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NON-LYTIC, NON-IONIC DETERGENT EXTRACTION OF PLASMA MEMBRANE CONSTITUENTS FROM NORMAL AND TRANSFORMED FIBROBLASTS

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SUMMARY

A technique has been developed for the selective extraction of plasma membrane protein constituents from normal and transformed cells employing non-ionic detergents. The extraction procedure does not damage cells as judged by cell viability, ^{51}Cr release, and trypan blue staining. Lactoperoxidase-catalyzed iodination followed by detergent extraction permits demonstration of a 100 000 dalton protein which is found on the surface of normal but not transformed hamster and mouse fibroblasts.

INTRODUCTION

Progress in the elucidation of membrane composition and structure has been aided by the use of neutral detergents and bile salt anions [1]. These substances can disrupt membrane organization and selectively solubilize membrane constituents. For the most part, solubilization of membrane constituents has been achieved either by solubilizing the entire membrane and specifically assaying for the component of interest [2–4] or by first isolating the membrane and subsequently solubilizing with detergent [5]. The latter approach has been successfully employed in the sequential solubilization of proteins from erythrocyte ghosts [6, 7].

We have employed an alternative method for extracting proteins from the plasma membrane of normal and transformed fibroblasts by using non-lytic concentrations of detergent to effect solubilization of protein constituents. A similar method has been used to successively solubilize proteins from Semliki Forest virus [8]. In the experiments with the virus, however, final detergent concentrations were employed that completely disrupted the virus. By using non-lytic concentrations of detergents, we have been able to reduce the complexity of the protein mixture extracted from the membranes without causing an increase in permeability of the membrane to macromolecules. In addition, electrophoretic analysis of detergent-solubilized material reveals the presence of a protein of approximately 100 000 daltons, which is present in extracts of normal cells but not transformed cells.

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MATERIALS AND METHODS

Cell culture. Cells employed in these experiments were obtained from established lines maintained at the Imperial Cancer Research Fund Laboratories: baby hamster kidney cells (BHK) and a polyoma virus-transformed derivative (PyBHK) and Balb/C 3T3 and Swiss 3T3 mouse fibroblasts and SV 40-transformed derivatives of each type. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % calf serum in a 5 % CO_2 incubator at 37 °C.

Chemicals. Detergents listed in Table I were obtained from Sigma, except NP-40 which was obtained from the Shell Chemical Co. Glucose oxidase was purchased from Calbiochem and lactoperoxidase from Sigma. Calf serum, Dulbecco's modified Eagle's medium and phosphate-buffered saline were from Gibco. All other chemicals were of the best grade commercially available.

Radiolabelling of cells. Cells were iodinated directly on the dish, using the lactoperoxidase-catalyzed method as previously described [9]. Briefly, before iodination, a confluent monolayer of cells was washed twice at room temperature with phosphate-buffered saline (pH 7.2). To each 5 cm dish was added 0.5 ml of phosphate-buffered saline containing D-glucose (0.9 mg/ml) and carrier-free Na ^{125}I (400 μCi/ml). 10 μl of an enzyme mixture containing lactoperoxidase (1 mg/ml) and glucose oxidase (0.1 unit/ml) was added and the reaction allowed to proceed for 10 min at room temperature. The reaction was stopped by the addition of 5 ml of 0.17 M NaI in 0.01 M phosphate buffer (pH 7.2). The cell monolayers were then washed twice with phosphate-buffered saline and extracted with detergent.

Extraction of cell monolayers with NP-40. Confluent monolayers of cells on 5 cm dishes, either radiolabelled or not, were extracted with non-ionic detergent as follows. The cell layer was washed twice with 5 ml of serum-free medium (37 °C) followed by two washes with cold medium (4 °C). The cells, in 5 ml cold medium, were placed in the cold for 15 min, the medium removed and replaced with 1 ml of cold 0.01 % NP-40 in phosphate-buffered saline. The detergent remained in contact with the monolayer for 30 min at 4 °C with occasional gentle rocking of the dish. After 30 min, the detergent was removed, centrifuged for 2 min at 2000 rev./min to remove any unattached cells, and the supernatant lyophilized.

Preparation of samples for gel electrophoresis. To lyophilized samples was added absolute ethanol (room temperature) and the protein allowed to precipitate overnight at −20 °C. The precipitate was spun at 10 000 rev./min for 15 min, the supernatant containing detergent was discarded, and 10 % trichloroacetic acid (0 °C) added to the precipitate. The mixture was kept on ice for 30 min and then centrifuged at 10 000 rev./min for 15 min. Again the supernatant containing trichloroacetic acid, phosphate and sodium salts was discarded, and the ethanol precipitation step was repeated to remove residual trichloroacetic acid. After discarding the final supernatant containing ethanol and trichloroacetic acid, the protein pellet was dried in vacuo and dissolved directly in electrophoresis buffer containing 2 % sodium dodecyl sulfate, 0.1 M Tris · HCl (pH 6.8) and 10 % glycerol.

Polyacrylamide gel electrophoresis. Prior to electrophoresis, the samples were adjusted to 0.1 M dithiothreitol and boiled for 2 min. Polyacrylamide gel electrophoresis [10, 11] was performed in a slab gel [12] at an acrylamide monomer concentration of 7.5 %. After completion of the electrophoresis the gels were stained

with Coomassie Brilliant Blue to visualize protein bands [13]. When radiolabelled proteins were electrophoresed, the slab gels were dried in vacuo and autoradiographed using Kodak X-ray film RP/R54 to locate the radioactively labelled components [12]. For stained gels, 100–150 μg sample protein was applied; for autoradiography equal amounts of radioactivity was applied for each sample.

^{51}Chromium release assay. Cells grown on 5-cm dishes were washed twice with medium containing 10 % calf serum, and 1 ml of medium containing 10 % calf serum and 200 μCi/ml ^{51}Cr was added. The dishes were incubated for 45 min at 37 °C, washed three times with medium (37 °C) and twice with cold medium (4 °C). Cells were then extracted with detergent as described and the supernatant counted to determine the radioactivity released. Counting of both ^{51}Cr and ^{125}I was done on a well-type crystal scintillation counter (Nuclear Enterprises).

Isolation of plasma membrane. Plasma membranes were isolated using the two-phase polymer system of Brunette and Till [14].

RESULTS

Cell viability

Several non-ionic detergents were tested to determine the highest concentration that could be mixed with BHK cells and not cause lysis after 30 min at 4 °C. As shown in Table I, detergents varied in their maximal pre-lytic concentrations at 0–4 °C. Similar determinations were made with PyBHK and Swiss and Balb/C 3T3 and SV 40-transformed derivatives of the mouse cell lines. The maximal pre-lytic concentration varied slightly with cell type but no consistent sensitivity to detergents could be correlated with transformation. For subsequent experiments, cold 0.01 %

TABLE I

DETERMINATION OF LYTIC CONCENTRATIONS OF NON-IONIC DETERGENTS ON BHK CELLS

Cells were exposed to detergent for 30 min at 0–4 °C and cell lysis judged microscopically. All detergent solutions were made in phosphate-buffered saline (w/w). −, no lysis. +, lysis.

Detergent	Concentration of detergent		
	0.01 %	0.1 %	1.0 %
Triton X-15	−	−	−
X-67	−	+	+
X-100	+	+	+
N-57	−	+	+
QS-15	−	+	+
CF-32	−	−	−
Tween 20	−	−	−
40	−	−	−
60	−	−	−
80	−	−	−
Lubrol Px	+	+	+
Nonidet P40	−	+	+

TABLE II

BHK AND PyBHK CELL VIABILITY AFTER EXTRACTION WITH 0.01 % NP-40

Experimental details are given in the text. Values given are averages of two determinations. Cell number is per 5-cm dish.

Cell type	Cell number after treatment with	
	0.01 % NP-40	Phosphate-buffered saline
BHK	$1.93 \cdot 10^6$	$1.89 \cdot 10^6$
PyBHK	$1.87 \cdot 10^6$	$2.06 \cdot 10^6$

NP-40 was employed. Following extraction with 0.01 % NP-40 or phosphate-buffered saline without detergent, cells were removed from the dish with 0.25 % trypsin-EDTA, counted in a Coulter counter and 10^6 cells were replated on a 5 cm dish in medium containing 10 % calf serum. Cells were incubated for 24 h, removed with trypsin-EDTA, and counted. The results, shown in Table II, indicate that after treatment with this concentration of NP-40 both normal and transformed cells possessed the same viability as cells treated with phosphate-buffered saline alone.

No morphological changes were apparent after detergent extraction of either normal or transformed cells. The cells maintained their flat, extended appearance and cell loss during extraction was less than 1 %. Occasionally, when cultures were very confluent, some cells floated off the dish during extraction but were subsequently removed by centrifugation.

To determine more sensitively if cell lysis was occurring during detergent extraction, cells were labelled with ^{51}Cr and extracted with 0.01 % NP-40. The results of this experiment, shown in Table III, indicated that there was no significant difference in the total radioactivity released after detergent treatment or after similar treatment with phosphate-buffered saline minus detergent. Treatment with 0.025 % trypsin at 20 °C released a significantly higher percentage of radioactivity than detergent treatment at 0 °C.

TABLE III

^{51}Cr RELEASE FROM BHK CELLS DURING EXTRACTION WITH 0.01 % NP-40

Results are averages of two determinations. ^{51}Cr-labelled BHK cells on a 5 cm dish were treated for 30 min with 1 ml of solution as indicated. The supernatant was removed from the dish, spun to remove any unattached cells, and 100 μl were counted to determine released radioactivity. The remaining monolayer was washed once with phosphate-buffered saline and lysed with 1ml of 2 % sodium dodecyl sulfate. A 100 μl aliquot was counted to determine the total radioactivity present. The % cpm was determined as 100(cpm released/cpm total).

Treatment	Radioactivity (cpm)		
	Released	Total	% cpm released
Phosphate-buffered saline at 4 °C	543	79 763	0.6 %
0.01 % NP-40 at 4 °C	777	105 405	0.7 %
Phosphate-buffered saline at 20 °C	907	92 513	1 %
0.025 % trypsin at 20 °C	2426	93 211	2.6 %

Trypan blue dye exclusion after detergent extraction again supported the conclusion that cells retained their viability. Less than 1 % of the cells took up dye after detergent treatment.

Protein extraction

After extraction of BHK and PyBHK cells, the supernatant was prepared for electrophoresis as described under Materials and Methods. Fig. 1 is a stained slab gel comparing BHK and PyBHK whole cells, isolated plasma membrane, detergent extracted proteins and a phosphate-buffered saline control. Due to the complexity of the pattern, many of the proteins appear to be present in all samples. There are, however, differences in protein composition between the whole cell, plasma membrane, detergent extracted material and saline control. It is probable that several of the proteins visualized here are serum proteins and experiments are underway with metabolically labelled cells to determine which are cellular proteins.

The arrow in Fig. 1 between samples D and E indicates a protein of approximately 85 000 daltons, which is extracted from normal but not transformed cells. This protein has the same molecular weight as a protein which purifies with plasma membranes isolated from normal but not transformed cells, and can be detected after lectin affinity chromatography (Smart, J. Pearlstein, E. and Waterfield, M. D., in preparation). This protein is probably derived from serum and preliminary experiments indicate it is not plasminogen.

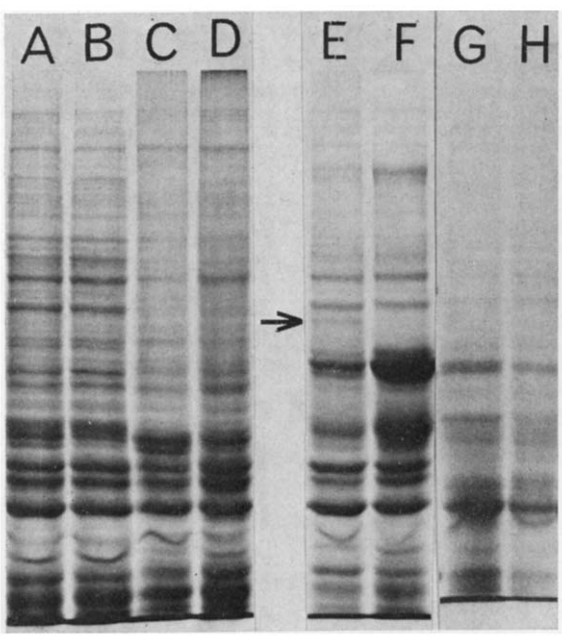

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of BHK and PyBHK cell proteins: whole cell (A) BHK and (B) PyBHK; isolated plasma membrane from (C) BHK and (D) PyBHK; detergent-extracted proteins from (E) BHK and (F) PyBHK; control phosphate-buffered saline extract of (G) BHK and (H) PyBHK. Arrow indicates 85000 molecular weight protein. Samples G and H were electrophoresed on a separate gel under identical conditions.

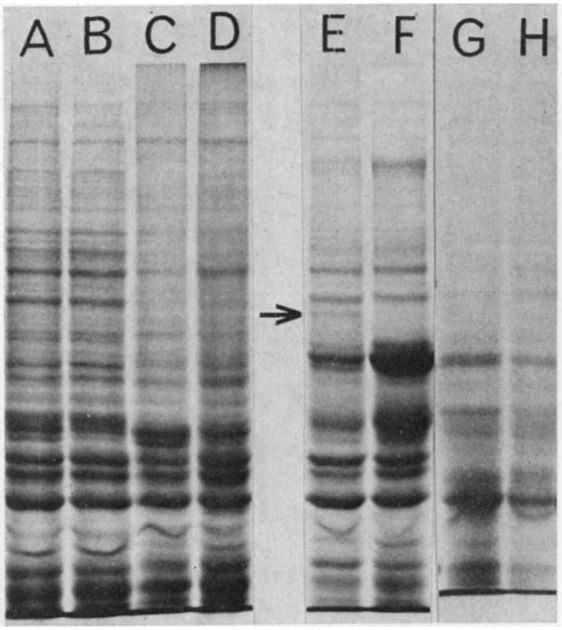

Fig. 2. Autoradiograph after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of iodinated (A) BHK and (B) PyBHK whole cells and detergent-extracted (C) BHK and (D) PyBHK cells. The molecular weight of the indicated proteins are: arrow 1, 250 000; arrow 2, 150 000; arrow 3, 100 000; arrow 4, albumin.

When BHK and PyBHK cells were labelled by lactoperoxidase-catalyzed iodination and subsequently extracted with detergent, 20–25 % of the total trichloroacetic acid-precipitable radioactivity associated with the cells was extracted by this procedure. Acrylamide gel electrophoresis, followed by autoradiography of labelled and extracted proteins, yielded the results shown in Fig. 2. Compared to whole normal (Fig. 2A) and transformed (Fig. 2B) cells which have been labelled and directly electrophoresed, the pattern of labelled proteins after detergent extraction (Figs. 2C and 2D) was clearly altered. The detergent extraction allowed demonstration of a protein of 100 000 molecular weight (Fig. 2C, arrow 3) which was present in the normal but not the transformed cell. Demonstration of this protein was difficult in whole labelled cells. Thus, detergent extraction facilitated its detection. A surface protein of 250 000 molecular weight described previously [15–17], which is heavily labelled on whole BHK cells (Fig. 2A, arrow 1), is not extracted by this procedure (compare Figs. 2A and 2C). When labelled cells were allowed to remain in phosphate-buffered saline under conditions identical to those employed during the detergent extraction, 85 % of the trichloroacetic acid-precipitable radioactivity released into the supernatant is associated with serum albumin. Thus, the labelled proteins extracted by detergent, with the exception of albumin (Fig. 2, arrow 4), are likely to be cell derived.

Fig. 3 shows the same experiment repeated with mouse fibroblasts. The results obtained with the hamster cells are also consistent with those obtained with the mouse cells. Thus, a protein of approximately 100 000 molecular weight (arrow 2) is present

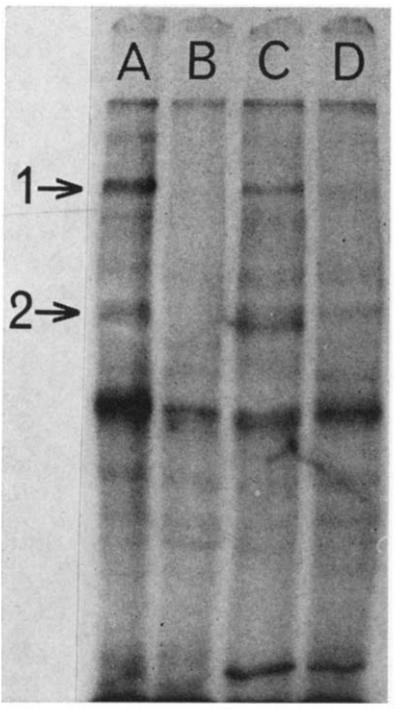

Fig. 3. Autoradiograph after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of iodinated and detergent-extracted (A) Balb/C 3T3, (B) SV Balb/C 3T3, (C) Swiss 3T3 and (D) SV Swiss 3T3. The arrows indicate proteins of molecular weight 150000 (arrow 1) and 100000 (arrow 2).

in both normal 3T3 Balb/C and 3T3 Swiss, but is lacking or greatly reduced in SV 40-transformed derivatives of both cell types. An additional protein of approximately 150 000 molecular weight is also present in the normal but not transformed cells (arrow 1). This is not identical to the 250 000 molecular weight surface protein discussed above, but may be similar to another surface glycoprotein previously described [18].

DISCUSSION

A novel method has been developed for extracting protein constituents from mammalian cell plasma membranes using sub-lytic concentrations of non-ionic detergents. In the experiments reported here, 0.01 % NP-40 was employed. Similar results have also been obtained with other non-ionic detergents, for example those listed in Table I, at their maximal non-lytic concentration and with the anionic detergent sodium deoxycholate (unpublished results). At 0–4 °C, 0.01 % NP-40 does not cause cell damage as judged by cell viability (Table II), ^{51}Cr release (Table III) and trypan blue staining. These measurements do not exclude the possibility of increased uptake or loss of ions. The proteins extracted by this procedure differ from those isolated by purifying the entire plasma membrane (Fig. 1) and the process is therefore selective. The selectivity of the extraction procedure is further demonstrated

by pre-labelling cells using lactoperoxidase catalyzed iodination followed by the detergent extraction procedure.

A comparison of labelled and detergent-extracted material with labelled whole cells (Fig. 2) demonstrate a specificity of the solubilization procedure for certain proteins. A large external transformation sensitive protein of 250 000 daltons previously demonstrated to be present on normal fibroblasts but missing from transformed derivatives [9, 15–19] (Fig. 2, arrow 1) is not selectively solubilized by this procedure. It has been shown that this protein is extracted from normal cell plasma membranes in highly purified form by 0.2 M urea [20].

Plasma membrane proteins have been classified as either integral or peripheral depending on physical parameters [21–23]. Integral [21] or intrinsic [22] proteins are firmly imbedded in the lipid bilayer and require detergents or organic solvents for their solubilization. Peripheral [21] or extrinsic [22] proteins are less hydrophobic and can usually be dissociated from the membrane in soluble form by using variations in ionic strength, pH changes or chelating agents. The failure of the 250 000 dalton glycoprotein to be extracted with detergent and its ease of solubilization with urea allow this protein to be categorized as peripheral.

The extraction procedure described in this report is effective in solubilizing a protein of 100 000 daltons (Fig. 2, arrow 3 and Fig. 3, arrow 2). This protein is apparently an integral component of normal cells but is reduced or missing from transformed cell plasma membranes. It has been demonstrated in BHK hamster cells and two normal mouse cell lines (Balb/C 3T3 and Swiss 3T3). Several alterations in plasma membrane composition subsequent to transformation have recently been demonstrated. Stone et al. [24] using ts mutants of RSV in chick embryo fibroblasts have shown changes in three plasma membrane proteins following transformation. A glycoprotein of 250 000 molecular weight decreases after transformation (probably identical to the glycoprotein discussed above). Two components of 73 000 and 95 000 molecular weight increase in amount after transformation. Since the protein of 100 000 molecular weight we describe here decreases following transformation, it represents an additional component of the plasma membrane quantitatively affected by transformation.

Another plasma membrane component of approximately 150 000 molecular weight selectively solubilized by the detergent extraction procedure (Fig. 2, arrow 2 and Fig. 3, arrow 1) and also displaying the characteristic of being present in the normal but not transformed cell plasma membrane, is similar in molecular weight to a protein previously described [18, 25]. If these components are identical, it is of interest that in the previous report [25] antibody directed against the 250 000 molecular weight glycoprotein co-precipitated this component. We have been able to extract this component free of the 250 000 molecular weight glycoprotein, just as others [20] have been successful in purifying the 250 000 dalton glycoprotein free of this component. This would support the idea [25] that, although the proteins are related immunologically, they may not be structurally associated in the plasma membrane. More work is needed to clarify the relationship between these two proteins.

The technique described here may be useful in investigating the degree of hydrophobic association between specific proteins and other constituents of the plasma membrane. This could be achieved by a careful application of detergents with

different hydrophilic-lipophilic balance to a selective extraction of membrane components. To this end, we have shown (Table I) that cells will remain viable in prelytic concentrations of detergents whose hydrophilic-lypophilic balance numbers vary from 13.1 (NP-40) to 16.7 (Tween 20). Assessment of specific protein extraction would be facilitated by the use of immunoprecipitation, a technique still possible in the presence of non-ionic detergents [26].

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