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A COMPARISON OF SURFACE PROTEINS IN EMBRYONAL CARCINOMA CELLS AND THEIR DIFFERENTIATED DERIVATIVES

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Summary

Surface proteins from five cell lines (three embryonal carcinoma cell lines (F9, PCC4 and PCC3), teratocarcinoma-derived endodermal cells (PYS) and fibroblasts (line 3/A/1-D-3 differentiated from PCC3)) were compared by two-dimensional polyacrylamide gel electrophoresis after selective iodination with ^{125}I in the presence of lactoperoxidase. The labeled proteins were solubilized either in Nonidet P40/urea/ampholyte/mercaptoethanol solution or in Nonidet P40 only. In total, about thirty major ^{125}I-labeled surface proteins were identified by their isoelectric point and molecular weight. 14 proteins are present in all five cell types, although their quantity or accessibility for labeling differs between differentiated and undifferentiated cells. Three proteins (200, 160 and 150 kilodaltons) are present in undifferentiated cells only. Two of them (160 and 150 kilodaltons) were solubilized by Nonidet P40/urea/ampholyte/mercaptoethanol, but not by Nonidet P40. One protein (50 kilodaltons) was found in nullipotent F9 cells only. About 14—15 proteins (including fibronectin) were released by Nonidet P40/urea/ampholyte/mercaptoethanol but not by Nonidet P40. They are presumably bound to submembrane or cytoskeleton structures by non-covalent bonds.

Introduction

Data indicating that the cell surface may play an important role in the cell life and, in particular, in cellular and embryonic differentiation have been accumulated [1—7] but the predominant number of surface proteins has not yet been biochemically and functionally identified. With the discovery of the teratoma system, pure cell lines at different stages of differentiation have

become available in quantities sufficient for biochemical studies. In contrast to most other tumors, teratomas contain different cell types corresponding to derivatives of the three embryonic germ layers [8]. The transplantable forms of teratomas, called teratocarcinomas, contain in addition early embryo-like cells called embryonal carcinoma cells. These cells possess some particular features: firstly, their injection into syngeneic adult mouse leads to the formation of highly malignant tumors and, secondly, they are able to differentiate into derivatives of three germ layers [9] which with few exceptions are non-malignant. Embryonal carcinoma cells can take a part in the formation of a normal animal when injected into a blastocyst [10,11]. In other words, the contact with normal embryonic cells can redirect embryonal carcinoma cells to differentiate in an ordered way. Their properties and further development are therefore closely related to the environmental conditions. Consequently these cells represent an interesting model for the study of cell surface proteins and their role in the cell differentiation.

The aim of this study is firstly to compare the pattern of cell surface proteins in embryonal carcinoma cells and their differentiated derivatives, and secondly to identify those surface proteins which occur on undifferentiated cells only.

Materials and Methods

Cell cultures

All cells used in this study were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum in an atmosphere of 12% CO_2.

Embryonal carcinoma cells F9 and PCC4-Aza which have lost the capacity to differentiate in vitro [12], as well as multipotent PCC3 cells were plated at high densities (10^6 per 10 cm diameter tissue culture dish) and replated every 48 h. Under these conditions, the cells remain in the exponential phase and do not differentiate in culture [13,14].

The other mouse cell lines employed in this study were: PYS, a teratocarcinoma derived endodermal cell line [15] and 3/A/1-D-3 fibroblasts differentiated from PCC3 embryonal carcinoma cells [16].

Iodination of surface proteins

For the iodination experiments three types of buffer were used: 0.2 M NaCl, 3.2 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.6 mM KCl, pH 7.4 (buffer A), the same buffer was made 10 mM in EDTA (buffer B) and 5 mM in glucose (buffer C). Cells were dissociated after incubation for 7 min with buffer A (F9 and 3/A/1-D-3 cells with buffer B). The dissociated cells were washed three times in 10 ml of the Hanks medium and resuspended in buffer C. The lactoperoxidase/glucose oxidase procedure of iodination was used [17—20] with the following modification: $1.5 \cdot 10^8$ cells were suspended in 5 ml of buffer C; then 10 μg of glucose oxidase (277 units/mg, Sigma), 100 μg of lactoperoxidase (67 units/mg, Sigma) and 2 mCi of carrier-free $Na^{125}I$ (219 Ci/l, C.E.A., Sorin) were added. The iodination was performed at 22°C for 20 min. The cells were then centrifuged and washed three times in buffer A containing 50 mM unlabeled NaI. By this procedure less than 1% of the cells were lysed.

Solubilization of cell surface proteins

The iodinated cells were resuspended in 1 ml of buffer A, divided into two samples, centrifuged, and treated by the following two methods.

1. The cells ($0.75 \cdot 10^8$) were suspended in 300 µl of 0.005 M Tris-HCl, pH 7.2, containing 2% Nonidet P40 and 1 mM diisopropylphosphorofluoridate. The dilution of diisopropylphosphorofluoridate from a stock solution (10^{-1} M in isopropanol) was made prior to use in order to prevent its decomposition in aqueous solution. After 15 min of incubation at 0°C they were centrifuged for 10 min at 10 000 × *g* at 4°C and the resulting supernatant was quickly frozen.

2. The cells ($0.75 \cdot 10^8$) were suspended in 300 µl of a solution containing 9.5 M urea, 2% Nonidet P40, 1.6% carrier ampholyte pH 5—7, 0.4% carrier ampholyte pH 3—10 and 5% 2-mercaptoethanol ('lysis buffer'). The supernatant was freeze-thawed rapidly three times and kept at —20°C for less than 1 week until electrophoresed.

Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional gel electrophoresis (on slab gels containing 10% acrylamide and 0.17% bis-acrylamide) was performed according to O'Farrell [21]. The standard mixture used in the second direction contained β-galactosidase, phosphorylase, bovine serum albumin, aldolase (subunit) and chymotrypsinogen (M_r 130 000, 96 000, 69 000, 40 000 and 25 000, respectively). Gels were stained with Coomassie brilliant blue and dried. Autoradiographs were prepared with Kodak X-ray film. For the pH measurement of the first dimension gel, a control gel after isoelectric focusing was cut into 1 cm pieces and incubated with 3 ml of distilled water overnight at room temperature, and pH was measured with a Metrohm Herisau pH meter. In all figures only those regions of the two-dimensional gels which contained the spots of the iodinated proteins are compared.

Results

A comparison of cell surface proteins solubilized by 'lysis buffer' in undifferentiated and differentiated cells

The results of two-dimensional electrophoretic analysis of three undifferentiated embryonal carcinoma cell lines (F9, PCC4 multipotent in vitro and PCC3 multipotent), teratocarcinoma-derived endodermal cell line (PYS) and fibroblasts differentiated from PCC3 (3/A/1-D-3) are shown in Fig. 1.

The electrophoretic patterns obtained for each cell line were essentially identical in three independent experiments. This reproducibility was due to the highly denaturating conditions of the 'lysis buffer' which eliminated all hydrolytic activity.

A schematic representation of the total number of major iodinated proteins presented in the five studied cell lines is given in Fig. 3 which summarizes the arbitrary numbering assigned to major proteins and their approximate molecular weights and isoelectric points. It shows that about 30 different surface proteins were iodinated in the five cell lines studied. Their molecular weights have rather high values, mostly between 40 000 and 200 000. The isoelectric points of all surface proteins analyzed here are situated in the acidic to neutral

pH region, between pH 4.8 and 6.8. No basic proteins were observed under our experimental conditions.

14 out of 33 surface proteins are common to all cell lines analysed (Fig. 1). These common proteins are: 7, 9—17, 20, 24, 27, 28. 18 proteins were iodinated in the case of undifferentiated cell lines F9 and PCC3, and 17 were found on PCC4 cells. 16 proteins were labeled on the surface of endodermal PYS cells and 25 on fibroblasts 3/A/1-D-3.

The protein 2 (200 kdaltons and isoelectric point 5.5—5.6) comigrated with the monomer of fibronectin which was isolated by affinity chromatography on a gelatin/Sepharose column according to Engvall and Ruoslahti [22]. Spot 2

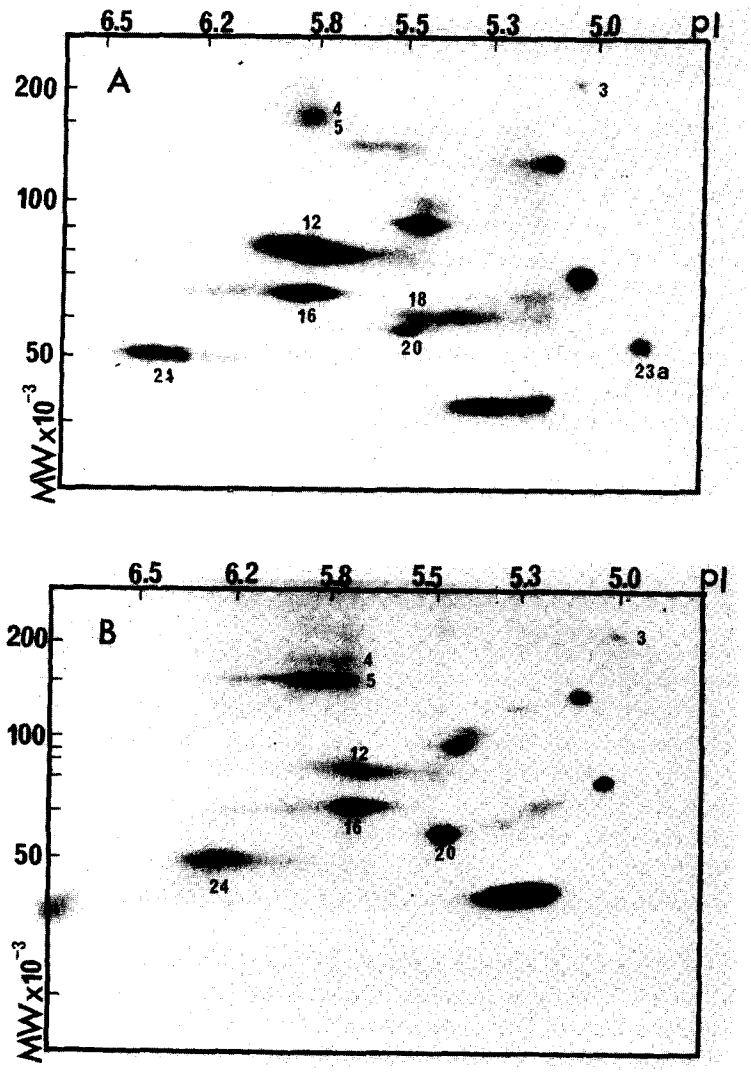
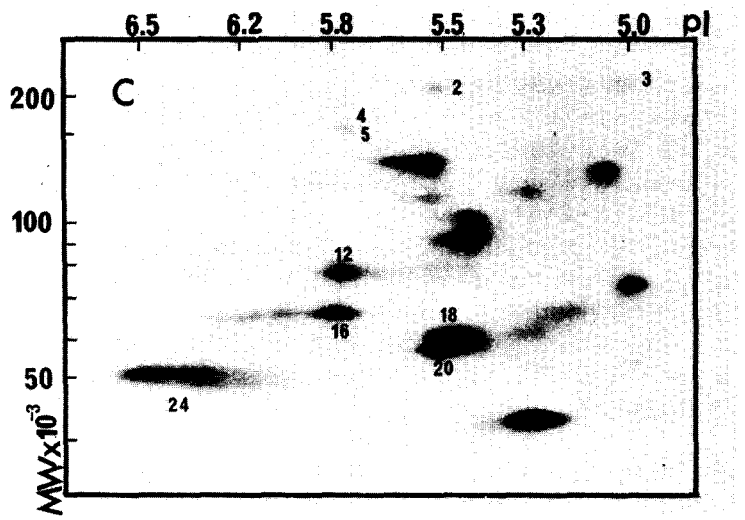


Fig. 1. Two-dimensional gel electrophoresis of the lactoperoxidase ^{125}I-labeled proteins solubilized by NP40/urea/ampholyte/mercaptoethanol from F9 (A), PCC4 (B), PCC3 (C), PYS (D), 3/A/1-D-3 fibroblasts cell lines (E).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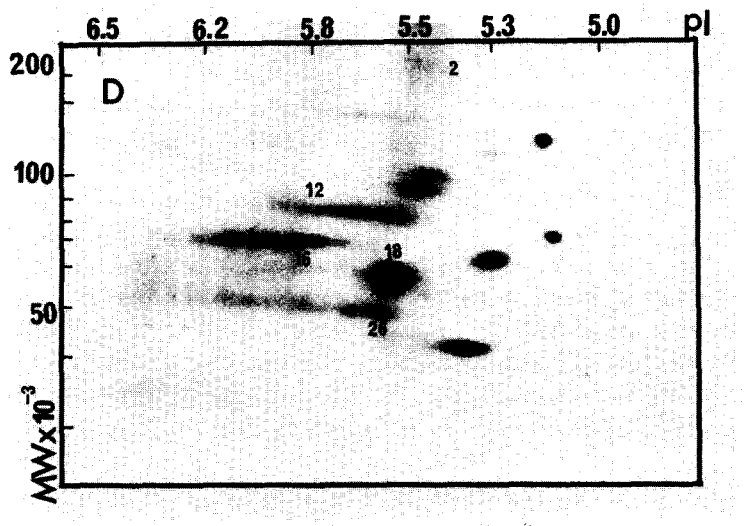
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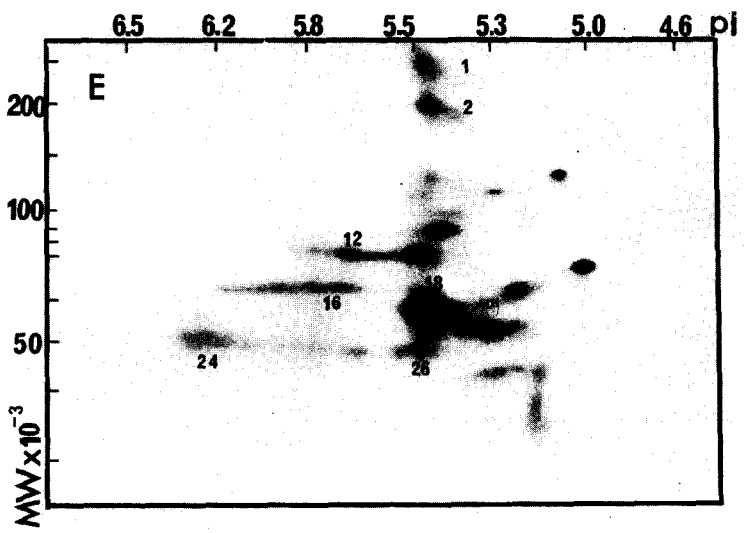
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Fig. 1. C, D and E.

corresponding to the monomer was the most intensively iodinated on 3/A/1-D-3 fibroblasts, but traces were also found on PCC4, PCC3 and PYS cell lines. Protein 1 which has the same isoelectric point but an apparent molecular weight higher than 400 kdaltons may possibly be the dimeric form of fibronectin. It was found on 3/A/1-D-3 cells only.

An increased accessibility to iodination in differentiated cells was also observed for protein 18. On the contrary, the availability for iodination of proteins 12, 16 and 24 decreased in differentiated cells.

The results of Fig. 1 show that three proteins, 3 (200—220 kdaltons and isoelectric point 5.1), 4 and 5 (150—160 kdaltons and isoelectric point 5.9), are iodinated on undifferentiated cells only.

A comparison of iodinated proteins solubilized by neutral detergent Nonidet P40 with those solubilized by 'lysis buffer'

The presence of proteolytic activity in the samples solubilized by Nonidet P40 was the major difficulty which will be discussed in the next paragraph. In order to eliminate the proteolysis, the surface proteins were solubilized by Nonidet P40 which was 1 mM in diisopropylphosphorofluoridate and the samples were immediately electrophoresed. Fig. 2 shows the proteins extracted by Nonidet P40. All of them are present in the samples extracted by lysis buffer from the same cell lines (Fig. 1), but the number of iodinated proteins solubilized by Nonidet P40 is reduced in comparison with those solubilized by 'lysis buffer'. The proteins 1, 2, 4, 6, 8, 10, 19, 21, 22, 23a, 24, 25, 27 and 28 (Fig. 3) are absent in the Nonidet P40 samples. We suggest that those proteins which resist Nonidet P40 extraction may be bound to some submembrane or cytoskeleton structures by non-covalent bonds. In this group of surface proteins (absent in Nonidet P40 extracts) it is important to mention that proteins 4 and 5 were found in undifferentiated cells only. To the proteins which were not extracted by Nonidet P40 in the absence of urea belong also proteins 1 and 2

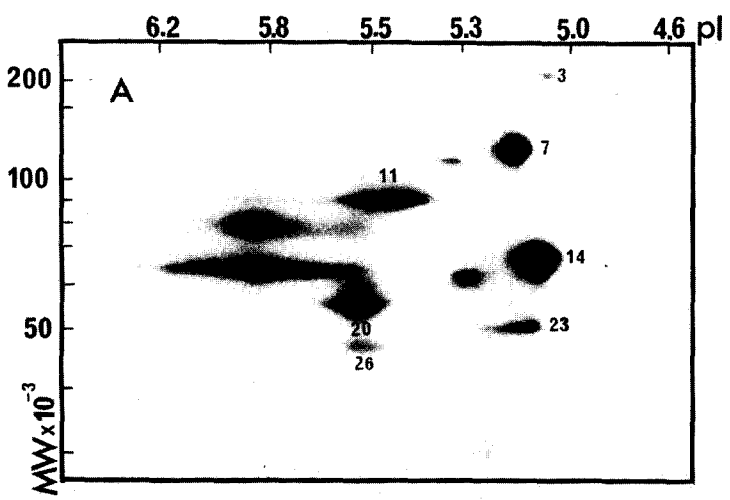

Fig. 2A.

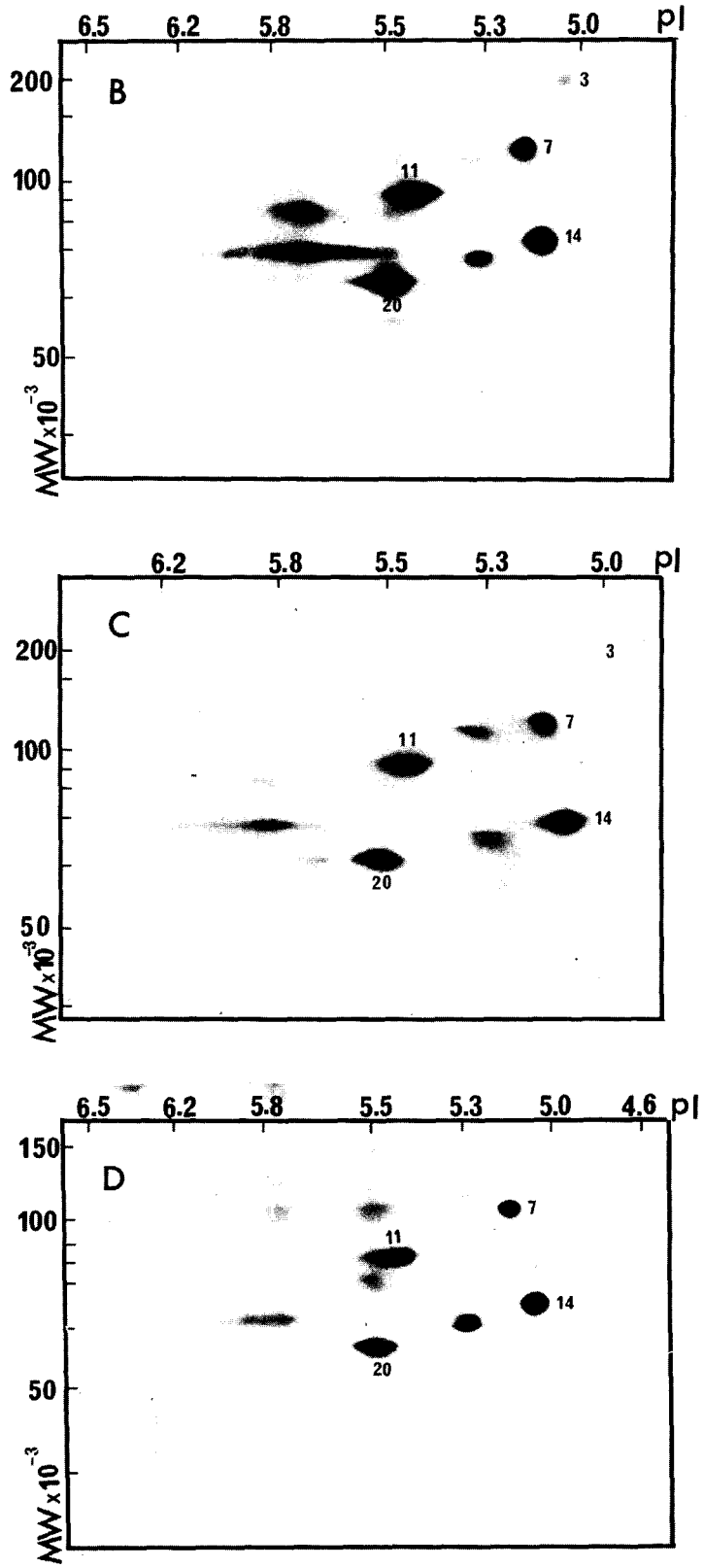
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Fig. 2. B, C and D.

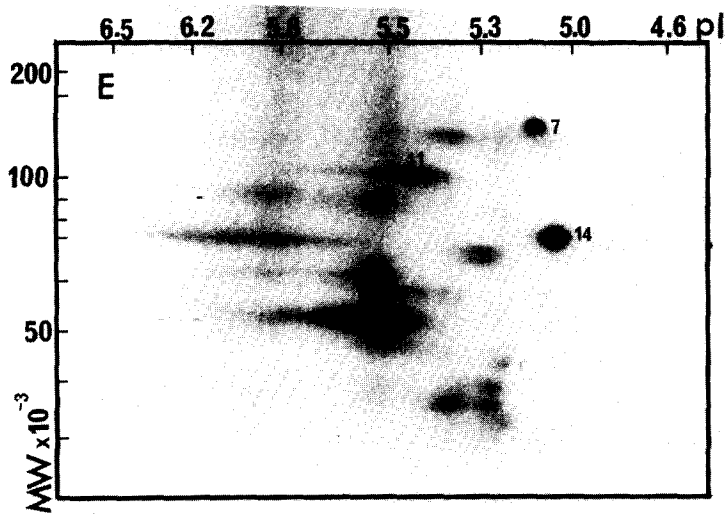


Fig. 2. Two-dimensional gel electrophoresis of lactoperoxidase ^{125}I-labeled proteins solubilized by 2% NP40, from F9 (A), PCC4 (B), PCC3 (C), PYS (D) 3/A/1-D-3 fibroblasts cell lines (E).

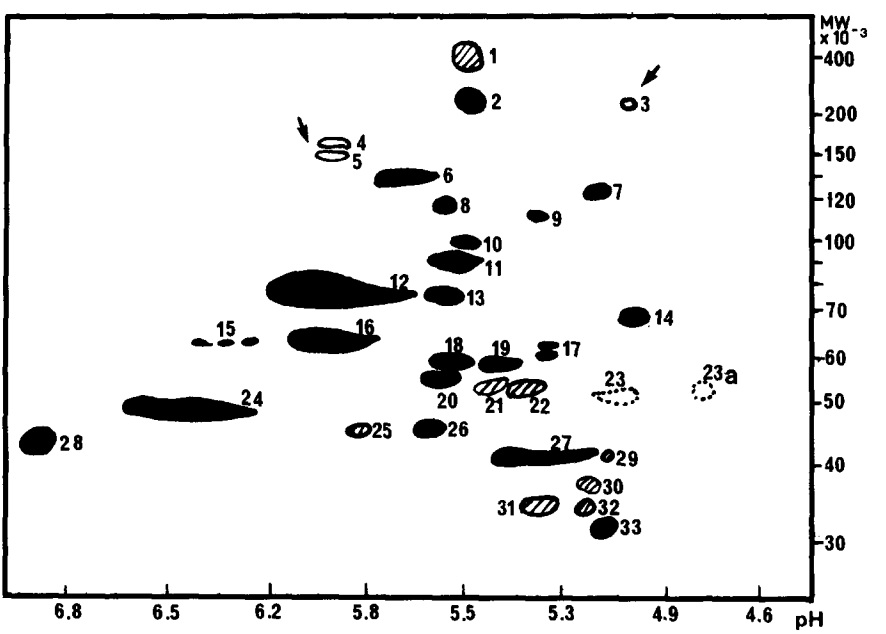


Fig. 3. A diagrammatic representation of the proteins shown in Figs. 1 and 2. The proteins which were found in both differentiated and undifferentiated cells are shown in solid figures. The proteins which were found in undifferentiated cells only are shown by open figures and arrows. The proteins which were found on F9 cells only (23 and 23a) are shown by open figures, encircled by dashed lines. The proteins which were found in differentiated cells only are shown by hatched spots.

(fibronectin). It has been shown that the presence of urea is indispensable for dissociation of fibronectin from the cell [22].

Proteins 23 and 23a were found in nullipotent F9 cells only. Protein 23 was found in the Nonidet P40 extract, whereas protein 23a was found in the case of solubilization by lysis buffer. Both proteins have the same size (50 kdaltons), but the isoelectric point of 23 is more basic (I = 5.1 instead of 4.8). These two spots could correspond to the same protein which has changed its charge under action of hydrolytic enzymes which can be active in Nonidet P40 solution, like neuraminidase (loss of sialic acid) or aminopeptidase (possible loss of aspartic or glutamic acid).

A comparison of iodinated surface proteins solubilized by the two different methods from undifferentiated embryonal carcinoma cells, and their differentiated derivatives has shown that among three proteins which are present in undifferentiated cells only, for example, 3 (200 kdaltons and isoelectric point 5.1) 4 and 5 (150—160 kdaltons and isoelectric point 5.7), two (4 and 5) were solubilized by 'lysis buffer', but not by Nonidet P40.

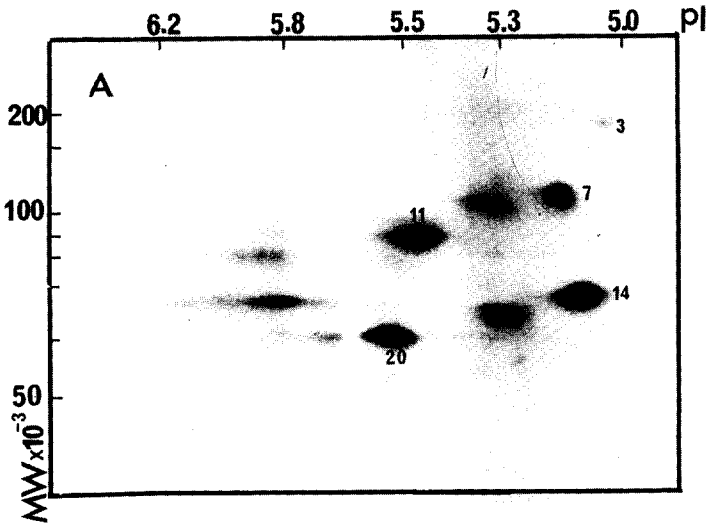
A study of possible proteolytic digestion or other changes under the different conditions of surface protein solubilization

The only criterion for the identity of the proteins in this work was their position in two-dimensional electrophoresis; in other words, their molecular weights and isoelectric points. Therefore it was essential to ascertain that these values were not altered by any secondary degradation.

In the case of the solubilization by 'lysis buffer' the possibility of proteolytic activity is considerably diminished due to the presence of such denaturing reagents as 9.5 M urea and reducing reagents such as 5% β-mercaptoethanol. When the samples were frozen in small aliquots and thawed once before use, the electrophoretic pattern did not change after two weeks of storage. On the other hand, several cycles of thawing and freezing during one month led to the appearance of some additional spots.

More important changes during storage were observed in the samples of surface proteins solubilized by Nonidet P40. A comparison of the electrophoretic patterns of PCC3 surface proteins solubilized by Nonidet P40 and conserved in the presence and in the absence of 1 mM diisopropylphosphorofluoridate is given in Fig. 4. A change in the electrophoretic pattern marked by the appearance of new iodinated proteins of lower molecular weights (Fig. 4 B, C) indicates a proteolytic degradation which could be considerably diminished by the addition of diisopropylphosphorofluoridate (Fig. 4A). Therefore serine protease(s), at least in part, participates in the proteolysis of surface proteins extracted by 2% Nonidet P40. The same protection by diisopropylphosphorofluoridate was observed in the case of F9, PCC4 and PYS cell lines.

In order to see which proteins are digested first, the surface proteins freshly extracted by Nonidet P40 from F9 cells were incubated for 2 h at 37°C. The samples before and after incubation were compared by two-dimensional electrophoresis. The results of Fig. 5 show that, after incubation, protein 3 which is present in undifferentiated cells only, 23 which was found exclusively in F9 cells, as well as proteins 9 and 17 common for all five cell lines, com-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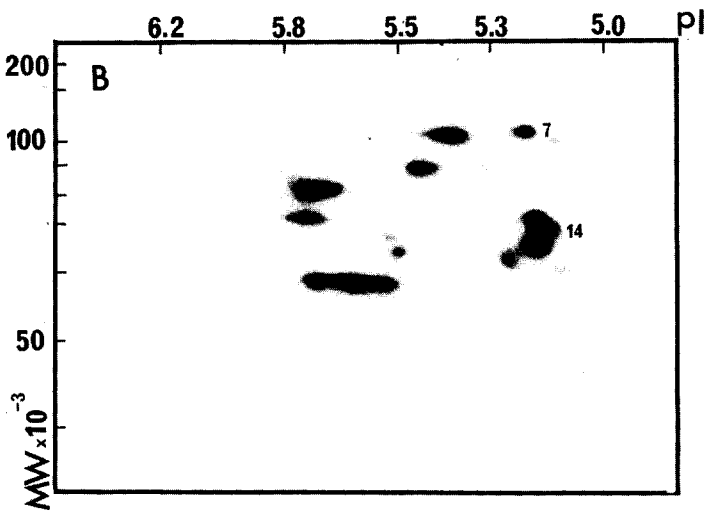
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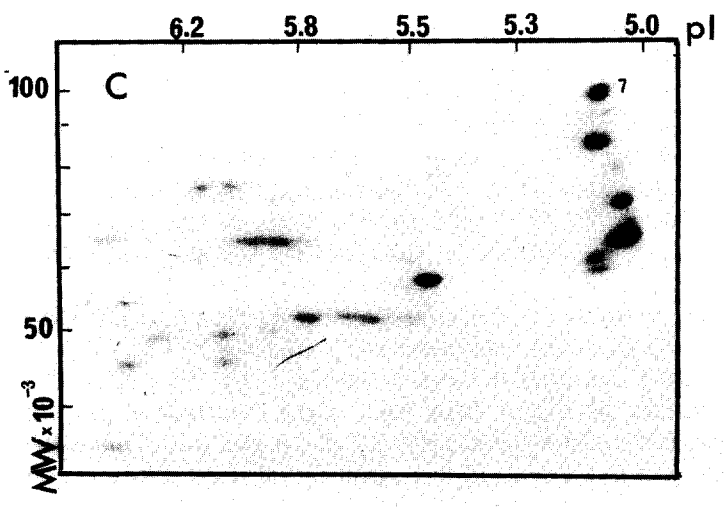
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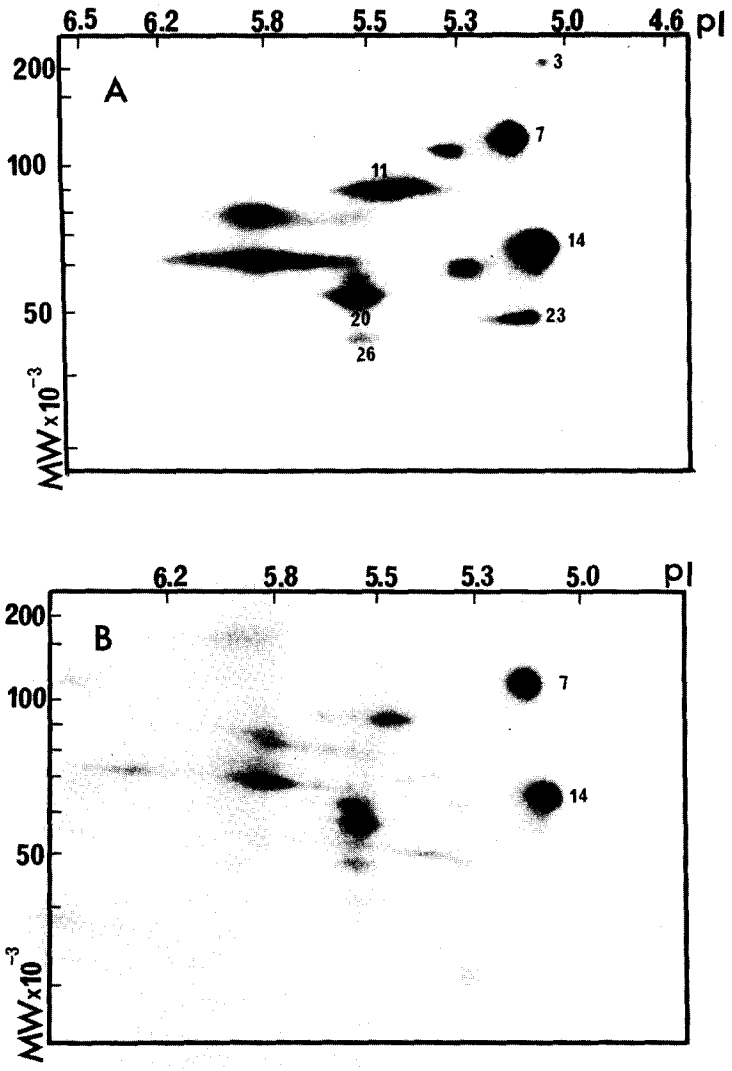

Fig. 5. Two-dimensional gel electrophoresis of F9 surface proteins (A) freshly solubilized by NP40, with diisopropylphosphorofluoridate (1 mM) added immediately to the sample, (B) aliquot of the same sample without diisopropylphosphorofluoridate incubated 2 h at 37°C. The samples A and B were analyzed in the same electrophoretic run.

pletely disappeared during incubation. The quantities of proteins 11, 12, 16 and 20 decreased. These results show that proteins 3 and 23 which were found in F9 cells exclusively are both sensitive to proteolytic activity in solution of Nonidet P40.

Fig. 4. Two-dimensional gel electrophoresis of PCC3 surface proteins solubilized by 2% NP40 (A) after their solubilization by NP40 containing 1 mM diisopropylphosphorofluoridate and one week of storage at −20°C, (B) after solubilization by NP40 without diisopropylphosphorofluoridate and one week of storage at −20°C, (C) after 1 month of storage without diisopropylphosphorofluoridate, including once-a-week thawing and freezing.

Discussion

A comparison of iodinated cell surface proteins in embryonal carcinoma lines and their in vitro differentiated derivatives has shown that the main pattern (14 out of a total number of 33 iodinated proteins) is similar in all investigated cells.

All the proteins are characterized by a high molecular weight and rather acidic isoelectric point. The same character of surface glycoproteins was reported for baby hamster fibroblasts BHK21 [23]. In that case the acidic isoelectric point was changed for most of the BHK surface glycoproteins after treatment by neuraminidase which removes sialic acid residues. More than 20 major lactoperoxidase radioiodinated components were found in the case of BHK fibroblasts by two-dimensional electrophoresis. In our experimental conditions we found 25 radioiodinated proteins in the case of the fibroblasts 3/A/1-D-3 which were differentiated from PCC3 embryonal carcinoma cell lines. On the surface of undifferentiated F9, PCC4, PCC3 and endodermal PYS cells, about 16—18 surface proteins were iodinated. Nevertheless the quantities or availability to iodination of certain proteins considerably change in the case of differentiated cells. We have found the closest similarities between three undifferentiated cell lines: F9 nullipotent, PCC4 nullipotent in vitro and multipotent in vivo and PCC3 multipotent in vivo and in vitro. Three proteins, 3, 4 and 5, were found on the surface of undifferentiated cells only, although their iodination is less extensive in the case of multipotent cells PCC3.

Only one protein 3 (200 kdaltons and isoelectric point 5.1) could be solubilized by Nonidet P40, two others, 4 and 5, were extracted by 'lysis buffer' only. The results obtained in Fig. 5 have shown that protein 3 is very sensitive to proteolysis, at least after its solubilization by neutral detergent Nonidet P40. It is evident that solubilization by detergent could be followed by conformational changes making surface proteins less resistant to proteolysis than they are in the intact cell. The proteases could also play a role in the disappearance of protein 3 during differentiation. Our results (Fig. 4) have shown that proteases entirely change the electrophoretic pattern of membrane proteins extracted by Nonidet P40 and that proteolysis is considerably diminished in the presence of 1 mM diisopropylphosphorofluoridate (inhibitor of serine proteases). The sensitivity of surface proteins to proteolysis is consistent with the concept of protease participation in the cellular differentiation. A general participation of proteases, particularly serine proteases such as plasminogen activator, in the development and differentiation of cultured mouse embryos, tissue remodeling and cell migration is already known [24,25].

A comparison of proteins solubilized by 'lysis buffer' with proteins solubilized by Nonidet P40 has shown that six proteins out of 30 could be solubilized under the strong denaturing conditions only; they are probably bound to some undermembrane structure, like cytoskeleton. In this group of proteins the protein 2 was identified as fibronectin.

Our results have shown that iodination of fibronectin (protein 2) is substantially increased in differentiated cells-fibroblasts 3/A/1-D-3, and only traces are iodinated in PCC3, PCC4 and PYS cell lines. This is in agreement with results obtained by Wolfe et al. with different cell lines of mouse terato-

carcinoma [26]. These authors have found that although undifferentiated nullipotent and multipotent cell lines and PYS synthesize fibronectin and release it into the medium, they do not retain it on their surface.

In the evaluation of results obtained in the present work certain limitations of methods must be considered. Firstly, the intensity of iodinated spots does not necessarily correspond to the quantity of protein but to the number of tyrosine residues available for iodination by lactoperoxidase. Secondly, certain changes, like subunit association or dissociation, could be induced by ampholyte, urea or β-mercaptoethanol in the conditions of two-dimensional gel electrophoresis [27,28]. The precision of the estimation of the molecular weights and isoelectric points under study is not absolute. It is well known that most of the membrane proteins contain sugars [1,29] which influence their mobility in SDS electrophoresis. The pH gradient was determinated in one tube in the set of twelve. Consequently, some deviation in parallel samples cannot be excluded. Therefore in this work we prefer to use the term 'approximate' for the values of molecular weights and isoelectric points.

The disappearance or appearance of the iodinated products in the differentiated cells is not direct evidence for a change in proteosynthesis. It could also reflect postranslational changes in the protein such as glycosylation, phosphorylation, partial proteolytic degradation or other secondary reactions. Only the isolation and identification of individual surface proteins could answer the question of their structural relationship to cell differentiation.

Acknowledgments

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