeled proteins were present in the membrane preparation from uninfected cells (Fig. 3A), and separation of individual components was not complete. The electrophoretic pattern of RSV(RAV-1)-transformed cell membrane proteins (Fig. 3B) was not regularly different from that of uninfected cells, except in the position designated membrane protein 1 (MP-1). The radioactive protein component MP-1 was regularly present in uninfected cells as a major membrane component. Its relative amount was the same in membrane preparations isolated from uninfected cells which were rapidly growing, uninfected cells in confluent monolayers, and cells infected with RAV-1, an avian leukosis virus which replicates to high titers in CEF but which, unlike RSV, does not alter morphology or growth properties of infected cells (28). The

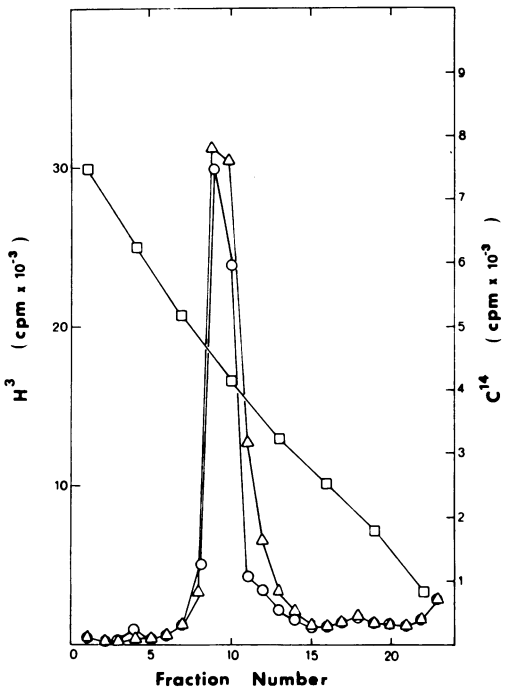

FIG. 2. *Centrifugation of radioactive membranes in a sucrose density gradient. RSV (RAV-1)-transformed cells were incubated with ^{14}C-amino acids (Δ) and uninfected cells with ^{3}H-amino acids (O), and membranes were prepared as described in Materials and Methods. Purified membranes of each type were mixed and layered over a preformed, linear 15 to 60% sucrose density gradient and centrifuged at 40,000 rpm and 2 C for 4 h in a Spinco SW40 rotor. Fractions were collected from the bottom of the tube, and solution density and trichloroacetic acid-precipitable radioactivity were determined.*

amount of radioactive MP-1 relative to other proteins in membrane preparations from uninfected cells was the same after incubation of uninfected cells with ^{3}H-amino acids for times from 15 min to 12 h. Because of the large number of unseparated proteins in the membrane preparation from RSV(RAV-1)-transformed cells resulting in a significant level of radioactivity over almost the entire length of the gels and because no individual radioactive component could be distinguished in the position of MP-1, it was difficult to estimate the amount of radioactive MP-1 in membrane preparations from RSV(RAV-1)-transformed cells. The radioactive material in the position of MP-1 in such gels may not be MP-1, but other proteins with similar electrophoretic mobilities. The amount of radioactivity migrating in the region of MP-1 in RSV(RAV-1)-transformed cell membranes was close to the background level of minor radioactive proteins migrating with MP-1. Improved protein separation will be required to determine the precise amount of MP-1 in membrane preparations from RSV(RAV-1)transformed cells.

When cells, after incubation with ^{3}H-amino acids, were removed from dishes by incubation with 0.25% crude trypsin for 10 min at 37 C and membranes were isolated, the SDS-gel patterns of ^{3}H-labeled membrane proteins in uninfected and RSV(RAV-1)-transformed cells and the relative amount of MP-1 in uninfected cell membranes were indistinguishable from those in cells removed with EDTA.

Unfractionated homogenates and membrane preparations from NDV-infected cells (Fig. 3C) contained about 10% of the total amount of radioactive protein present, respectively, in homogenates and membrane preparations of uninfected or RSV(RAV-1)-transformed CEF. The major radioactive proteins in the membrane preparation were from NDV-infected cells, indicating that synthesis of these proteins (including MP-1) was greatly reduced by NDV infection. In contrast, there was no apparent inhibition of synthesis of cellular proteins (except for MP-1) in membrane preparations from RSV(RAV-1)-infected cells (Fig. 3A and B).

Several attempts were made to detect newly synthesized virion structural proteins in membrane fractions from RSV(RAV-1)-infected cells by using radioactive virions for coelectrophoresis in double-isotope experiments. Although RSV(RAV-1)-infected cells were synthesizing large amounts of viral protein and whole virus, no viral proteins could be detected among the large number of other labeled proteins in membrane fractions from these cells.

Coelectrophoresis of radioactive membrane proteins from uninfected and RSV-(RAV-1)-transformed cells. Although the difference in membrane proteins from uninfected and RSV(RAV-1)-transformed cells (Fig. 3A and B) was quite reproducible and was observed in many individual experiments, in order to clearly demonstrate the difference, the two membranes were analyzed together by electrophoresis in the same gel. Uninfected cells were incubated for 2 h with ^{3}H-amino acids, and RSV(RAV-1)-transformed cells were incubated for 2 h and ^{14}C-amino acids as described in Materials and Methods. Samples of the membrane preparations derived from each cell type were mixed; SDS, urea, and mercaptoethanol were added; and the sample was heated and subjected to gel electrophoresis as described in Materials and Methods. MP-1 was demonstrated in the membrane material from uninfected cells and was not detected as a distinct

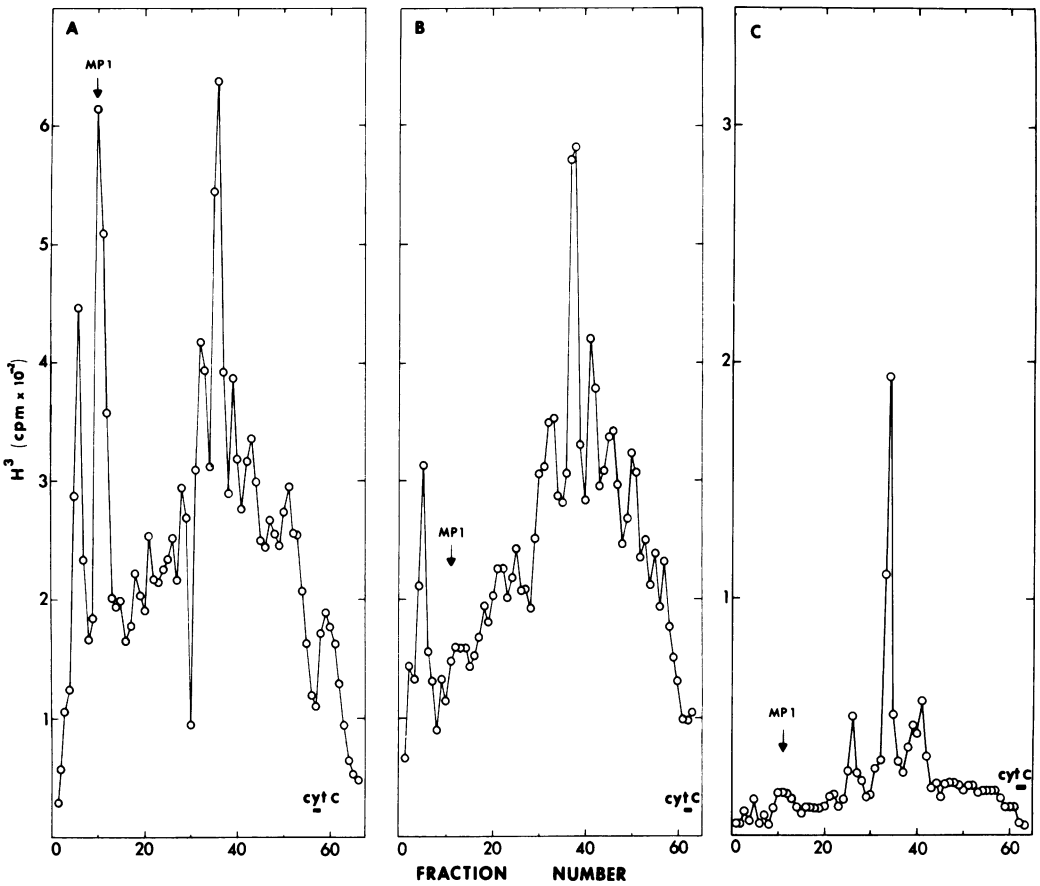

FIG. 3. *Electrophoresis of ^{3}H-amino acid-labeled proteins of membranes from uninfected (A), RSV(RAV-1)-transformed (B), and NDV-infected (C) cells. Cultures of uninfected chicken embryo cells, RSV(RAV-1)-transformed cells, and cells 10 h after infection with NDV (3.8 × 10^7 cells) were incubated for 1 h with a mixture of ^{3}H-amino acids as described in Materials and Methods. Cell membranes were prepared and membrane proteins were dissociated in sodium dodecyl sulfate, 2-mercaptoethanol, and urea. Electrophoresis in sodium dodecyl sulfate-gels was carried out on equal samples from the three preparations.*

component in transformed cell membranes (Fig. 4).

Estimation of the size of MP-1. The molecular weight of MP-1 was estimated by the method of Shapiro et al. (29) to be 1.42×10^5, based on its electrophoretic mobility in SDS-gels relative to proteins of known molecular weight (Fig. 5). It is not known how accurate this method is for glycoproteins, so that if MP-1 is a glycoprotein, the above estimate may not be as accurate as for a nonglycoprotein.

Occurrence of MP-1 in different cell fractions. Uninfected (Fig. 6A, C, E, and G) and RSV(RAV-1)-transformed (Fig. 6B, D, F, and H) cells were incubated with ^{3}H-amino acids for 1 h, disrupted in a Dounce homogenizer, and fractionated by centrifugation as described in Materials and Methods. MP-1 was clearly present only in the large membrane preparation from normal cells recovered from the 30 over 45% interface of the discontinuous sucrose density gradient (Fig. 6A). Figure 6B shows a typical electrophoretic pattern of proteins of the same membrane fraction from RSV(RAV-1)-transformed cells in which MP-1 is absent or present in a much smaller amount. None of the other cell fractions, including the 230 × *g* pellets (Fig. 6C and D) and the 100,000 × *g* supernatant fluids (Fig. 6G and H) from uninfected and RSV(RAV-1)-transformed cells, contained discernible amounts of MP-1. This result suggests that MP-1 is present in the greatest amount in the large membrane fraction of uninfected cells.

DISCUSSION

We have prepared a membrane fraction from uninfected chicken embryo tissue culture cells

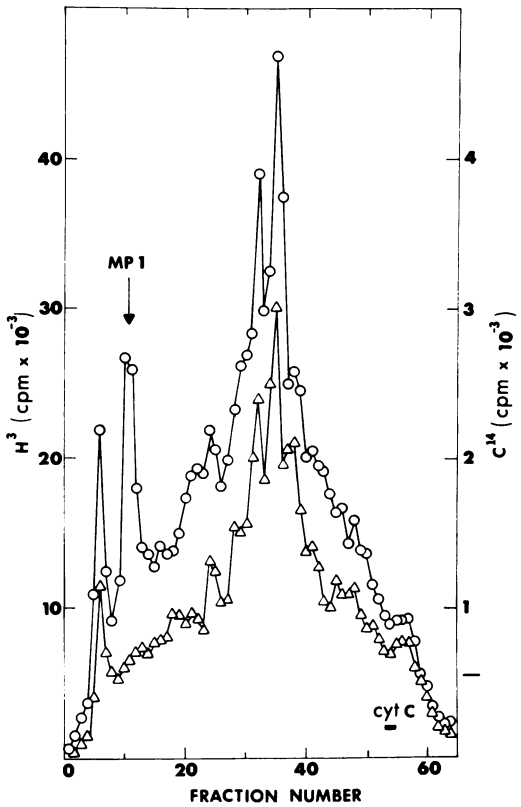

FIG. 4. *Coelectrophoresis of membrane proteins from uninfected and RSV(RAV-1)-transformed cells. Uninfected cells were incubated with ^{3}H-amino acids (O), RSV(RAV-1)-transformed cells were incubated with ^{14}C-amino acids (Δ) for 2 h, and membranes were prepared as described in Materials and Methods. Samples of the two membrane preparations were mixed and sodium dodecyl sulfate, urea, and mercaptoethanol were added, and the sample was heated and subjected to gel electrophoresis as described in Materials and Methods.*

and from cells after transformation with RSV(RAV-1) by a method similar to that reported by Atkinson and Summers (2). Cells were disrupted by Dounce homogenization, and cell homogenates were fractionated by centrifugation, by using conditions which yielded membranes with characteristic size and buoyant density. Microscopic examination of the final membrane preparation revealed large membrane fragments, many cell ghosts, and no unbroken cells or nuclei. Unbroken cells and nuclei were eliminated by low-speed centrifugation, and any remaining nuclei were removed from the large membranes during the sucrose gradient centrifugation. Less than 0.5% of the DNA in a cell homogenate was recovered from the interface between 30 and 45% sucrose (Fig. 1), where the large membranes were found after centrifugation, indicating that nuclear contamination of the final membrane preparation was very low. Soluble proteins and very small particulate structures remained above the 30% sucrose layer under the conditions used for the sucrose gradient centrifugation (7,000 × *g* for 20 min) step in membrane isolation.

These experiments were initiated in an attempt to study synthesis of RSV(RAV-1) virion proteins and assembly of virions in cell membranes. Newly synthesized virion polypeptides can be detected in membrane preparations from cells infected with NDV (Fig. 3C), influenza virus (18, 21), and vesicular stomatitis virus (10), because these viruses appear to switch off synthesis of most normal membrane proteins. This is not the case in cells infected with RSV(RAV-1), in which most of the same membrane proteins were synthesized at the same relative rates as in uninfected cells (Fig. 3A and B). Probably because of the active synthesis of normal cell membrane proteins in RSV(RAV-1)-transformed cells, synthesis of virion polypeptides was not detected.

A change in the membrane proteins of uninfected cells was regularly observed after transformation with RSV(RAV-1). A major radioac-

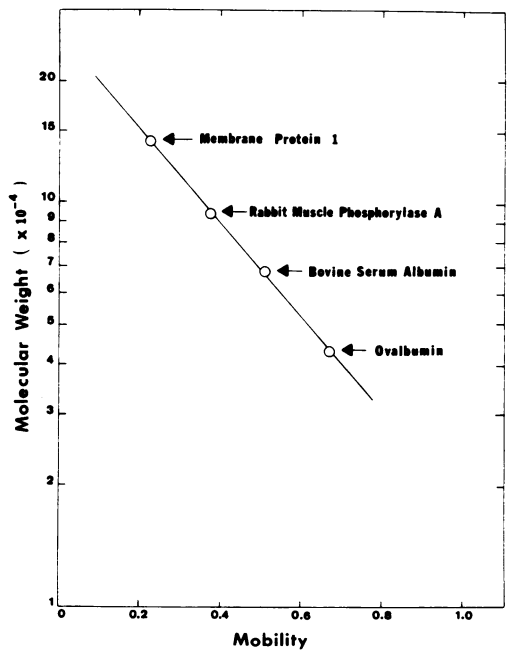

FIG. 5. *Plot of electrophoretic mobility in sodium dodecyl sulfate-gels against molecular weight for three standard proteins for estimation of membrane protein 1 molecular weight by the method of Shapiro et al. (29).*

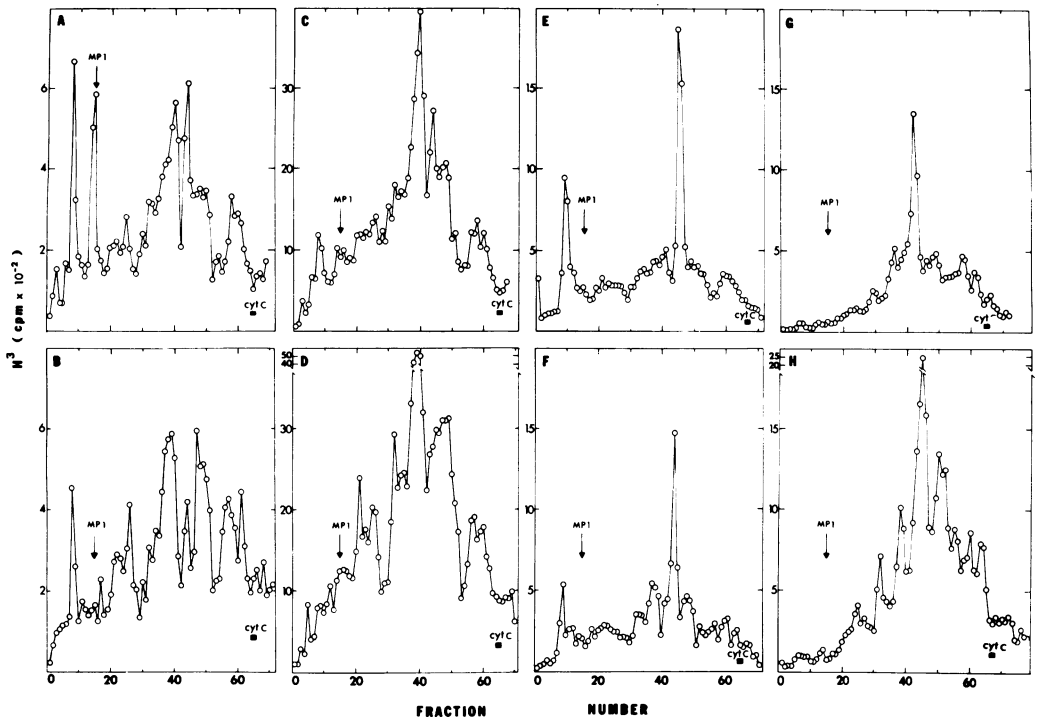

FIG. 6. *Electrophoresis of proteins in fractions from ^{3}H-amino acid-labeled, uninfected (A, C, E, and G) and RSV(RAV-1)-transformed (B, D, F, and H) cells. Cultures of uninfected chicken embryo fibroblasts (4.0 × 10^6 cells) and RSV(RAV-1)-transformed chicken embryo fibroblasts (2.2 × 10^6 cells) were incubated for 1 h with a mixture of ^{3}H-amino acids as described in Materials and Methods. Cells were then removed from the culture dishes, disrupted by Dounce homogenization, and fractionated by centrifugation as described in Materials and Methods for preparation of membranes. Electrophoresis in sodium dodecyl sulfate-gels was carried out on equal samples from the 230 × g pellets (E and F), the membrane fractions from the 30 over 45% sucrose gradients (A and B), the 100,000 × g pellets (C and D), and the 100,000 × g supernatant fractions (G and H).*

tive protein component of uninfected cells, with a molecular weight of about 142,000, was absent or greatly reduced in amount in membrane preparations from RSV(RAV-1)-transformed cells. The radioactive membrane component (MP-1) was present in the same amount relative to other membrane proteins in populations of rapidly growing CEFs and in confluent monolayers in which most cells were not dividing, which suggests that cell growth rate does not differentially affect the synthesis of this component. Infection of cells with a transforming virus, RSV(RAV-1), but not with a nontransforming virus such as RAV-1, was associated with reduction or disappearance of this component. This suggests that viral transformation of cells, rather than avian leukosis virus replication in cells, is necessary for the observed alteration in MP-1.

Experiments with radioactive fucose and glucosamine suggest that the large membrane protein MP-1 is a glycoprotein (unpublished results), although further characterization of the protein will be required to unequivocally establish this. MP-1 is in the size range of membrane components found by Chiarugi and Urbano (9) to have altered glycosylation in polyoma virus-transformed hamster cells. They did not, however, observe a reduction or disappearance of a normal cell, radioactive amino acid-labeled component in virus-transformed cells as we have found in RSV(RAV-1)-transformed cells. Buck et al. (4) also did not detect differences between uninfected and RSV-transformed cell membrane proteins after incubation of cells with radioactive amino acids.

How the change in MP-1 after cell transformation by RSV(RAV-1) may be related to changes in other cell membrane properties and cell behavior after viral transformation is not clear. However, the change in MP-1 supports the concept that viral transformation involves structural changes in cell membranes. One property of the cell surface that is altered by viral transformation is its reactivity with plant lectins. Tissue culture cells transformed by RSV

(8) or other tumor viruses (7, 20) are agglutinated by wheat germ agglutinin and concanavalin A. Treatment of nontransformed cells with proteolytic enzymes, such as trypsin, makes them as agglutinable as transformed cells (6, 7, 20). We have treated normal cells with trypsin and found no effect on MP-1. This suggests that proteolytic action on MP-1 is not involved in the cell surface change caused by trypsin, which results in agglutination by plant lectins.

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