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SEPARATION OF MAMMALIAN CELL SURFACE PROTEINS BY A TWO-DIMENSIONAL GEL ELECTROPHORESIS SYSTEM

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Summary

Separation of externally exposed plasma membrane proteins of mammalian cells has been achieved by a new two-dimensional gel electrophoresis system. The proteins were separated in the first dimension on cylindrical polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) and in the second dimension on polyacrylamide slab gels containing 9 M urea, 0.1% SDS, and 0.1% Triton CF10. Using this method we have obtained reproducible high-resolution patterns of cell surface proteins of differentiated rat neuro-tumor cells in culture and of normal rat retinal cells. Different cell types show characteristic cell surface proteins in addition to ubiquitous ones. The number of common surface proteins between two cell types account for approximately half of the total surface proteins. By immunoprecipitation we have also found that rabbit anti-serum against a rat neuronal cell line can recognize most of these external proteins. Since the separation in the first dimension is done in the presence of SDS and the second dimension in the presence of SDS, a non-ionic detergent, and urea, the technique is particularly suitable for proteins that are of poor solubility. In addition to size, net charge and hydrophobicity appear to be important factors in the separation. Virtually all of the proteins that run in the first dimension can be recovered and further separated in the second.

Introduction

Characterization of cell surface proteins is of considerable importance in the study of cell differentiation and cell-to-cell interactions. At present, however,

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Abbreviations: SDS, sodium dodecyl sulfate; TEMED, *N,N,N',N'*-tetramethylethylenediamine; PMSF, phenylmethylsulfonyl fluoride.

assays for biological or immunological activity are available only for some of the cell surface proteins. As a part of our project on neural cell specificity, we have developed a two-dimensional gel electrophoretic technique which is suitable for separating external cell membrane proteins. Though a number of techniques have been available for two-dimensional separation of proteins contained in isolated membrane [1—5], separation of the surface proteins on two-dimensional gels has not been reported. Analyses of external membrane proteins specifically labeled with ^{125}I, by some of the available methods [4,6] gave us unsatisfactory results in the extent of both resolution and recovery. In general, a major difficulty in analyzing these proteins has been their poor solubility. By using SDS polyacrylamide gel electrophoresis in the first dimension and a polyacrylamide gel system containing SDS, Triton CF10, and urea in the second, we have achieved reproducible, high-resolution separations of cell surface proteins. The use of SDS in the first as well as in the second dimension ensures that the membrane proteins dissolve and remain in solution. Using this method, surface proteins of intact rat retinal cells and those of differentiated neural cells in culture have been separated into discrete spots. The results show that different cell types have characteristic as well as common surface proteins and that rabbit anti-serum against a rat neuronal cell line can precipitate most of the surface proteins of the same cell line.

Materials and Methods

First dimensional gel electrophoresis

First dimensional electrophoresis is performed using the SDS (0.1%) polyacrylamide gel electrophoresis system of Laemmli [7]. The gels are prepared in glass tubes (0.2 cm inner diameter, 17 cm long) with the separating gel occupying 12 cm and the stacking gel 3 cm. A constant current of 0.1 mA/gel is applied overnight (approximately 15 h) followed by a current of 0.5 mA/gel until the marker dye is approximately 1 cm from the bottom of the gel. First-dimensional gels are extruded from the glass tubes by hydraulic action [8]. They are then equilibrated in a buffer containing 46 mM Tris (pH adjusted to 6.74 with HCl), 5% β-mercaptoethanol, 0.1% SDS and 0.1% Triton CF10 for 1.5 h with gentle shaking. The gels can be frozen before or after equilibration and stored at −20°C.

Second dimensional gel electrophoresis

Two-dimensional gel electrophoresis glass plates (170 × 160 mm) [6] are used. Compositions of the gel layers and running buffers are given in Table I. The separating gel solution is polymerized chemically with 18 μl of TEMED and 0.21 ml of 10% ammonium persulfate per 30 ml of the acrylamide solution. The stacking gel solution is photopolymerized under daylight fluorescent lamps with a mixture of catalysts consisting of 1.5 μl of TEMED, 12 μl of 10% ammonium persulfate and 75 μl of 0.4 mg/ml riboflavin per 6 ml of the solution. The separating gel solution is poured to a level 35 mm below the notch and overlyed with 0.5 ml of water. After completion of polymerization (1 h) the water is removed, the stacking gel solution is poured to the notch and overlayed with water. Polymerization of the stacking gel is usually completed within 2 h.

The layer of water is removed and the first-dimensional sample gel is fixed to the upper surface of the second-dimensional gel in the manner described in ref. 6, in 1.5 ml of an agarose solution consisting of 1% agarose, 5% β-mercaptoethanol, 2% SDS, 0.5% Triton CF10, 46 mM Tris (pH adjusted to 6.74 with HCl) and 0.001% Bromophenol blue. Electrophoresis is performed with the apparatus of Studier [9] at a constant current of 1.5 mA/gel for approximately 20 h until the marker dye (Bromophenol blue) reaches the bottom of the gel. The anode is connected to the lower buffer chamber, the cathode to the upper buffer chamber. If an accelerated run is desired, the current can be boosted to 3 mA/gel when the marker dye reaches the halfway point of the second-dimensional gel.

Drying gels and autoradiography

After the second-dimensional electrophoresis the gel slabs are placed on individual screens (glass fiber screens coated with polyvinyl chloride, available in hardware stores), and equilibrated in 50% ethanol/10% acetic acid for 3 h, and then in 33% ethanol/10% acetic acid for 3 h. The use of alcohol at concentrations lower or higher than 33% in the second solution causes expansion or shrinkage of the gels, respectively. The gels are dried on 0.5 mm blotting paper (A.H. Thomas) in the apparatus of Fairbanks et al. [10]. Autoradiograms are made by placing the dried gels in contact with Kodak X-ray films (SB-5). The films are processed according to standard procedures.

Labeling cell surface proteins

Two methods of lactoperoxidase-catalysed radioiodination were used. For neural retinal cells (Fig. 1), the method of Hubbard and Cohn [11] was used. And for neural cells in culture (Fig. 2), the procedure of Vitetta et al. [12] was followed, with some modification.

Rat neural retinal tissue was dissected from both eyes of an adult rat in the cold and washed three times with cold phosphate-buffered saline [13]. Radioiodination was carried out in 0.2 ml of phosphate-buffered saline containing 5 mM glucose, 5.4 μM KI, 500 μCi carrier-free $Na^{125}I$ (Amersham Searle), 16 μg of lactoperoxidase (B grade, Cal Biochem.) and 1 unit of glucose oxidase (Sigma), according to ref. 11. After 30 min at room temperature the reaction was stopped by adding 1 ml of phosphate-buffered saline containing 0.4 mM PMSF and 10 μM sodium thiosulfate. The tissue was collected by centrifugation (250 × *g* for 15 min) and the cells were ruptured by 5 rounds of quick freezing and thawing in 2 ml of phosphate-buffered saline. A crude membrane fraction was obtained by centrifugation (35 000 × *g* for 30 min), washed once in the same buffer and dissolved in 0.1 ml of SDS gel electrophoresis sample buffer [7] for 2 to 5 min at 100°C with occasional shaking. One fifth of the sample was analyzed by the gel electrophoresis.

RT4-71-D2 and RT4-E4 cells were cultured to confluency on a 35 mm culture dish (approximately 10^6 cells) according to the conditions described in ref. 14, washed 3 times with 2 ml of phosphate-buffered saline and iodinated for 10 min at room temperature in 0.5 ml of phosphate-buffered saline with 750 μCi $Na^{125}I$. 10 μg of lactoperoxidase and 75 μl of 0.03% H_2O_2 were used. H_2O_2 was added in 3 aliquots of 25 μl at 3 min intervals [12]. After the reac-

tion, cells on the plates were washed 4 times with 5 ml of phosphate-buffered saline with 0.1 M KI. The cells were lysed in 0.5 ml of sample buffer [7] containing 0.4 mM PMSF. The samples were heated at 100°C for 2—5 min with occasional shaking until the cells were dissolved. The viscous lysate was then centrifuged for 1 h at room temperature at 100 000 × *g* to remove DNA, and an aliquot was analyzed by the gel electrophoresis.

Antiserum against a neuronal cell line and immunoprecipitation

Rat neuronal cell line B50 [15] was cultured in Dulbecco modified Eagle's medium with 10% fetal calf serum in 100 mm tissue culture plates. Confluent cells were washed twice with 5 ml of saline (0.155 M NaCl) and harvested with a rubber policeman. These cells were used for preparing antiserum and for labeling cell surface proteins with ^{125}I.

Rabbit anti-B50 serum was prepared by the method of Stallcup and Cohn [16]. Approx. $2—3 \cdot 10^7$ saline-washed cells were injected intravenously into a New Zealand white rabbit on days 0, 7, 14 and 28. The rabbit was bled on day 35. The srrum was heated at 56°C for 30 min and stored at −20°C. For iodination, $1 \cdot 10^7$ saline-washed cells were suspended in 0.5 ml phosphate-buffered saline and iodinated using lactoperoxidase and H_2O_2 as described above. The labeled cells were then lysed in 1 ml of 0.5% NP40. The particulate matter was removed by centrifugation at 12 000 × *g* for 30 min at 4°C.

For immunoprecipitation, the procedure described by Kessler [17], using heat-inactivated Staphylococcus, was followed. Briefly, 100 μl aliquots of the NP40 lysate (approximately $1 \cdot 10^6$ trichloroacetic acid-precipitable counts) were incubated with 10 μl of antiserum at 4°C for 2 h. Heat-inactivated Staphylococcus (200 μl of a 10% (v/v) solution) were added and incubated for 15 min at 4°C. The adsorbent was washed 4 times with 2 ml of phosphate-buffered saline containing 0.5% NP40 by centrifugation (2000 × *g*, 6 min). The pellet was resuspended and mixed with vortex at each washing. After the final washing, the adsorbent was resuspended and boiled in 100 μl of sample buffer [7]. The suspension was centrifuged by a Beckman microfuge B, and the supernatant was assayed for the radioactivity by the gamma-counter and applied to the gel.

Chemicals

Acrylamide (Eastman Kodak Co., no. 5521) is recrystalized at least twice from ethyl acetate. TEMED and bisacrylamide (*N,N'*-methylene-bisacrylamide) from the Eastman Kodak Co., SDS (British Drug House Chemicals, Ltd.) from Ballard-Schlesinger Chemical Manufacturing Co., ultra-pure urea from Schwartz-Mann, and Triton CF10 (benzyl ether of octylphenol condensed with ethylene oxide) from Sigma Chem. Co. are used. All other chemicals are reagent grade and obtained from several commercial sources.

Results

Compositions of the second-dimensional gel system

The system contains 0.1% SDS, 0.1% Triton CF10 and 9 M urea. This achieves high resolution and quantitative recovery of proteins in the second

TABLE I

THE COMPOSITION OF THE SECOND DIMENSIONAL GEL SYSTEM

The buffer concentrations are expressed in molar concentrations of Tris. The percentage concentrations are expressed by w/v except for Triton CF10, which is expressed by v/v.

	Buffer (mM)	pH	Acryl-amide (%)	Bis-acryl-amide (%)	SDS (%)	Triton CF10 (%)	Urea	Glycerol (%)
Upper electrode buffer	Tris · glycine *	8.91 *			0.1	0.1		
Stacking gel	Tris · HCl (46)	6.74	2	0.2	0.1	0.1		10
Separating gel **	Tris · HCl (375)	9.60	4	0.03	0.1	0.1	9 M	
Lower electrode buffer	Tris · HCl (120)	8.07			0.1	0.1		

* Contains 46 mM glycine. No further pH adjustment was made.

** In several cases the gel was polymerized in the presence of 0.13 mg/ml of bovine γ-globulin (Cohn fraction II: Sigma Chem.). The rationale for this is given in the text.

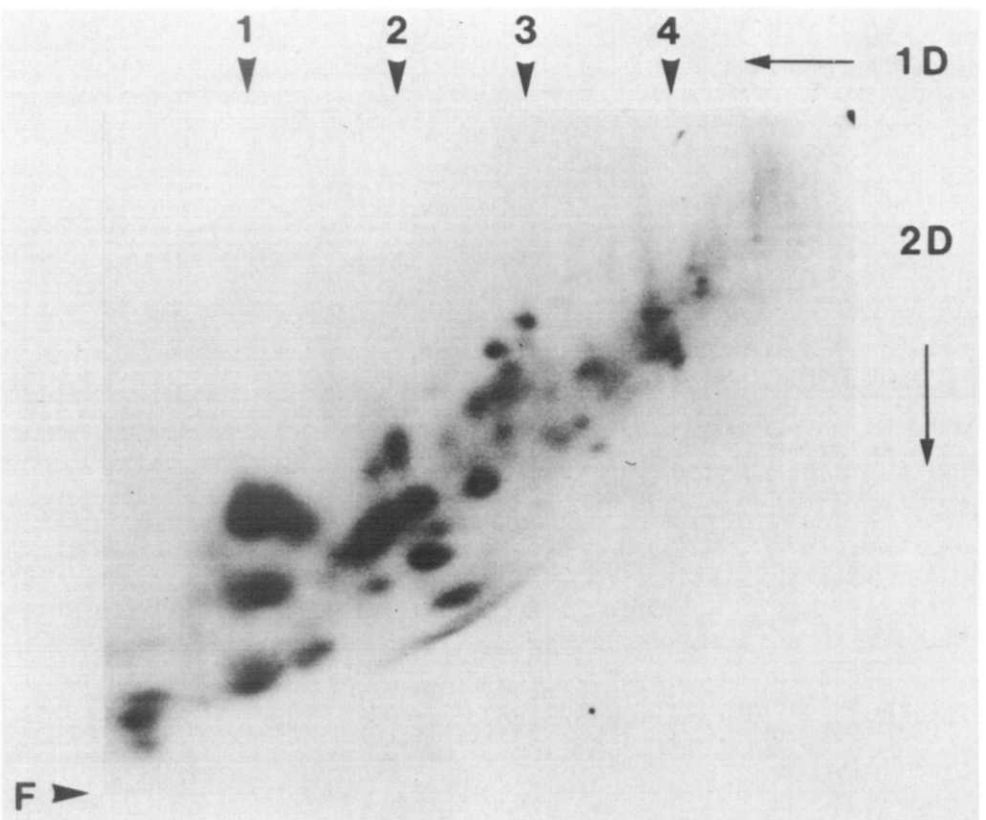

Fig. 1. Two-dimensional separation of external plasma membrane proteins of rat retinal cells. An aliquot (20 μl, approximately 200 000 cpm) of ^{125}I-labeled rat retina was separated in the first dimension on an SDS exponential gradient polyacrylamide gel of 8 to 14% from the top to the bottom of the gel, respectively. The autoradiogram was obtained with three weeks of exposure. The molecular weights of 4 major sample proteins were estimated from a separate analysis using SDS gel electrophoresis of the sample as well as standard proteins of known molecular weights. These are indicated above the gel pattern (1, 34 500; 2, 52 000; 3, 70 000; 4, 96 000). The directions of the electrophoretic migration is shown by arrows and "1D" and "2D" for the first- and second-dimensional directions, respectively. The marker dye front indicating the extent of migrating in the second dimensional analysis is shown by arrows and "F".

dimension. Sieving effects in the second dimension are minimized by low concentrations of acrylamide and bisacrylamide. Discontinuity in pH and anions (Table I) through the gel layers and electrode buffers contributes to a high degree of resolution [8]. We have tried a number of non-ionic detergents,

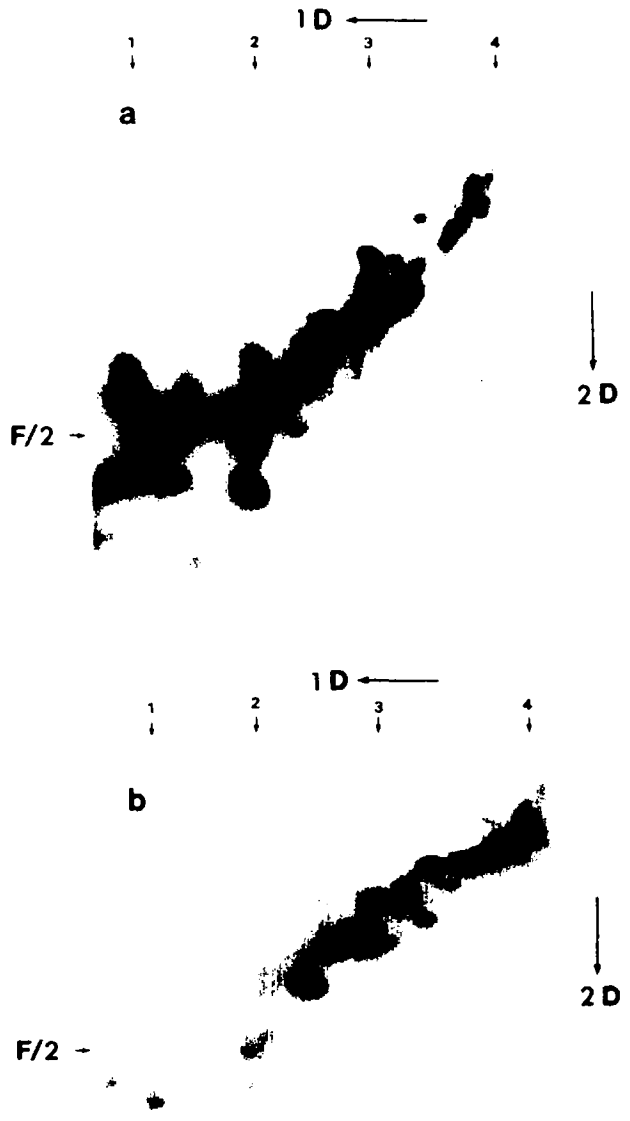

Fig. 2. Two-dimensional separation of the external plasma membrane proteins of rat neurotumor cells. Aliquots of the lysate of ^{125}I-labeled cells containing approximately 10 μg protein and $3.6 \cdot 10^6$ cpm were separated in the first dimension on a 7.5% polyacrylamide gel. The autoradiogram was prepared after 2 weeks exposure. The separating gels contained bovine γ-globulin (Table I). Numbers 1, 2, 3 and 4 in the figure indicate positions of proteins in the first-dimensional analysis with molecular weights 47 000, 78 000, 113 000 and 250 000, respectively. The midway point to the marker dye front in the second-dimensional migration is shown by arrows and "F/2". a, RT4-71-D2; b, RT4-E4.

including Triton X100. Nonidet P40, Brij 35, and Brij 58 in place of Triton CF10. Triton X100 or Nonidet P40 at 0.1% also gives satisfactory results. Addition of γ-globulin to the separating gel layer helps to eliminate artificial tailing of proteins, as discussed later.

External plasma membrane proteins of rat retinal cells

Under appropriate conditions, lactoperoxidase-catalyzed iodination using ^{125}I has been shown to label only external plasma membrane proteins of intact cells (see a review article, ref. 18). Rat retina labeled by this method was subjected to the two-dimensional analysis. More than 60 proteins of molecular weight between 20 000 and 100 000 were separated (Fig. 1).

External plasma membrane proteins of cultured cells

We have analyzed surface proteins of neurotumor cell lines in culture. The results of electrophoresis of rat neurotumor cell lines, RT4-71-D2 and RT4-E4 (Imada, M. and Sueoka, N., unpublished), are shown in Fig. 2. These cell lines were isolated from a single neurotumor, RT4, but exhibited distinct cellular

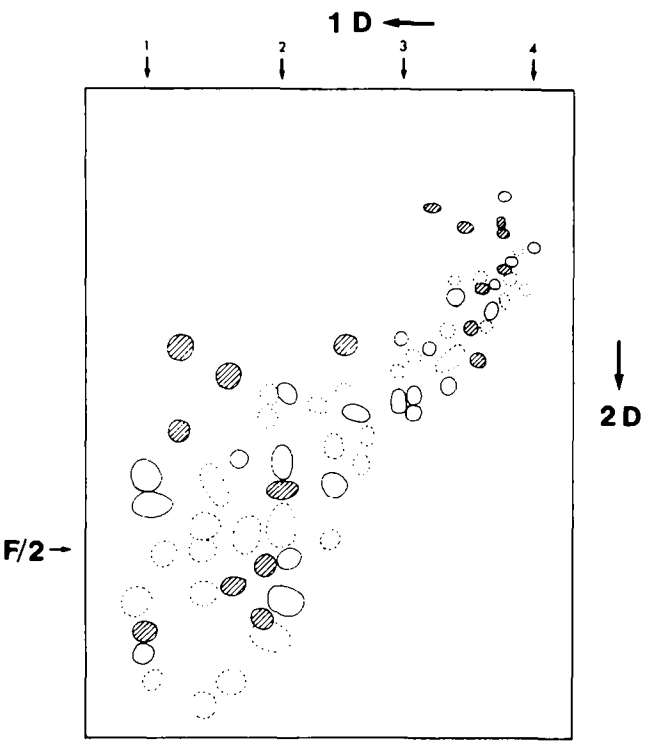

Fig. 3. Diagramatic representation of proteins found in RT4-71-D2 and RT4-E4 cells. The surface proteins of RT4-71-D2 and RT4-E4 cells shown in Fig. 2 are traced from the original autoradiograms and a combined picture with normalization in both coordinates is presented. The quantitative difference of the spots between the two cell lines are not represented in the diagram. The proteins found in both cell types are shown by solid-line circles, and those found exclusively in RT4-71-D2 cells and in RT4-E4 cells are indicated by dotted-line and hatched circles, respectively. Numbers 1, 2, 3 and 4 indicate positions of proteins with different molecular weights as shown in Fig. 2.

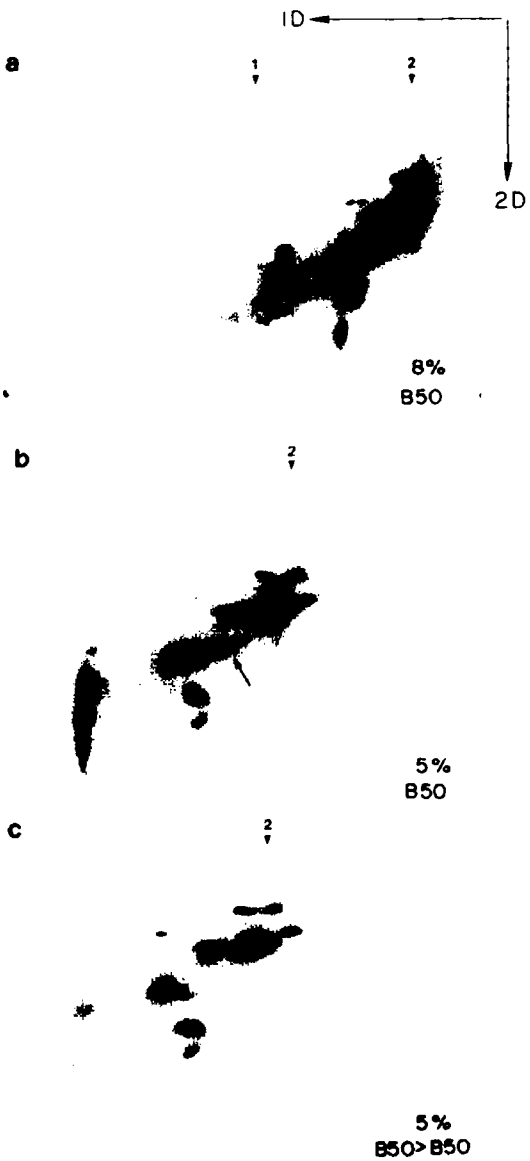

Fig. 4. Two-dimensional gel electrophoresis of rat neuronal cell line (B50) surface proteins with and without specific immunoprecipitation. B50 cells were labeled with ^{125}I and 100-μl aliquots of their lysate (approximately $6 \cdot 10^5$ trichloroacetic acid-precipitable counts) were mixed with equal volumes of double-strength sample buffer [7], boiled for 2 min and applied to first-dimensional gels of 8% (a) and 5% (b). The autoradiogram was obtained after an exposure of 5 days. In c, an immunoprecipitate of the lysate with rabbit anti-B50 (80 000 cpm) was applied to a 5% gel. The autoradiogram was obtained after an exposure of 14 days. A spot indicated by an arrow in b is missing in c. Numbers 1 and 2 indicate estimated positions corresponding to molecular weights 60 000 and 120 000, respectively.

characteristics in culture. Cell surface proteins in a molecular weight range of 45 000—250 000 are compared. The results are also diagramatically shown in Fig. 3.

Immunoprecipitation and gel analysis of cell surface proteins

We have analyzed cell surface proteins of rat neuronal and glial cells by combining immunoprecipitation and the two-dimensional electrophoresis (Hsieh, P. and Sueoka, N., unpublished). Because of the general applicability of this approach, an example is presented here in Fig. 4.

A rat neuronal cell line, B50 [15] was cultured and labeled with ^{125}I. Figs. 4a and 4b show the gel patterns of the lysate which was analyzed by using 8% and 5% gels in the first dimension, respectively. The pattern in Fig. 4c was obtained when the above lysate was precipitated with a rabbit anti-serum prepared against cultured B50 cells and the precipitate analyzed by the gel system.

Discussion

In this system, the best second-dimensional separation has been achieved by using the amounts of SDS, Triton CF10 and urea specified in Table I. Different concentrations of SDS or Triton CF10 resulted either in poorer separations or in a failure to extract proteins from the first-dimensional gel. A poor resolution also resulted from the elimination of urea. The parameters involved in the second dimensional separation are as yet not clearly understood. Differences in hydrophobicity and ionic properties of proteins are likely to affect this separation. However, since the protein distribution are roughly diagonal in every case, migration in the second dimension evidently depends to some extent upon molecular weight as well. The first-dimensional sample gel showed virtually no radioactivity after the second-dimensional electrophoresis, indicating that all proteins had entered the second-dimensional gel. A small amount of protein with a molecular weight of about 250 000, however, was occasionally retained in the first-dimensional gel.

When a minute amount of protein was analyzed, artifactual trailing of some proteins was observed in the second dimension. It was found that the trailing could be eliminated by the addition of carrier proteins to the separation gel. The artifact was probably caused by non-specific adsorption of proteins to the gel matrix; a carrier protein competes with the sample protein for such adsorption sites. Hence, in our most recent work (Figs. 2 and 4) the second-dimensional gels were run in the presence of bovine γ-globulin (Table I).

Neurotumor cells lines TR4-71-D4 and RT4-E4 were isolated from a rat injected with a carcinogen, ethylnitrosourea [19]. Although they are clonal sublines established from a single tumor, they differ in morphologies, tumorigenicity, and neuro-specific S100 proteins [20] production (Imada, M. and Sueoka, N., unpublished). RT4-71-D2 cells are spindle shaped, not contact inhibited, tumorigenic and produce S100 protein. On the other hand, RT4-E4 cells are flat, contact inhibited, non-tumorigenic, and do not produce S100 protein. Two-dimensional analysis shows that each line possesses several unique proteins and a considerable number of common proteins. We detected 53 and 39 surface

proteins of RT4-71-D2 and RT4-E4 cells, respectively, in a molecular weight range of 45 000—250 000. Twenty-two of them were found to be present in both cell types, accounting for 42% and 56% of the total number of cell surface proteins of RT4-71-D2 and RT4-E4, respectively (Fig. 3). It is interesting to note that in RT4-E4 proteins of molecular weight below 80 000 are considerably less than those of larger molecular weight, while in RT4-71-D2 this does not occur. These cell type specific differences are reproducible.

The combination of specific immunoprecipitation and two-dimensional gel electrophoresis provides a powerful method for the analysis of cell surface proteins. Particularly encouraging is the fact that a rabbit immunized with intact cultured cells is capable of generating antibodies to the majority of the cell surface proteins. As is clear from Fig. 4, the antiserum precipitates most of the major ^{125}I-labeled proteins, except for the one, which is present in Fig. 4b and missing in Fig. 4c.

Different methods of two-dimensional gel electrophoresis have been used for separating membrane proteins [1—5]. Ames and Nikaido [4] modified O'Farrell's system [6] so that more membrane proteins dissolve in first-dimensional isoelectrofocusing. The system, however, employs a limited pH range of 5—7.5 [4]. We tried to use these techniques [4,6] with ^{125}I-labeled mammalian surface proteins, but many proteins failed to separate and most spots showed extensive streaking. A further modification of this system widens the pH and gives better resolution (O'Farrell, P.Z., O'Farrell, P.H. and Jones, P., personal communications). Another potentially interesting system, using chloral hydrate in the first-dimension electrophoresis and organic-base dodecyl sulfate in the second dimension, has recently been reported [5]. Applicability of this technique on externally labeled cell-surface proteins is not clear.

Our technique, developed primarily for cell surface proteins, separates membrane proteins by principles hitherto unexploited. Partial displacement of SDS from proteins in the second dimensions by Triton CF10 and urea is a likely cause for the separation. The extent of the displacement of SDS probably depends on the hydrophobicity of the proteins. The technique may be applied to the study of various aspects of cell-surface differentiation and function.

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