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Immunochemical Purification of Probe-Labeled Plasma Membrane Proteins: An Approach to the Molecular Anatomy of the Cell Surface

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The probe 2,4,6-trinitrobenzene sodium sulfonate may be used under appropriate conditions for selective labeling of plasma membrane proteins exposed at the outer cell surface. Labeled proteins, solubilized by detergents, can be purified by reverse immunoadsorption using antiprobe antibodies covalently linked to Sepharose 4B. This method has been applied to an investigation of the outer cell surface structure of chicken embryo and hamster fibroblasts. Coelectrophoresis in sodium dodecyl sulfate-polyacrylamide gels of probe-labeled membrane proteins purified from baby hamster kidney fibroblasts have shown that 7 major protein groups of different molecular weight are exposed on both control and Rous sarcoma or polyoma virus-transformed cells. Moreover, the transformed cells display a nonvirion component of 80–100 k daltons that is not labeled by the probe in normal cells. In fibroblasts transformed by a temperature sensitive Rous sarcoma virus mutant, that transforms at 37°C but not at 41°C, the expression of this component is related to the expression of the transformed phenotype.

Key words: affinity chromatography, plasma membrane, neoplastic transformation

The selective labeling of molecules exposed at the outer surface of intact cells has proved to be a suitable method for investigating the molecular structure of plasma membranes of eukaryotic cells (for review see Ref. 1). In the past few years this laboratory has developed a method that is both analytical and preparative. It involves the binding of the chemical hapten-2,4,6-trinitrophenol (TNP) to lysine ϵ-amino groups exposed at the outer cell surface on plasma membrane proteins, and the purification of labeled molecules by affinity chromatography with insolubilized antihapten antibodies.

We have previously shown that selective binding of TNP groups to proteins exposed at the cell surface may be achieved by labeling, under appropriate conditions, living cells with 2,4,6-trinitrobenzene sulfonic acid (TNBS). This has been confirmed by a variety of techniques, including immunofluorescence and immunoelectronmicroscopy with fluorescein or horseradish peroxidase-coupled anti-TNP antibodies, subcellular fractionation, digestion

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of cell surface by proteolytic enzyme, and comparative analysis of proteins labeled by TNP or radioiodinated with lactoperoxidase (1–7).

This paper reviews briefly the method devised and its application to the study of the structure of plasma membranes of normal fibroblasts grown in vitro as primary explants or as continuous cell lines. The structural alterations found by this method in membranes of virus-transformed cells will also be discussed.

MATERIALS AND METHODS

All the fibroblast lines used in this experiment were grown to subconfluency in Eagle's minimal essestial medium (MEM) supplemented with 10% calf serum (EuroBio, Paris, France), penicillin (50 IU/ml) and streptomycin (100 μg/ml). Cultures were incubated at 37°C in a 5% CO_2 atmosphere and periodically checked for pleuropneumonia-like organism (PPLO) infection (8). C13 cells were a subline of baby hamster kidney (BHK) fibroblasts derived from the BHK 21/C13 line established by Macpherson and Stoker (9). PY and B4 cells were sublines of BHK 21/C13 cells transformed in vitro by polyoma virus and Rous sarcoma virus (RSV) of the Bryan strain, respectively. Neither subline produced virus particles. Each had lost topoinhibition and acquired a "malignant" phenotype (10). 14B was a BHK 21/C13 fibroblast line transformed by the FU19 temperature-sensitive mutant of RSV (11); the full virus genome persisted in FU19-transformed fibroblasts since the virus could be rescued by fusing them with chicken cells (12). The FU19 mutant does not replicate in hamster cells but transforms them at 37°C.

Chicken embryo fibroblasts were primary cultures obtained by repeated passages of glass-adherent cells dissociated by trypsin digestion from 11-day-old embryo skeletal muscles. Selection and enrichment of myoblasts was performed as described in detail elsewhere (6).

Anti-DNP sera were prepared in rabbits immunized by 3 monthly injections of 1 mg of DNP_{47}-human serum albumin, in Freund's complete adjuvant. Anti-DNP antibodies were purified using an immunoadsorbent made with Sepharose 4B coupled to DNP_{60}-bovine gamma globulin by the cyanogen bromide technique (13). Fifteen milligrams of antigen were coupled to each gram of activated Sepharose. Purified antibodies were eluted by 100 mM dinitrophenol buffered at ph 8.0 chromatographed on Dowex 1-X8 ion-exchange resin (Cl^- form; 20–50 mesh), dialyzed against 0.1 M sodium bicarbonate, pH 9.0, and used to prepare reverted immunoadsorbents. Twenty-five milligrams of purified antibodies were reacted overnight at 4°C with each gram of activated Sepharose. Under these conditions, 80% of the antibodies were covalently linked to the resin. The same procedure was used to link to Sepharose immunoglobulins purified by DEAE-cellulose chromatography (2) from anticalf serum rabbit antisera.

Acrylamide gel electrophoresis was performed by the SDS-disk gel method of Maizel et al. (14), using 9% or 7.5% acrylamide gels with a 3% upper spacer. Purified TNP-labeled surface proteins solubilized from control and transformed cells and radiolabeled with ^{131}I or ^{125}I were coelectrophoresed in the same gel. Each gel was sectioned in 2-mm slices and the radioactivity of both isotopes counted in each slice. The cpm values of ^{125}I were corrected for interference of ^{131}I. Molecular weight calibration of the system was achieved by simultaneously running the following standards: egg albumin (43 k daltons); rabbit immunoglobulin μ chains (73.5 k daltons), γ chains (50 k daltons), and light chains (23.5 k daltons); bovine serum albumin (67 k daltons); lactoperoxidase (92 k daltons).

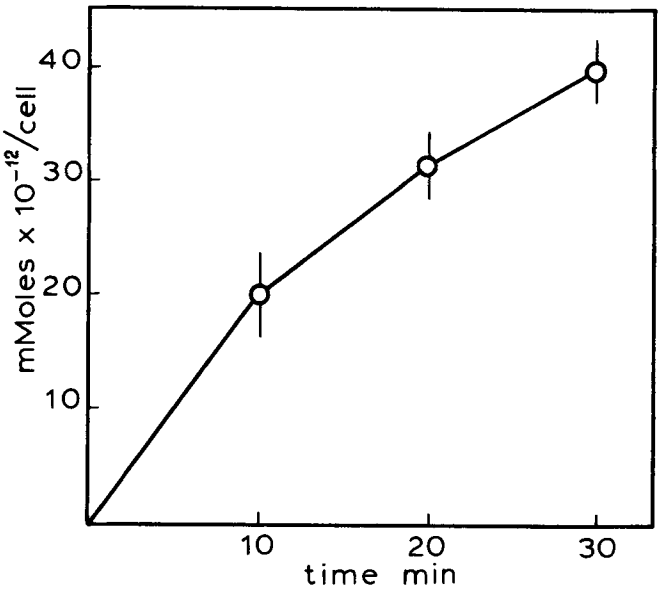

Fig. 1. Kinetic curve of the reaction between TNBS and its binding groups exposed on the outer surface of RSV-transformed BHK fibroblasts (B4). The number of TNP groups linked to the cells was measured by light adsorption at 348 nm of labeled cell preparations (5–10 X 10^6 cells) dissolved in 10% SDS, 2 M mercaptoethanol at 100°C for 3 min. Blanks were prepared by dissolving an identical number of unlabeled cells in the same way. The molar extinction coefficient of TNP-lysine (E_M^{1cm} = 15,400) was used in calculations.

Slab gel electrophoresis was performed according to Studier (15) with 3-mm thick 9% acrylamide plates. Autoradiography of dried slabs was performed with ^{131}I-labeled samples and Gevaert Curix RP-2FW x-ray film.

RESULTS AND DISCUSSION

Selective Labeling of Proteins Exposed at the Plasma Membrane Outer Surface by TNBS

Cell monolayers were carefully washed and incubated at 37°C with 5 mM trinitrobenzene sulphonic acid (Pierce) in Earle's solution buffered at pH 7.4. The reaction was stopped with iced 750 mM glycine diluted 1:1 with Earle's solution. Figure 1 shows a representative experiment in which the kinetics of the reaction between TNBS and its binding groups exposed at the cell surface of B4 hamster fibroblasts was measured. Saturation was not reached within 30 min of incubation; however, labeling was not further carried on because prolonged incubation in the absence of serum was found to result in loss of cell viability and changes in membrane impermeability to TNBS. Trypan blue was at any event excluded from more than 97% of the cells up to 45 min. Detachment of cells from tissue culture flasks by treatment with ethylenediaminetetraacetic acid (EDTA) or trypsin or by gentle scraping with a rubber policeman before reaction with TNBS always resulted in significant loss of cell viability and alteration in membrane permeability. Ten minutes of incubation of monolayers under the conditions described above were sufficient to bind a suitable number of TNP groups to surface proteins to allow their purification by

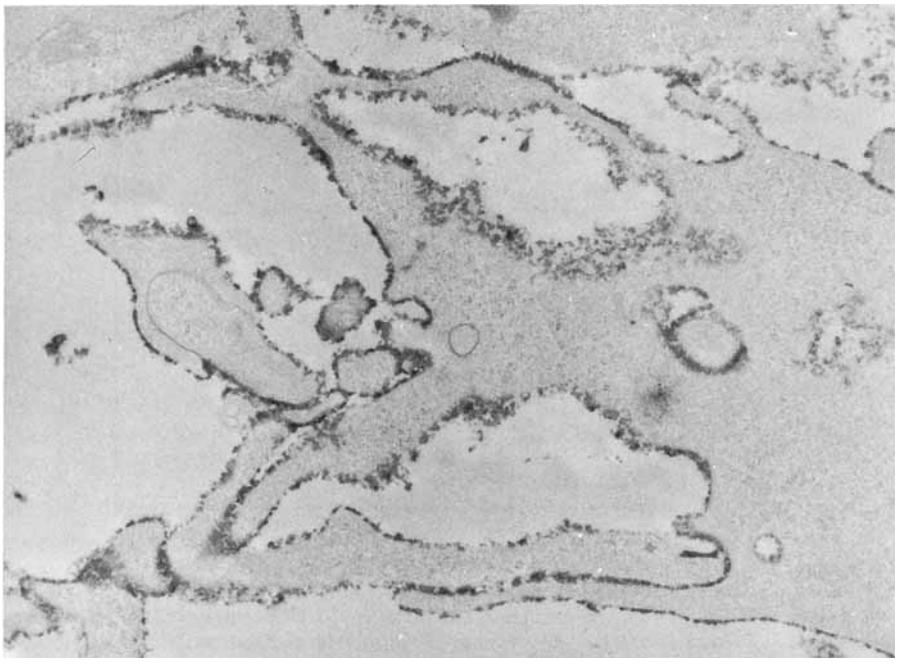

Fig. 2. Visualization of TNP groups bound to chicken embryo myoblasts by immunoelectronmicroscopy using anti-TNP antibodies conjugated with horseradish peroxidase. Spread cells in monolayers were fixed and sectioned parallel to the plane of cell growth. Peroxidase activity was developed by 15 min incubation with 3,3′-diaminobenzidine (0.5 mg/ml) in 0.1 M Tris-HCl, pH 7.4, in the presence of 0.01% hydrogen peroxide. (Courtesy of Dr. S. Sartore and Dr. S. Schiaffino).

immunoadsorption. This labeling protocol was followed in all experiments reported here. Under these conditions binding for TNP groups was restricted to the outer cell surface. This has been shown by a variety of techniques reported in previous papers (1–7). Moreover, direct visualization of cell bound TNP groups has been also recently achieved by immunoelectronmicroscopy, using goat anti-TNP antibodies coupled with horseradish peroxidase. The pattern obtained with chicken embryo fibroblasts and myoblasts (Fig. 2) confirmed the results obtained previously with hamster fibroblast and fluorescein-isothiocyanate labeled anti-TNP antibodies. All detectable TNP groups were found on the outer cell surface.

Purification of TNP-Labeled Surface Proteins

TNP-labeled cells were solubilized (in ice) with 1% of sodium dodecyl sulfate (SDS) in 0.25 M Tris-HCl buffer, pH 8.0. Tosylphenylalanylchloromethylketone, tosyllysyl-chloromethyl ketone, tosyl-L-arginine methyl ester and diisopropyl fluorophosphate were added, at final concentrations of 30, 200, 100, and 1,000 μM respectively, to prevent proteolytic degradation. After reduction with 1% mercaptoethanol and 50 mM dithiothreitol (DTT) for 3 h at 0°C in a nitrogen atmosphere, and alkylation with twofold molar excess of iodoacetic acid, samples were centrifuged at 150,000 × g for 1h. The supernatant was dialyzed against 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 0.05% sodium deoxycholate and proteins were labeled with radioactive iodine by the chloramine-T method (16).

TABLE I. Binding of Solubilized Membrane Proteins (SMP) to Rabbit Antibody Immunoadsorbents

Sample	Adsorbed on:	% binding (total)	% binding (specific)[a]
TNP-labeled SMP	IgG[b]-Sepharose	0.7 ± 0.1	0
Unlabeled SMP	anti-DNP[c]-Sepharose	0.9 ± 0.1	0
TNP-labeled SMP	anti-DNP-Sepharose	5.5 ± 0.5	4.6
TNP-labeled SMP preadsorbed[d]	anti-DNP-Sepharose	3.6 ± 0.3	2.7

[a]cpm bound by the antibody-Sepharose column after subtraction of the cpm bound by the IgG-Sepharose column.

[b]Nonimmune rabbit immunoglobulins bound to Sepharose.

[c]Purified rabbit anti-DNP antibodies bound to Sepharose.

[d]Chromatographed through an immunoadsorbent made of Sepharose coupled to IgG purified from anti-calf serum proteins antisera.

Under these conditions 80% of the membrane proteins were solubilized from all cell types, as measured in experiments performed with plasma membranes isolated by sucrose gradients described elsewhere (17).

TNP-labeled surface proteins were purified by affinity chromatography on a column of rabbit anti-dinitrophenyl (DNP) antibodies covalently linked to Sepharose 4B, or by double immunoprecipitation using rabbit anti-DNP and goat antirabbit immunoglobulin antiserum. Anti-DNP antibodies cross-react with TNP and were used instead of anti-TNP antibodies to allow specific elution with DNP-glycine as discussed in previous papers (3, 4).

For the column procedure, 1 ml of packed immunoadsorbent was washed with 1 M acetic acid and equilibrated with 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 0.05% sodium deoxycholate. It was found that membrane proteins solubilized by detergents have a certain affinity for Sepharose bound immunoglobulins (18). For this reason the immunoadsorbent was presaturated with excess nonradiolabeled proteins solubilized from the membrane of unrelated cells. Solubilized membrane proteins were slowly passed through the column; after extensive washing at 4°C to prevent proteolytic degradation, TNP-labeled molecules were eluted either by 100 mM DNP-glycine or by boiling Sepharose beads in 5% SDS plus 10% mercaptoethanol.

When double immunoprecipitation was used, 10 μl of rabbit anti-DNP antiserum were added to radiolabeled membrane samples in the presence of excess unlabeled proteins solubilized from mouse liver membranes and incubated for 1 h in ice. Goat antirabbit immunoglobulin G antiserum was then added at the equivalence and incubated for 15 min; the precipitate was washed twice with saline and eluted with DNP-glycine, or dissolved by boiling in SDS-mercaptoethanol as above.

About 5% of the proteins solubilized from cells were TNP labeled and specifically adsorbed on the anti-DNP immunoadsorbent. Controls were performed both with membranes not labeled with TNBS and with nonimmune rabbit immunoglobulins bound to Sepharose (Table I).

Removal of Exogenous Serum Proteins Adsorbed Onto the Cell Surface

It was found that about 40% of the TNP-labeled surface proteins reacted with antibodies prepared against the calf serum of the tissue culture medium (Table I). This showed that serum components were tightly adsorbed to the cell surface of fibroblasts grown in vitro. Immunochemical purification and analysis by gel electrophoresis showed that these were predominantly low-molecular-weight polypeptides, undetectable when native calf

serum was coelectrophoresed for reference. As discussed elsewhere (7, 18) these polypeptides probably originate from the proteolytic degradation of higher-molecular-weight serum proteins. Since these polypeptides were still bound by specific antisera they were removed from solubilized membrane samples by chromatography on rabbit anticalf serum antibodies covalently linked to Sepharose, as described in detail previously (18). After removal of exogenous proteins adsorbed onto the cell surface, 2–3% of the membrane proteins solubilized from the TNP-labeled cells were specifically bound by the anti-DNP immunoadsorbent (Table I). The amount of proteins bound was virtually identical in all the cell lines studied, either control or virus-transformed. Similar results were obtained after purification of TNP-labeled surface proteins with double immunoprecipitation.

Exposed proteins are thus no more than 3% of the total cell proteins. Similar values have been obtained in other laboratories by cell surface iodination with lactoperoxidase under conditions where binding of exogenous serum proteins was cut down to the minimum (19).

Analysis of TNP-Labeled Membrane Proteins Purified From Control and Virus-Transformed Fibroblasts

TNP-labeled surface proteins eluted from the anti-DNP immunoadsorbent or from specific immunoprecipitates were analyzed in SDS-electrophoresis after removal of contaminating serum proteins. Determination of the radioactivity in the gel slices revealed a pattern which, though complex, was sufficiently reproducible to enable the detection of several major component classes of molecular weight 20 to over 150 k daltons. Figure 3 shows the TNP-labeled cell surface proteins purified from BHK and chicken embryo fibroblasts.

Peak radioactivity distribution varied from one cell preparation to another, whereas peak mobility was constant and reproducible. Further resolution in the electrophoretic separation of TNP-labeled surface proteins was achieved with slab gels. These showed that most peaks obtained in tube gels were composed of more than one electrophoretic band (Fig. 4).

The electrophoretic pattern of TNP-labeled proteins showed a small amount of radioactivity in components weighing more than 150 k daltons in either cells (Fig. 3). Several laboratories have used lactoperoxidase catalyzed surface radioiodination to identify a membrane glycoprotein, known as cell surface protein (CSP) or as large external transformation-sensitive (LETS) protein, of molecular weight 250 k daltons (20–23). CSP is present in large quantities in nongrowing cells and its expression is reduced in transformed cells; it is probably involved in intercellular adhesion (24, 25). The possibility that TNBS for some reason fails to label this protein has been ruled out by experiments on chicken embryo fibroblasts and myoblasts (Fig. 3 and Ref. 6). Moreover it is known that CSP is labeled by amino group-specific reagents (26). We concluded that the expression of CSP is low in our BHK-C13 subline.

Comparison between surface proteins purified from nontransformed C13 fibroblasts and those transformed by RSV or polyoma was carried out by running ^{131}I- and ^{125}I-labeled proteins on the same acrylamide gel. Perfect coincidence was noted in the case of 6 out of the 7 peaks, with slight differences in the radioactivity distribution. However,

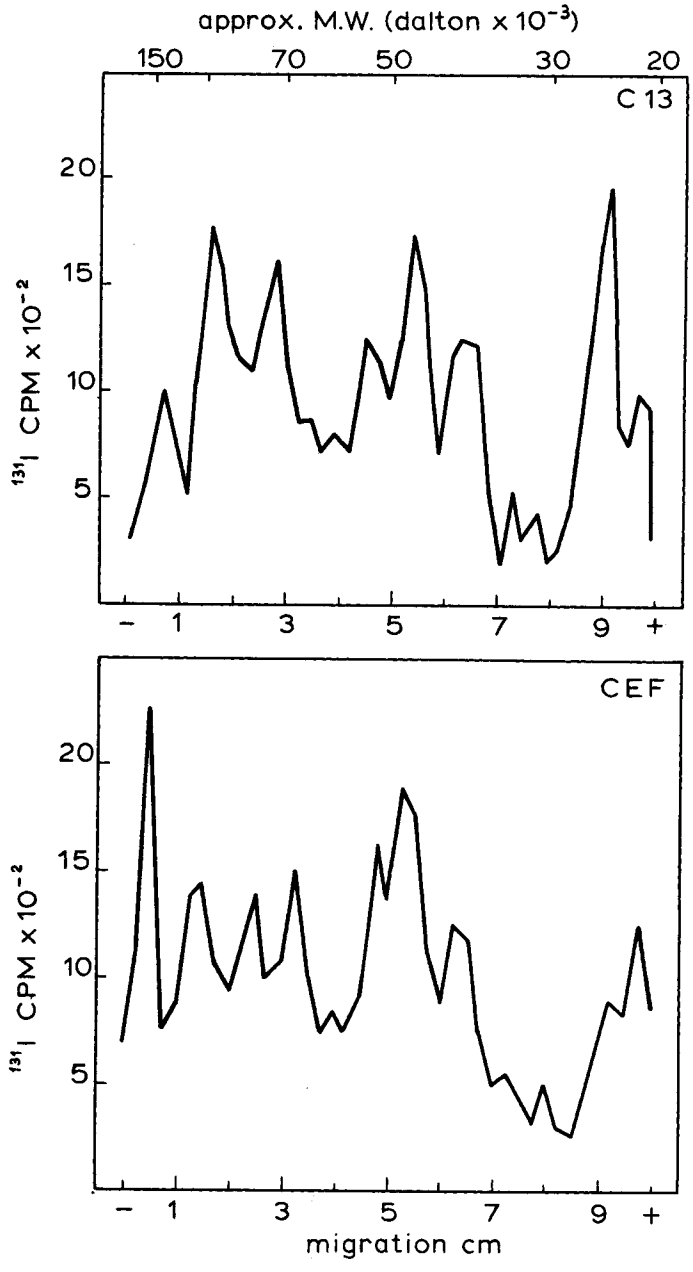

Fig. 3. Electrophoretic profile of TNP-labeled surface proteins purified by affinity chromatigraphy from baby hamster kidney (BHK) fibroblasts (C13) and from chicken embryo fibroblasts (CEF) after removal of membrane-bound serum components of the cutlure medium. Electrophoretic separations shown here and in Fig. 5 were carried out on SDS-9% polyacrylamide tubes under the conditions described in the Materials and Methods section.

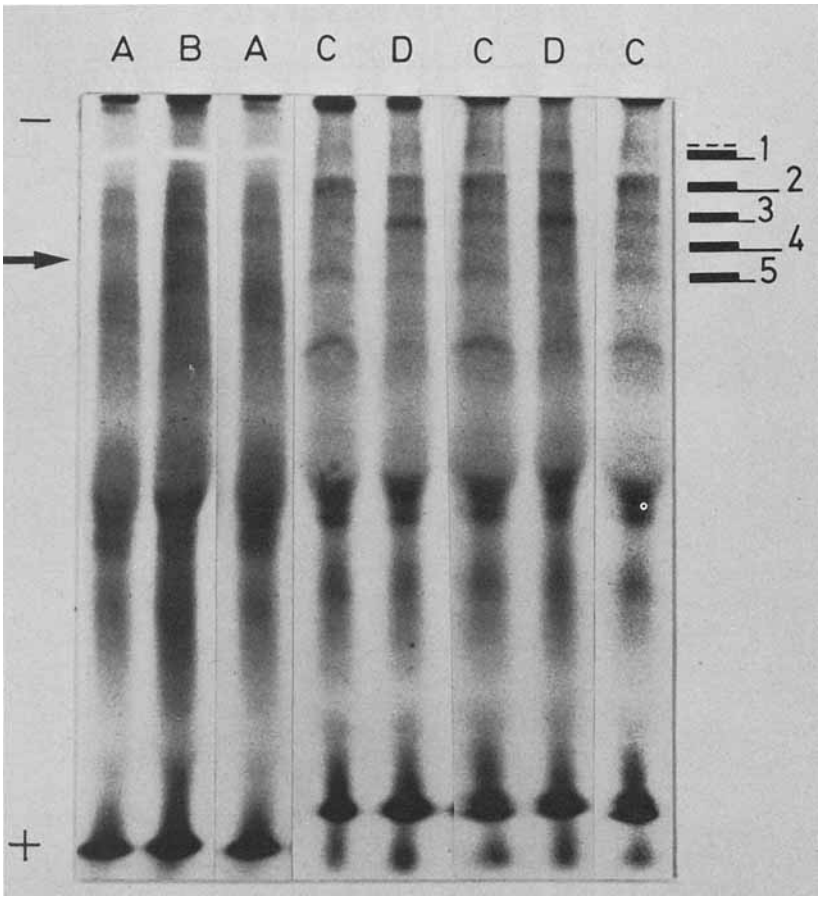

Fig. 4. Autoradiography of purified TNP-labeled surface proteins separated in SDS-9% polyacrylamide slab gel electrophoresis. A) C13 control hamster fibroblasts, B) PY-transformed fibroblasts, C) B4-transformed fibroblasts, D) B4-transformed fibroblasts after Vibrio cholerae neuraminidase digestion of the cell surface (50 IU/5 × 10^6 cells for 1 h at 37°C). Numbers indicates the major high-molecular-weight protein bands; the arrow shows the position of band 4 which is undetectable in control fibroblasts.

one of the peaks (peak C, Fig. 5) was displaced (peak C′) in the patterns of both transformed cell lines. As can be seen in Fig. 5, peak C′ of B4 cells migrates 4 mm behind peak C of C13 cells. This is a difference of 4% in relative mobility. The same result was obtained when surface proteins purified from PY cells were coelectrophoresed with those purified from C13 cells. On the other hand, analysis of transformed B4 and PY cells by co-electrophoresis showed perfect coincidence of the relative mobility of all 7 peaks. Slab gel experiments showed that in both cases the displacement was caused by the presence of an extra band, in the transformed cell pattern of approximately 80–100 k daltons (Fig. 4). Comparison of the relative mobility of tube peaks and slab bands showed that both in control and transformed cells peak B is composed of 2 bands (number 2 and number 3). This is also true for peak D and probably peak F. On the other hand peak C is composed

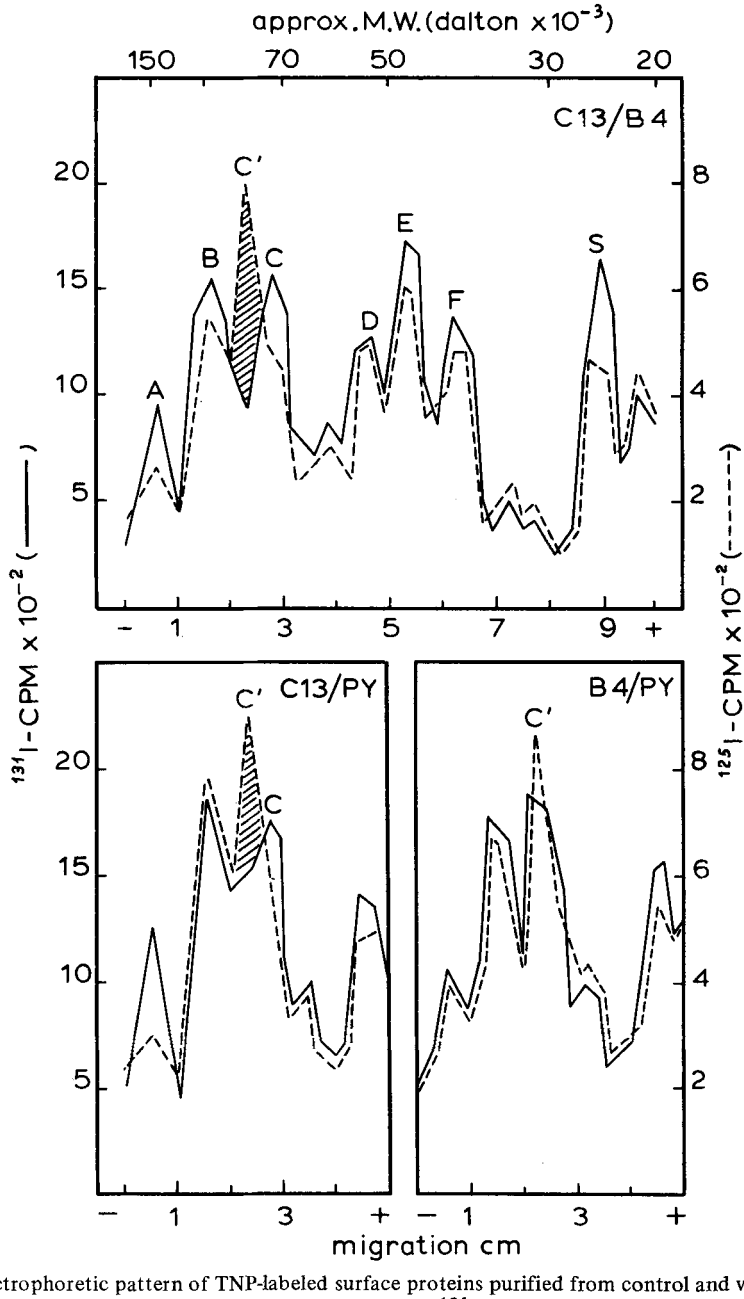

Fig. 5. Electrophoretic pattern of TNP-labeled surface proteins purified from control and virus-transformed baby hamster kidney fibroblasts. Upper plot: ^{131}I-labeled sample purified from control cells (C13) coelectrophoresed in the same gel with ^{125}I-labeled sample purified from RSV-transformed cells (B4). Lower plot on the left: ^{131}I-labeled sample purified from control cells (C13) coelectrophoresed with ^{125}I-labeled sample purified from polyoma-transformed cells (PY). Lower plot on the right: ^{125}I-labeled samples purified from RSV-transformed cells (B4) coelectrophoresed with ^{131}I-labeled samples purified from polyoma-transformed cells (PY). In lower plots only peaks found in the first 5 cm of the gels are shown.

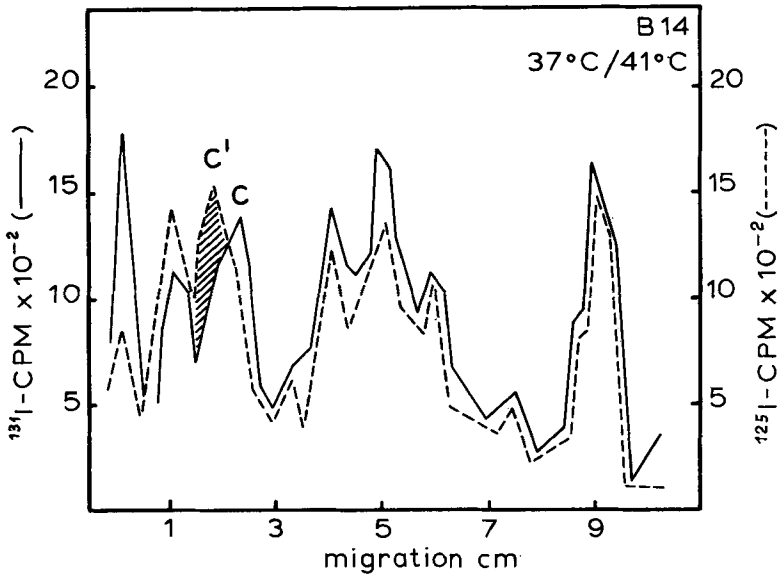

Fig. 6. Electrophoretic pattern of TNP-labeled surface proteins purified from hamster fibroblasts transformed by a temperature-sensitive mutant of RSV. Samples purified from cells grown at the permissive temperature (37°C) were labeled by ^{125}I and coelectrophoresed with samples purified from cells grown at the restrictive temperature (41°C) which were labeled by ^{131}I.

of 2 bands (number 4 and number 5) both in B4 and PY cells, but only one (number 5) in C13 control cells. It thus seemed likely that peak C of control cells exactly coincided with band 5, whereas the extra band 4 in transformed cells – not resolved from the band 5 in tubes – caused displacement of the peak to C'.

To investigate whether the displacement of peak C' in B4 and PY cells was related to transformation or to cell growth, surface proteins purified from C13 and B4 cells in logarithmic phase of growth or in resting condition (confluent monolayers) were analyzed. Peak C was not displaced during the transition of C13 cells from the growing to the resting phase. Peaks C' of growing and resting B4 cells were similarly coincident (7).

Further support for the view that displacement of peak C' was related to malignant transformation came from experiments with the 14B line of BHK fibroblasts transformed by the FU19 temperature-sensitive mutant of RSV. TNP-labeled surface proteins purified from cells grown at 37°C or 41°C were analyzed in SDS-acrylamide gels. At the permissive temperature of 37°C the electrophoretic pattern showed the same displacement of peak C to C' observed in B4- and PY-transformed cells, whereas at 41°C the pattern was similar to that obtained with control C13 cells (Fig. 6 and Ref. 7). Thus in this cell line the extra-protein responsible for the displacement of peak C is exposed at the cell surface at permissive but not at restrictive temperatures, showing correlation with the expression of the transforming gene of RSV.

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