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TWO-DIMENSIONAL ELECTROPHORESIS OF SURFACE GLYCOPROTEINS OF NORMAL BHK CELLS AND RICIN RESISTANT MUTANTS

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Summary

The surface glycoproteins of baby hamster kidney (BHK) cells were iodinated by lactoperoxidase and submitted to a two-dimensional electrophoresis procedure involving isoelectric focusing in the first dimension and SDS gel electrophoresis in the second dimension. After autoradiography a complex but reproducible pattern was obtained. The technique was then applied to the study of three ricin-resistant mutant clones with reduced rates of cell-cell and/or cell-substratum adhesion. Abnormal patterns were observed in all three mutant clones indicating different mechanisms of ricin resistance and identifying glycoproteins which may be involved in cellular interactions.

Introduction

A good understanding of the nature of the recognition processes involved in cell-cell and cell-substratum interactions should form the basis for any rational approach to the investigation of complex biological phenomena such as contact inhibition of growth, metastasis and morphogenesis. Although it is generally believed that cell surface glycoproteins may participate in mediating these interactions [1,2] we lack precise knowledge of the structure and function of the individual macromolecules involved.

Recently, several mammalian mutants have been isolated which are resistant to the toxic effects of ricin, the lectin of castor beans (*Ricinus communis*) [3—5].

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Abbreviations: TEMED, *N,N,N',N'*-tetramethylethylenediamine; SDS, sodium dodecyl sulphate; CAPS, cyclohexylaminopropanesulphonic acid; PMSF, phenylmethylsulphonylfluoride; DATD, *N,N'*-diallyltartardiamide.

Several of these mutants display decreased binding of ricin to the cell surface and can thus be presumed to have architectural or structural alterations in carbohydrate-containing plasma membrane components. In general, the mutants with decreased ricin binding exhibit reduced rates of cell-cell and cell-substratum adhesion [6] and their availability provides a good opportunity to investigate the biochemical basis of cell interactions.

We report here on the application of a two-dimensional macromolecular mapping procedure involving isoelectric focusing in the first dimension and SDS gel electrophoresis in the second dimension to perform a comparative study of the surface anatomy of baby hamster kidney (BHK) cells and three ricin-resistant mutant clones, two of which bind ricin very poorly.

Materials and Methods

Chemicals. Nonidet P-40 (NP-40), urea (Aristar grade), acrylamide, *N,N'*-methyl-bisacrylamide, *N,N,N',N'*-tetramethylenediamine (TEMED), sodium lauryl sulphate (SDS, specially pure) and ammonium persulphate were purchased from British Drug House Chemical Ltd. Glycine, aspartic acid, glutamic acid, Tris base, cyclohexylaminopropanesulphonic acid (CAPS) and phenylmethylsulphonylfluoride (PMSF) were obtained from Sigma Chemical Corp. Ampholines were obtained from LKB Produkter, agarose (Indubiose A37) from L'Industrie Biologique Francaise and *N,N'*-diallyltartardiamide (DATD) from Bio-Rad Laboratories.

Enzymes. Trypsin (twice crystallized, type III), lactoperoxidase (from milk, 40—50 units/mg) and glucose oxidase (Type V) from *Aspergillus niger*, 200 units/mg) were purchased from Sigma Chemical Corp. Neuraminidase (from *Vibrio cholerae*, 500 units/ml) was obtained from Behringwerke AG.

Solutions and buffers. A, phosphate-buffered saline: NaCl (8.0 g), KCl (0.29 g), Na_2HPO_4 (1.16 g), KH_2PO_4 (0.29 g), $CaCl_2$ (0.19 g) and $MgCl_2$ (0.19 g) diluted to 1 l with H_2O, pH 7.04. B, phosphate-buffered iodide: it contained the same salts as phosphate-buffered saline except that NaI (8.0 g) was substituted for NaCl, C. trypsin/EDTA solution: trypsin (4 mg/ml), EDTA 4 mg/ml), NaCl (7.6 mg/ml), KCl (0.224 mg/ml), glucose (1.8 mg/ml) and Na_2HPO_4 (0.268 mg/ml) adjusted to pH 7.0. This stock solution was diluted 1 : 100 with phosphate-buffered saline containing no calcium or magnesium just before use. D, lysis buffer: 9.5 M urea, 2% (v/v) NP-40, 2 mM PMSF, 10 mM CAPS, pH 11.0. E, 30% acrylamide stock for isoelectric focusing: 28.38% acrylamide and 1.62% bisacrylamide. F, stock NP-40 solution: 10% (v/v) NP-40 in H_2O. G, 0.05 M aspartic acid, 0.05 M glutamic acid in H_2O, H, ammonium persulphate: 10% (v/v) in H_2O prepared just before use. I, Anolyte: 5% (v/v) phosphoric acid in H_2O, J, catholyte: 1% (v/v) ethylenediamine in H_2O degassed by stirring under vacuum for 2 h before use. K, SDS sample buffer: 2.3% SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 0.0625 M Tris-HCl, pH 6.8. L, Agarose gel: 1% agarose in buffer K. M, staining solution: 0.2% Coomassie blue in 46.5% methanol and 7% acetic acid in H_2O. N, destaining solution: 5% methanol, 7% acetic acid in H_2O.

Cell lines and culture conditions. Baby hamster kidney fibroblasts BHK 21, clone 13 and ricin-resistant mutants (Ric^R mutants) are described fully else-

where [4,5]. In total, 24 resistant clones were isolated; these could be classified in four broad classes according to their ability to bind ricin to the cell surface and their cross-resistance to *Phaseolus vulgaris* agglutinin, a lectin which has a similar specificity as ricin towards β-galactosyl and *N*-acetylgalactosaminyl residues [5]. For this study we chose one clone each from classes II, III and IV, respectively Ric^R 19, Ric^R 17 and Ric^R 14. Clones from classes III and IV have decreased binding of ricin, while clones from class II, although binding normal levels of ricin are also resistant to *P. vulgaris* lectin and may thus also be defective in some receptor activity [5]. Wild type and ricin-resistant mutants were grown routinely in Glasgow modified minimal essential medium (Flow Laboratories) supplemented with 10% (v/v) foetal calf serum, 10% tryptose phosphate broth, $NaHCO_3$ (2 g/l) and gentamycin (50 μg/ml). Cells were transferred every 4—6 days at an inoculation density of approx. 10^3 cells/cm^2 in Falcon plastic culture bottles. Cells were removed for subculturing by treatment with a trypsin/EDTA solution (C) at 37°C.

Surface labelling. The lactoperoxidase-glucose oxidase procedure of radioiodination of cell surface components was used [7—10]. Intact confluent monolayers of wild type or Ric^R cells growing on 60 mM Falcon plates were rinsed four times with phosphate-buffered saline at room temperature. The labelling reaction was carried out in phosphate-buffered saline (2 ml) containing 2.5 mM glucose, 20 μg/ml lactoperoxidase, 0.2 units/ml glucose oxidase and 250 μCi of carrier-free sodium [^{125}I]iodide (1 MS 30, ⩾0.1 Ci/ml, Radiochemical Centre, Amersham, U.K.). Cells were incubated for 15 min at room temperature and the reaction was stopped by aspirating the labelling mixture and washing the monolayers twice with cold (2—4°C) phosphate-buffered iodide. The cells were then washed twice with cold phosphate-buffered saline and removed by scrapping with a rubber policman. After several washes with cold phosphate-buffered saline by centrifugation at 2°C the cell pelletes were either treated with enzymes or stored at −10°C until electrophoresed. Control experiments were performed in which the lactoperoxidase or the glucose oxidase were omitted or in which the reaction mixture was incubated for 20 min in the absence of cells, mixed with phosphate-buffered iodide and then added to cell monolayers for a further 20 min; these control experiments consistently did not lead to any labelled components in gels.

Enzyme treatments. For neuraminidase treatment the cell pellets were suspended in 1 ml of a 50 U/ml solution of the enzyme in 0.05 M acetate, 0.15 M NaCl and 0.01 M $CaCl_2$, pH 5.5 and incubated at 37°C for 30 min [11]. For mild trypsinization cells were suspended in 1 ml of a 9 μg/ml solution of trypsin in Ca^{2+} + Mg^{2+}-free phosphate-buffered saline and incubated at 25°C for 5 min. Then 200 μl of a solution of soybean trypsin inhibitor (100 μg/ml in phosphate-buffered saline, Sigma Chemical Corp.) were mixed in and the cells washed twice in cold phosphate-buffered saline.

Solubilization of cell pellets. Cell pellets were suspended in 250 μl of lysis buffer (D) and freeze-thawed rapidly five times. The resulting homogenous solution was kept at −10°C until electrophoresed, usually no more than 1 week. Occasionally cell pellets were extracted with NP-40 in the absence or urea by suspending them in 1 ml 0.5% NP-40, 1 mM PMSF, 10 mM Tris-HCl, pH 8.2 and incubating at 2°C for 30 min with frequent mixing. The suspension

was then spun at 1000 $\times g$ for 5 min at 4°C and both pellet and supernatant stored until used.

Two-dimensional electrophoresis. Two-dimensional macromolecular mapping was done with isoelectric focusing in the first dimension and SDS gel electrophoresis in the second dimension by a modification of the method of O'Farrell [12] as follows:

(a) First dimension isoelectric focusing. Isoelectric focusing gels were cast in glass tubes (120 × 3 mm internal diameter) sealed at the bottom with Parafilm. The tubes had been previously cleaned with 10% H_2O_2 in concentrated H_2SO_4 and treated with ethanolic KOH for 2 h before use. 5 ml gel mixture were prepared by dissolving 2.75 g urea in 0.67 ml acrylamide stock (E), 1.0 ml NP-40 stock (F), 1.0 ml H_2O, 25 μl Ampholines, pH 3.5—10, and 7 μl TEMED. Alternatively a mixture of Ampholines comprising 100 μl, pH 3.5—10, 75 μl, pH 5—7, and 75 μl, pH 4—6 was used and 1.0 ml solution G was substituted for the 1.0 ml H_2O. The gel mixture was degassed for 10 min and then the sample (20—100 μl) of solubilized radioiodonated cells added. Finally, 10 μl of solution H were mixed in, the gels were cast and overlayed with H_2O. Isoelectric focusing was performed in a standard electrophoresis chamber and room temperature and with the anolyte at the top. Power was applied as follows: 50, 100, 150 and 200 V for 1 h each followed by 400 V for 16 h and 800 V for 1.5 h. Gels were then removed from tubes, shaken for 2 h at room temperature in 5 ml SDS sample buffer (K) and frozen until run in the second dimension. In every run at least one gel was sliced in 0.5-cm sections and pH was determined in each slice after 2 h incubation in H_2O.

(b) Second dimension, SDS electrophoresis. Electrophoresis was performed with the discontinuous buffer system of Maizel [13]. 3-mm thick gels containing 7.5% acrylamide and 0.2% bisacrylamide were polymerized in a modified version of the EC box-type slab gel apparatus. A complete description of the electrophoresis cell is given by Gordon [14]. Stacking gels contained 4.25% acrylamide and 0.75% DATD. Isoelectric focusing gels were thawed and placed horizontally on top of the stacking gel. Molten agarose (L) was used to ensure complete contact between the gels. Gels were run overnight at 12 mA and then stained, destained and dried for autoradiography. Standard protein markers (included in every gel) were a mixture of smooth muscle myosin (molecular weight 200 000) and the structural proteins of reovirus with molecular weights of 155 000, 80 000, 38 000 and 34 000 [15]. These markers were a gift from Dr. A. Hay.

Autoradiography of gels. Dried gels were autoradiographed at —70°C using Fuji RX films in the presence of Ilford Fast Tungstate intensifying screens as described by Laskey and Mills [16].

Results

Isoelectric focusing with pH 3.5—10 Ampholines

When the isoelectric focusing was done with the regular pH 3.5—10 Ampholines a fairly linear gradient was obtained extending from about pH 4 to 9, but occasionally some degree of plateau formation near pH 7 was observed. Macromolecular maps obtained after such isoelectric focusing showed a number

of iodinated spots crowded in the anodic half of the gel (Fig. 1A). One possible explanation for this behaviour is that most, if not all, iodinated surface proteins are glycoproteins and have an acidic isoelectric point because they contain sialic acid residues. This hypothesis was tested by treatment of cells with neuraminidase before solubilization. The resulting maps showed a cathodic shift of most spots (Fig. 1C), thus providing support to the above notion. A major iodinated species of lactoperoxidase labelled intact BHK cells is a glyco-

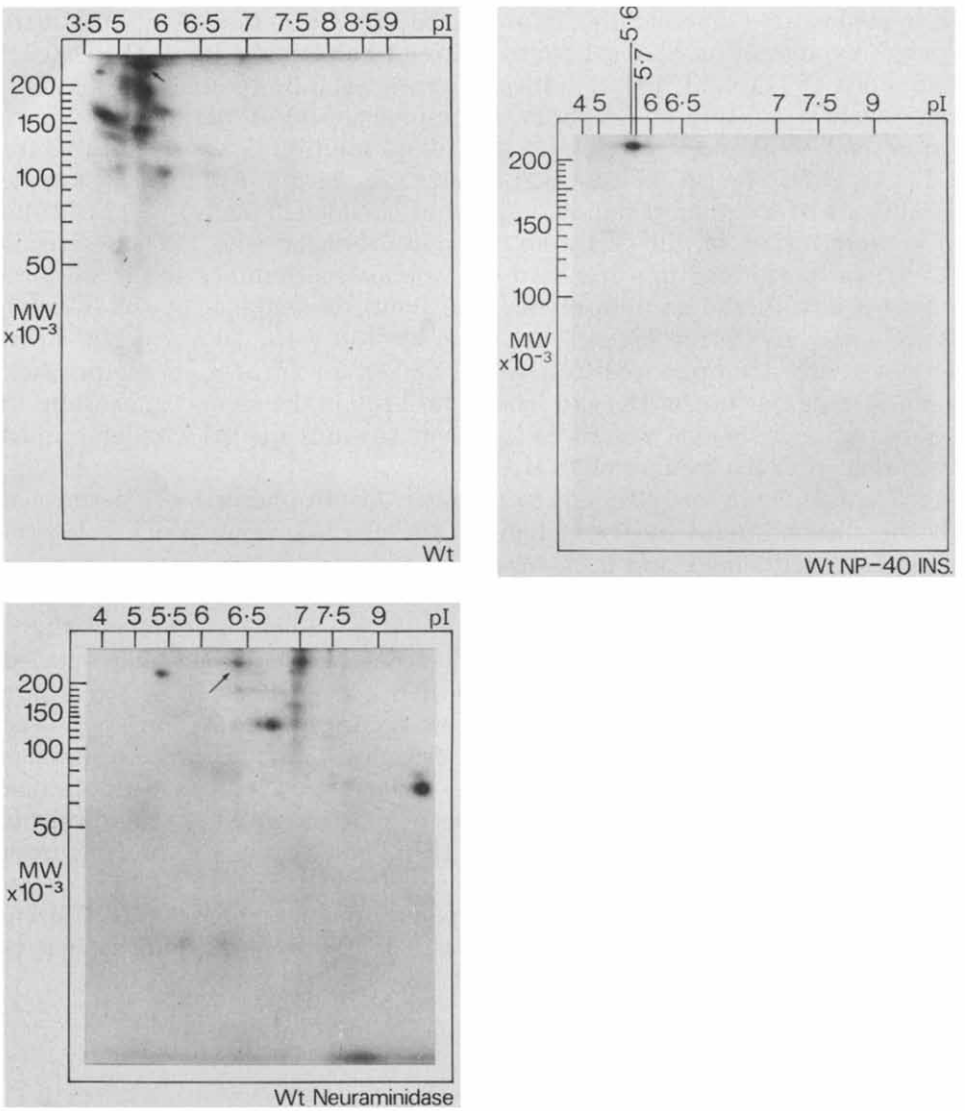

Fig. 1. Two-dimensional macromolecular maps of radioiodinated wild type BHK cells. Isoelectric focusing was performed with pH 3.5—10 Amopholines. A, normal wild type BHK cells. B, insoluble material after extraction of BHK cells with 0.5% NP-40, 1 mM PMSF, 10 mM Tris-HCl, pH 8.2, for 30 min. C, wild type BHK cells after treatment with neuraminidase. The arrows in A and C indicate the 250 000 dalton fibronectin component.

protein with an apparent molecular weight of approx. 250 000 [17]. This component is shown focusing as two closely spaced spots between pH 5.5 and 6.0 (Fig. 1A, arrow). This glycoprotein was the only major iodinated species not extracted from cells by NP-40 in the absence of urea (Fig. 1B) confirming earlier results [17] and was selectively removed by very mild trypsin treatment (not shown). These features characterized it as an important major surface glycoprotein of fibroblasts variously called fibronectin [18], LETS [19] or CSP [20]. After neuraminidase treatment the isoelectric point of fibronectin increased to approx. pH 6.4 (Fig. 1C, arrow) indicating that sialic acid residues are present and contribute significantly to the isoelectric point of the molecule.

Isoelectric focusing with mixtures of Ampholines

Although fibronectin was well resolved in the maps shown, the other components were too crowded thus limiting the usefulness of the procedure. By using a mixture of Ampholines, pH 3.5—10, pH 4—6 and pH 5—7 and by adding aspartic acid and glutamic acid to stabilize the acid end of the pH gradient [21] we were able to generate pH gradients more linear and expanded in the pH 4—7 region. The maps thus obtained showed a much more even distribution of spots and greatly improved resolution (Fig. 2A). Unfortunately, fibronectin no longer focused sharply but showed pronounced streaking (Fig. 2A, arrow). However, a consistent pattern of over 20 major well defined iodinated species was obtained (Fig. 2B).

Macromolecular maps of the ricin-resistant mutants

Fig. 3 shows the one-dimensional SDS-electrophoresis pattern of radioiodinated wild type BHK cells (track 4) and the three ricin-resistant mutants. Clone Ric^R 19 (track 1) had an essentially normal pattern while in Ric^R 17 (track 2) there was the conspicuous absence of the 250 000 dalton fibronectin

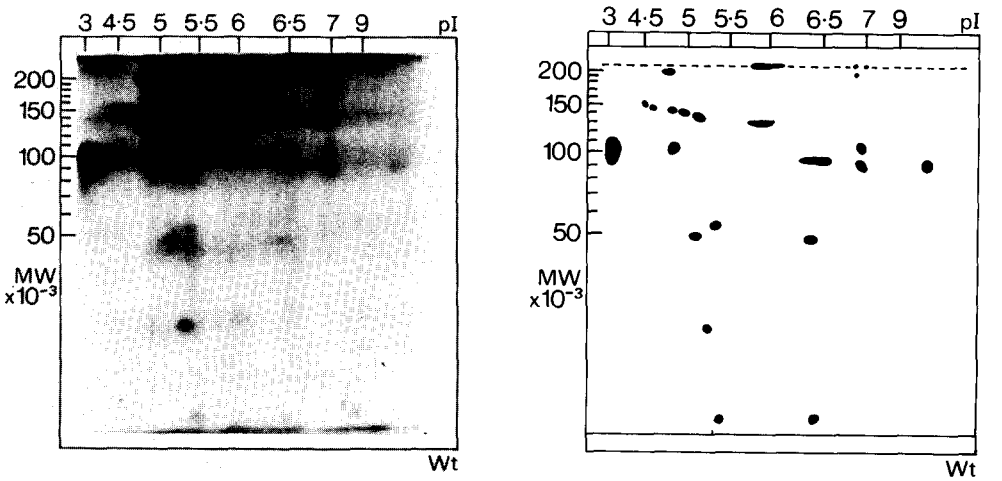

Fig. 2. Two-dimensional macromolecular maps of surface radioiodinated with type BHK cells using a mixture of pH 3.5—10, pH 4—6 and pH 5—7 Ampholines. The pattern seen in A is very reproducible, allowing the establishment of the standard reference map shown in B.

component (Fig. 3, arrow). In clone Ric^R 14 (track 3) the radioactive bands moved more rapidly than the radioactive bands of normal cells and fibronectin, although present at normal mobility, labelled somewhat more weakly than in wild type cells.

Much more precise structural information was obtained from the mutants with the two-dimensional maps shown in Fig. 4. Clone Ric^R 19 again had a pattern very similar to normal (Fig. 4A). However, besides some minor changes, one could observe the appearance of a new major spot at an isoelectric point of approx. pH 6.5 and an apparent molecular weight of about 180 000 (Fig. 4A, arrow). In the clone Ric^R 17 there was also appearance of several new spots, some of them extremely basic (Fig. 4B, arrows). However, the major change was the previously noted complete absence of the fibronectin component. The most drastic map alterations were seen in the clone Ric^R 14 (Fig. 4C). There was complete subversion of the normal patterns with most components assuming a more basic isoelectric point and an increased mobility in

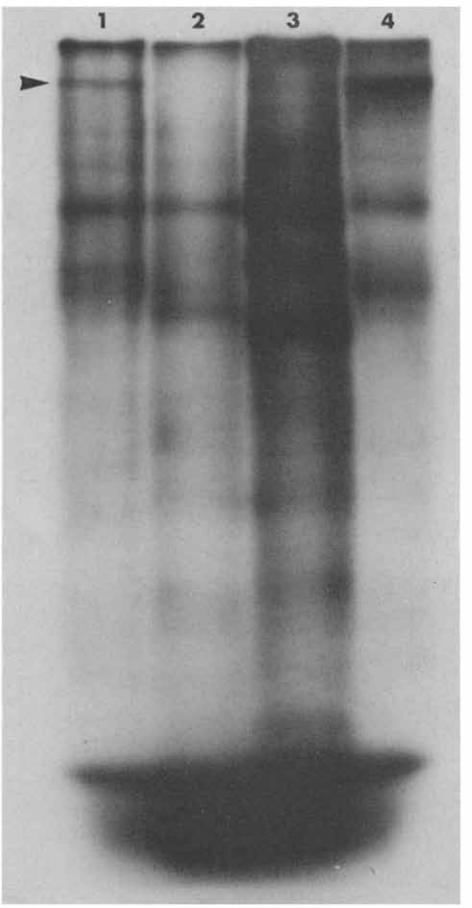

Fig. 3. One-dimensional SDS-electrophoresis of surface radioiodinated wild type BHK cell (track 4) and ricin resistant clones Ric^R 19 (track 1), Ric^R 17 (track 2) and Ric^R 14 (track 3). The arrow indicates the 250 000 dalton fibronectin component.

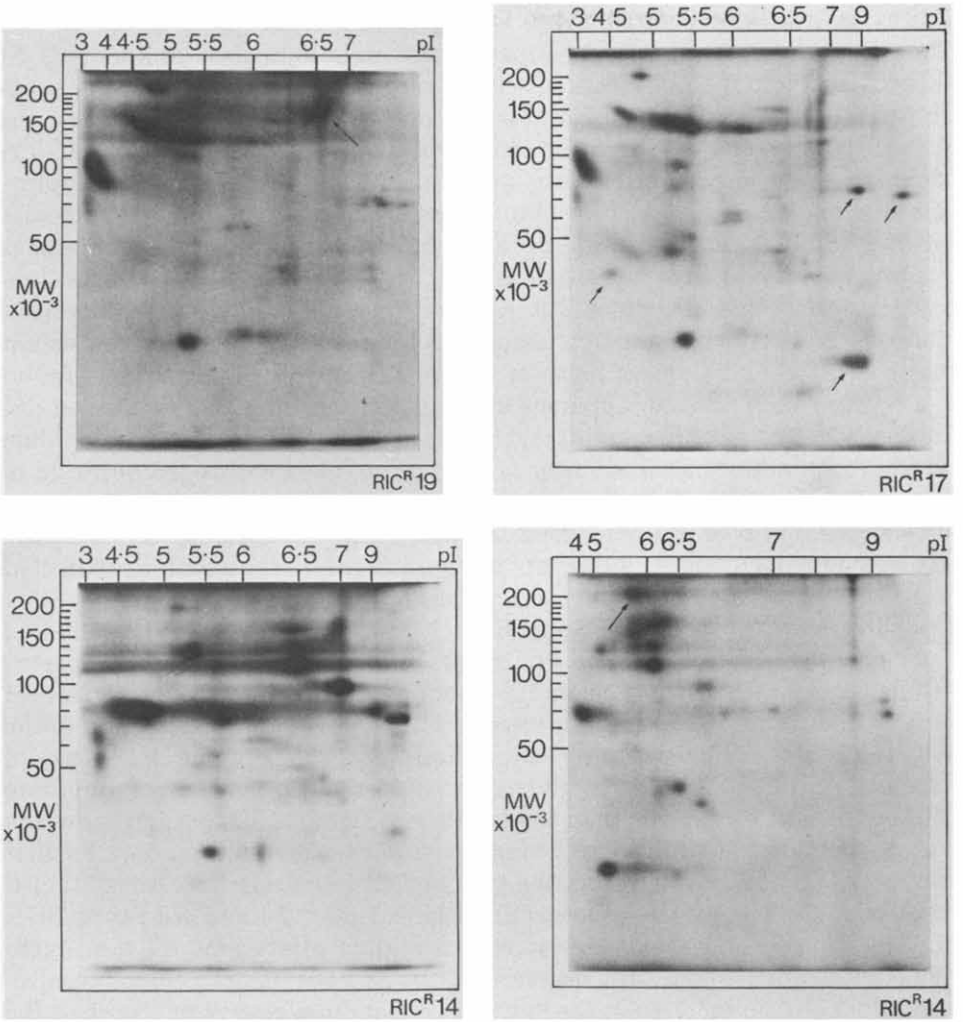

Fig. 4. Two-dimensional macromolecular maps of lactoperoxidase radioiodinated ricin-resistant mutant BHK cells using pH 3.5—10 Ampholines (D) or a mixture of pH 3.5—10, pH 4—6 and pH 5—7 Ampholines (A—C). Mutant clones shown were Ric^R 19 (A), Ric^R 17 (B) and Ric^R 14 (C and D). Arrows in A and B indicate some iodinated species not seen in maps of wild type BHK cells. The arrow in D indicates the 250 000 dalton fibronectin component.

the SDS-electrophoresis dimension. However, fibronectin (most clearly seen in the map with pH 3.5—10 Ampholines shown in Fig. 4D) did not appear altered in its isoelectric point or apparent molecular weight.

Discussion

Isoelectric focusing and SDS-electrophoresis are analytical procedures of high resolution which separate proteins according to almost independent parameters [22]. Consequently, two-dimensional macromolecular maps combining these techniques provide almost optimal separation of complex protein

mixtures as was demonstrated by O'Farrell [11]. The application of this method to the separation of lactoperoxidase radioiodinated components of BHK cells and mutant ricin resistant clones resolved more than 20 components and provided interesting new information about the surface of the mutant cells.

Although only two of the three ricin clones studied had decreased binding of ricin, all three showed alterations in the normal pattern of surface mapping. In clone Ric^R 19 a new surface component with an approximate molecular weight of 180 000 and isoelectric point of 6.5 was observed. This component was never seen by us in lactoperoxidase-labelled wild type BHK cells: presumably it is either not present or not exposed to radioiodination. A somewhat similar observation was made by Juliano and Ling [23] in several drug resistant mutants of BHK and Chinese hamster ovary (CHO) cells. These authors found that a glycoprotein with an apparent molecular weight of 170 000 labelled (by lactoperoxidase or galactose oxidase) much more intensely in the mutants than in the corresponding parental lines. A regulatory role for this glycoprotein in the membrane permeability to drugs has been proposed [23,24]. The abnormalities observed in the maps of the Clone Ric^R 19 suggest that structural rearrangements have taken place on the surface of this mutant as expected from its cross-resistance to *P. vulgaris* haemagglutinin [5].

The alterations observed in clone Ric^R 14 showed that the radioiodinated surface proteins of this mutant are more basic and have a lower apparent molecular weight than the analogous components present on the surface of wild type cells. Ric^R 14 cells are known to have a pronounced reduction in the activity of a *N*-acetylglucosaminyl transferase [4]. This enzyme defect would theoretically produce a block in glycosylation leading to smaller carbohydrate chain lengths and to a decrease in the number of terminal sialic acid residues in the glycoproteins, thus fully explaining the observed map alterations. Further support for this notion came from the study of glycopeptide fractions obtained by pronase digestion of [^{3}H]glucosamine-labelled Ric^R 14 and wild type BHK cells. This analysis revealed several compositional alterations of the glycopeptides including a significant decrease of the number of sialic acid and galactose residues and an increase in the content of mannose, as well as a shift of the glycopeptides towards lower molecular weight in gel chromatography [25]. Interestingly, the macromolecular maps of lactoperoxidase radioiodinated Ric^R 14 cells obtained in the present study did not show any alterations of the isoelectric point or apparent molecular weight of fibronectin, suggesting that this protein may not share the same *N*-acetylglucosaminyl transferase as the other membrane glycoproteins.

In clone Ric^R 17 the most striking observations was the absence of iodinated fibronectin from the macromolecular maps. This absence cannot be explained by postulating decreased synthesis of the protein because fibronectin seems to be present in apparently normal amounts in the spent media of Ric^R 17 cells [32]. Hence, alternative mechanisms have to be entertained. One possibility is that fibronectin has been rendered unavailable to lactoperoxidase-induced radioiodination by structural alterations at the periphery of the mutant cells. Alternatively, fibronectin may fail to form stable associations at the surface of Ric^R 17 cells. This latter possibility was made plausible by recent reports that fibronectin does not seem to be an intrinsic membrane protein, but rather an

extracellular component intimately associated with the cell surface [26,27]. Indeed, it is conceivable that the interaction between fibronectin and the cell surface might involve ricin receptors in the latter, thus providing a good explanation for the absence of fibronectin from Ric^R 17 and other ricin-resistant mutant clones [4,25]. It is also interesting to observe that virally-transformed fibroblasts share many of the characteristics of Ric^R 17 cells, namely decreased rates of cell-cell and cell-substratum adhesion, altered morphology and absence of iodinatable fibronectin from the cell surface [19,28,29]. However, it appears that the decrease in surface fibronectin in transformed cells is, at least partially, caused by reduced synthesis [30].

Fibronectin is currently believed to play an important role in cell-cell [18] and cell-substratum [31] interactions and when added exogenously it has been shown to restore normal morphology and adhesiveness to virally-transformed cells [20]. It is thus tempting to speculate that the disappearance of fibronectin from the surface of Ric^R 17 cells and to some extent from Ric^R 14 cells may be related to the observed decreased rates of adhesion in these cells [32].

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