Biochimica et Biophysica Acta, 332 (1973) 59–68

BBA 76520

IODINATION OF CELL MEMBRANES
II. CHARACTERIZATION OF HeLa CELL MEMBRANE SURFACE PROTEINS

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(Received June 18th, 1973)

SUMMARY

The molecular weights of the five iodinatable surface membrane proteins of HeLa cells were determined to be 170000, 145000, 130000, 93000 and 53000. The proteolytic digestion of these proteins with pronase, trypsin and chymotrypsin was also studied.

Metabolic studies showed that these iodinated surface proteins are released into the medium in both acid-soluble and acid-insoluble forms. Antibodies prepared towards these released membrane fragments as well as antibodies prepared towards whole membrane inhibit the growth of HeLa cells.

INTRODUCTION

In the previous paper of this series [1] we presented evidence that the lactoperoxidase–H_2O_2–I^- method of iodination of cell surface proteins of Phillips and Morrison [2] can be a very effective tool for the iodination of eukaryotic cells if used judiciously. We also showed how under certain conditions it can be used to label HeLa cells. We indicated some of the pitfalls which may be encountered and how these can be avoided or can be taken advantage of experimentally.

The work on HeLa cells indicated that five main protein bands were iodinated under optimal conditions on the HeLa cell surface.

In this article we wish to present a more detailed characterization of these proteins in terms of molecular weight and gross chemical composition. In addition we wish to present some studies on their metabolic turnover as well as the effect of antiserum prepared against these membrane fractions on the growth of HeLa cells.

MATERIALS AND METHODS

The iodination procedures, the methods for the isolation of plasma membranes as well as the sodium dodecylsulfate–polyacrylamide gel electrophoresis

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have been described [1]. The protein bands in the acrylamide gels were stained with Coomassie blue [3], the glycoproteins were stained with basic fuchsin [4]. In the protease experiments, trypsin and lima bean trypsin inhibitor was obtained from Worthington Biochemical Co.; α-chymotrypsin from Sigma Co.; the protein standards used for the determination of molecular weights were obtained from Schwarz and Mann; phosphorylase *a* was from Sigma Co. The plasma membranes were isolated as previously described [1]. During this isolation, and beginning with the discontinuous sucrose gradient, all solutions including the dialysis steps contained 2 mM phenylmethylsulfonylfluoride [5] to minimize protein degradation. After the trypsin digestion, an equal amount of lima bean trypsin inhibitor was added to the cell suspension before the cells were washed. The following precautions were taken to avoid extensive hydrolysis of the proteins after the isolation of the membranes. The cells were washed six times with complete medium (containing 10% horse serum) to compete for the added proteolytic enzymes so that they would not further degrade the membrane components. In addition, the cells were also washed and the membranes isolated in the presence of phenylmethylsulfonylfluoride to inhibit proteolytic activity. This latter treatment has been shown to maintain the integrity of the membrane proteins after proteolytic digestion [5].

Extraction of plasma membrane with organic solvents

Cell membranes were extracted with ethanol–diethyl ether (3:1, v/v) or acetone–ethanol (1:1, v/v) 3 times at −20 °C as described by Guidotti [6].

Release of ^{125}I-labeled surface proteins during growth of HeLa cells

HeLa cells were isolated and iodinated as usual under conditions which minimized bacterial contamination. They were then washed with sterile medium and suspended in medium ± fetal calf serum. Bacterial count detected by plating on nutrient agar was 20/ml culture at 0 h, 30/ml culture at 4 h and 10000/ml culture at 24 h, while HeLa cells were $3 \cdot 10^5$ to $5 \cdot 10^5$ cells/ml. Aliquots were removed at intervals and centrifuged for 10 min at 4 °C. The acid-insoluble radioactivity (5% trichloroacetic acid) was determined in both the supernatant and cell pellets. The kinetics of ^{125}I release do not respond to this change in bacterial population (Fig. 4).

Isolation of released ^{125}I surface proteins in non-protein containing media

The outline of the experiment was the same as above except that the ^{125}I-labeled cells were suspended in complete media in the absence of fetal calf serum. The cells, after 3–4 h tended to attach to the glass surface but remained trypan blue negative. After removing the cells, the medium was dialyzed for 24 h against repeated changes of 0.5 M NaCl to facilitate the removal of low molecular weight ionized compounds and then for 24 h against water. The medium was then lyophilized. This yielded the released membrane proteins referred to below.

Preparation of antisera

Rabbits were injected with released membrane proteins or isolated plasma membranes [1]. The first injection was given subcutaneously as an emulsion (1–2 mg protein/ml) with complete Freund's adjuvant while the next three, in 2-week intervals,

were given intradermally into the foot pads. Blood was taken from the ear vein two weeks after the last injection, sera were isolated, collected and stored at −40 °C in small portions.

The effect of antisera on the growth of cells

The HeLa cell cultures ($2 \cdot 10^5$–$3 \cdot 10^5$ cells/ml) were maintained in 25-ml Spinner flasks. To these were added antisera with or without prior heating at 56 °C for 30 min to destroy complement or sera from non-immunized rabbits as controls.

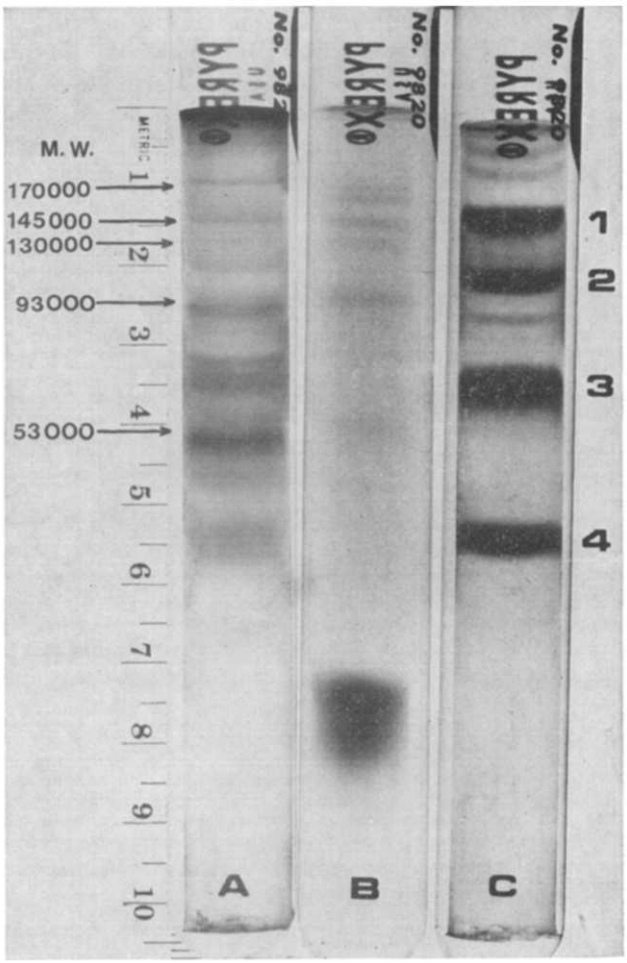

Fig. 1. Coomassie blue and Fuchsin staining pattern of HeLa membranes after sodium dodecyl-sulfate–acrylamide gel electrophoresis. ^{125}I-labelled HeLa cell membranes (100–250 μg) were electrophoresed on 7.5% acrylamide gel and stained for protein (A) and carbohydrate (B). The molecular weight standard on Gel C: Marker 1, human γ-globulin (160000); Marker 2, phosphorylase *a* (94000); Marker 3, bovine serum albumin (67000); and Marker 4, chymotrypsinogen (25000). The molecular weights identify the radioactive bands. The position of bromophenol blue was located by stabbing the gels with a needle, gel A 6.3 cm; Gel B, 6.5 and Gel C, 6.1 cm.

RESULTS

1. The radioactivity pattern and the molecular weight distribution of the ^{125}I-labeled cell membrane proteins

The HeLa cell membranes can be isolated in relatively pure form [7]. When intact HeLa cells are labeled with ^{125}I and the membranes isolated, solubilized and separated on sodium dodecylsulfate–acrylamide gels (Fig. 1) at least twenty bands can be detected by staining with Coomassie blue (Gel A), six bands stain for carbohydrate with the fuchsin stain (Gel B) while five bands are labeled with ^{125}I. The intense, fast-moving band staining for carbohydrate (Gel B) was associated with sodium dodecylsulfate front and probably is a glycolipid.

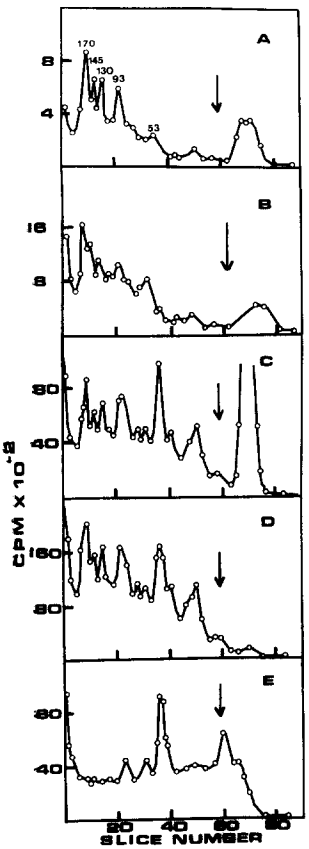

Fig. 2. ^{125}I profile of sodium dodecylsulfate–acrylamide gels of HeLa cell membranes obtained under different conditions. (A) Pattern observed when $2.0 \cdot 10^7$ HeLa cells/ml are iodinated, membranes extracted, etc., as per legend to Fig. 1. Included above each peak are the molecular weights in thousands corresponding to each peak. (B) Pattern observed when $5 \cdot 10^5$ HeLa cells/ml are iodinated, membranes extracted, etc., as per legend to Fig. 1. (C) Pattern observed when membranes are first isolated and then iodinated. (D) Pattern observed when membranes in C are extracted with alcohol–ether before acrylamide gel electrophoresis. (E) Pattern observed when membranes are first extracted with alcohol–diethyl ether and then iodinated. Protein, 10–50 μg. The arrow indicates the position of bromophenol blue marker.

Of the five ^{125}I-labeled bands, at least three coincide with the carbohydrate stain. The molecular weights of the ^{125}I-labeled bands, determined by sodium dodecyl-sulfate–acrylamide gel chromatography, based on known standards, are 170000, 145000, 130000, 93000 and 53000 ± 10% (Fig. 2) [8]. The 93000 molecular weight peak occasionally contains a lower molecular weight shoulder and at other times a distinct closely associated peak of approx. 80000 molecular weight. As discussed below, the low molecular weight radioactive peak beyond the bromophenol blue marker is extractable by alcohol–diethyl ether.

In contrast, when HeLa cell membranes are first isolated and then iodinated, the ^{125}I pattern of Fig. 2C is observed; note also that the extent of labeling is very much greater. Under these conditions, the predominant ^{125}I peak is one which moves ahead of bromophenol blue and has the following characteristics: (a) the molecular weight is extremely small, less than 10000; (b) iodination of isolated membranes followed by alcohol–diethyl ether extraction eliminates this low molecular weight peak, leaving the other peaks untouched (Fig. 2D); (c) extraction of isolated membranes with alcohol–diethyl ether followed by iodination yields a very different spectrum of iodinated proteins (Fig. 2E). This observation may be interpreted to indicate that the three dimensional configuration of the exposed membrane proteins has changed after alcohol–diethyl ether extraction.

Comparison of the two protein labeling patterns (obtained after iodination of whole cells versus that obtained after iodination of isolated membranes) shows that in the former case a very discrete labeling pattern is observed (Fig. 2A versus Figs. 2C, 2D and 2E). It is interesting to note that when a relatively small number of HeLa cells are labeled under these conditions, Fig. 2B, the acrylamide gel pattern becomes intermediate between that obtained when an optimal number of cells is labeled (Fig. 2A) and when isolated membranes are labeled (Figs 2C and 2D). This type of result is in agreement with the labeling of red blood cells in which it was clearly shown that additional membrane components can become labeled unless optimal labeling conditions are maintained.

2. Alteration of the ^{125}I protein pattern by treatment of ^{125}I HeLa cells with proteolytic enzymes

^{125}I-labeled HeLa cells were treated with pronase (Fig. 3A), trypsin (Fig. 3C) and chymotrypsin (Fig. 3D), respectively. The membranes were then isolated and the sodium dodecyl–sulfate–acrylamide gel patterns determined. For convenience, these are normalized with respect to the cell number and plotted within the pattern of the control sample so that the correspondence of the positions of the peaks can be determined as well as the relative loss of radioactivity. It should be noted at this time, that loss of radioactivity from the whole cells corresponds to a similar loss in radioactivity from the cell membrane. Pronase affects the four high molecular weight peaks more than do the other enzymes, but has relatively little effect on the small molecular weight components. Chymotrypsin has a primary effect on the high molecular weight component while trypsin has an intermediate type of action. It is interesting to note that the radioactivity pattern that emerges if the HeLa cells are first treated with pronase and then iodinated (Fig. 3B) is qualitatively similar to that obtained when this sequence is reversed (Fig. 3A); indicating that the families of exposed membrane proteins may be similar in both cases.

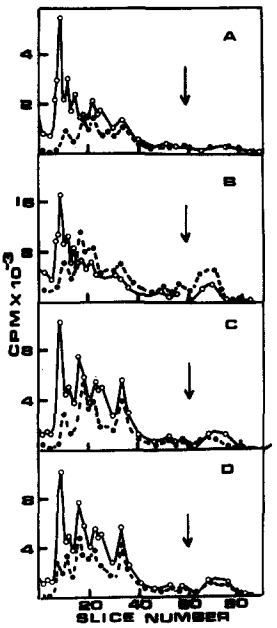

Fig. 3. The effect of proteolytic digestion on the ^{125}I profile of sodium dodecylsulfate–acrylamide gels of HeLa cell membranes. (A, C and D) After iodination, the HeLa cells ($6 \cdot 10^5$ cells/ml) were divided into two fractions — one was left untreated, the other treated for 30 min at 37 °C with pronase (25 μg/ml) trypsin (50 μg/ml) and chymotrypsin (50 μg/ml), respectively, (B) The HeLa cells ($6 \cdot 10^5$ cells/ml) were first treated with pronase (25 μg/ml, 30 min) and then iodinated. The solid lines in all cases represent the undigested control, the dashed lines represent the digested sample. The values within each of the four figures have been normalized with respect to the cell number to indicate the extent of digestion under these conditions. Consequently, the change in the radioactive profile per cell following proteolytic digestion can be seen. Membranes were isolated and treated as per legend to Fig. 1, 20–50 μg protein electrophoresed.

3. Are the exterior iodinated membrane proteins metabolically labile°

The labeled HeLa cells grow at the same rate as the control non-iodinated cells as shown in the preceding paper. ^{125}I-Labeled HeLa cells were incubated in complete medium and the acid-insoluble radioactivity of the whole cells and of the medium was monitored with time. Fig. 4A shows that the HeLa cells do lose radioactivity; however, only 20% of this loss can be accounted for by a corresponding increase of acid-insoluble radioactivity in the medium. Consequently, we have to conclude that most of these radioactive cell surface proteins are released as acid soluble fragments. Proteolytic activity under these conditions should be minimal because of the high (10%) competing concentration of serum proteins in medium. Deiodination, independent of turnover could also occur.

However, the question remains whether the degradation of the membrane proteins occurs by a reaction between cells and liberated proteins or whether it occurs during the process of extrusion of the cell membranes protein. This was done by incubating ^{125}I-labeled HeLa cells for 24 h in normal growth medium. The ^{125}I-labeled HeLa cells are then removed and the medium which now contained ^{125}I-labeled acid-insoluble proteins was incubated with an equivalent amount of

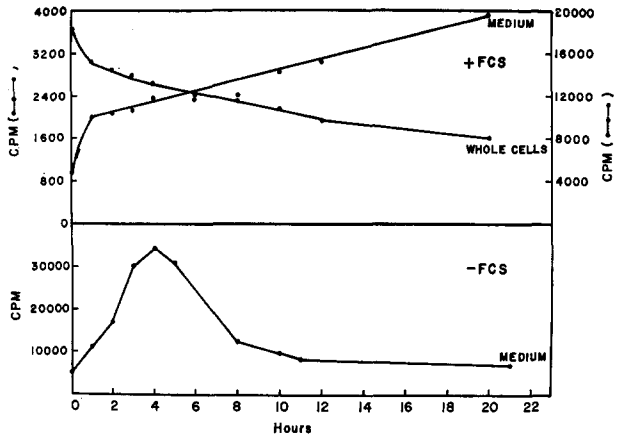

Fig. 4. Loss of acid-insoluble radioactivity from ^{125}I-labeled HeLa cells. (A) ^{125}I-labeled HeLa cells were incubated in the presence of complete medium containing foetal calf serum (FCS); the loss of acid-insoluble radioactivity from the cells and the appearance of acid-insoluble radioactivity in the medium were monitored. (B) A similar experiment in which the ^{125}I-labeled HeLa cells were incubated in the absence of foetal calf serum (FCS); because of experimental difficulties only the acid-insoluble radioactivity released into the medium was monitored. The cpm are normalized for 10^6 cells.

non-radioactive HeLa cells. Samples are removed over a period of 24 h and the acid-insoluble radioactivity was determined. It was found that in effect, continued degradation of the ^{125}I-labeled proteins occurred in the presence of HeLa cells as compared to control (no HeLa cells). This suggests that logarithmically growing HeLa cells can degrade the free proteins released from their surface. We have made attempts to identify the gel electrophoresis pattern of the released radioactive proteins; however, because of the large amount of serum present in the medium this has proved to be impossible.

4. Can the surface membrane proteins be isolated from the medium?

Incubation of ^{125}I-labeled HeLa cells in the absence of serum showed that in effect such ^{125}I-labeled acid-insoluble proteins can also be released in this case (Fig. 4B). However, the kinetics of the reaction show that this release is followed by a degradation after 4 h. In such a medium, HeLa cells start attaching to the glass surface and a number of aberrant reactions may occur although they remain trypan blue negative. We have grown large volumes of washed HeLa cells for 3 h in serum-free medium centrifuged off the HeLa cells and isolated the proteins released into the medium by lyophilization followed by dialysis and subsequent concentration (see Materials and Methods).

The radioactivity patterns are shown in Fig. 5B. Again, we see a multicomponent fraction; however, it shows a poly-disperse molecular weight pattern which is probably a reflection of the active degradation which is occurring as indicated by Fig. 5B. Administration of these fractions to rabbits with Freund's adjuvant has provided us with antiserum to these released membrane fractions.

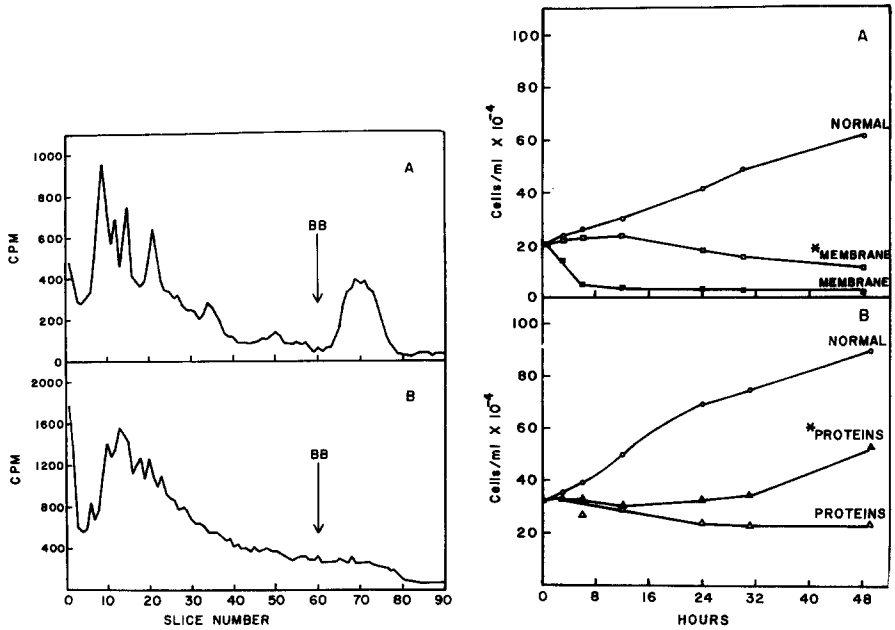

Fig. 5. Radioactivity profile of sodium dodecylsulfate–acrylamide gel electrophoresis patterns of labeled membrane proteins of HeLa cells. (A) The control membrane pattern of ^{125}I-labeled HeLa cells. (B) The pattern of radioactivity released into the medium from ^{125}I-labeled HeLa cells incubated for 3 h in the absence of foetal calf serum (FCS) (see Fig. 4). BB refers to the bromophenol blue marker.

Fig. 6. The growth pattern of HeLa cells in the presence of rabbit antiserum prepared against whole membranes and membrane fragments released from HeLa cells. (A) HeLa cells grown in the presence of "normal" rabbit serum, and serum of rabbits immunized with whole HeLa cell "membranes". The "*membrane" refers to serum which had been heated at 56 °C for 30 min to destroy complement. (B) A similar experiment in which serum of rabbits immunized with released HeLa cell membrane "proteins" was added (see Fig. 4, fraction obtained after 3 h incubation of HeLa cells). "*Proteins" refers to the same rabbit serum heated at 56 °C for 30 min. Amount of rabbit serum added in all cases is 70 mg to a 25-ml culture containing 200 mg foetal calf serum. Note that a slightly less inhibitory effect on the growth of HeLa cells occurs after incubating the serum at 56 °C for 30 min.

5. Effect of antiserum (prepared against released membrane proteins) on the growth of HeLa cells

When small amounts of such antiserum were added to HeLa cells growing in culture, the growth of the HeLa cells was inhibited. This inhibition was not noted with serum obtained from non-immunized rabbits but slightly decreased by heating the serum at 56 °C for 30 min to destroy complement (Fig. 6B). Fig. 6A shows that antiserum prepared against whole membranes is also inhibitory to HeLa cell growth. Consequently, we can conclude that this antiserum does affect the membrane surface of the HeLa cell as measured by this criterion. It is therefore also apparent that we are isolating fractions which are antigenically equivalent to sites on the cell surface.

DISCUSSION

The ^{125}I labeling experiments show that five proteins are reproducibly labeled by this method. Because of the method of labeling these must be relatively exposed proteins. Fig. 2B, shows that in HeLa cells as in red blood cells [1], extensive labeling of membrane proteins can occur if the correct iodination conditions are not maintained. There is an increased labeling of high molecular weight material and the distinct labeling of individual proteins is masked by an increased background of additional labeled proteins.

The two large molecular weight glycoproteins are also very sensitive to proteolytic digestion. We have found that upon proteolytic digestion, both the ^{125}I label and the glycoprotein staining disappear concurrently. The evidence is therefore reasonably good that these two, the ^{125}I label and the glycoproteins, coincide. However, this is not absolutely certain as yet. We anticipate that judicious use of degradative enzymes on whole cells suspensions combined with low temperatures and short incubation periods may yield these specific large molecular weight components in a relatively pure and undegraded form.

From the point of view of metabolic studies it is encouraging to know that this method of iodination does not interfere with cell growth. However, our studies on the degradation of these labeled proteins raise questions as to the significance of this release of trichloroacetic insoluble proteins. We are planning to study this question further in order to understand it in greater detail.

The inhibitory effect of the antiserum on cellular growth raises the question of the mechanism by which a membrane surface bound antibody inhibits the growth of the cell. From Fig. 6B it can be seen that this is a cellulostatic effect which can also be relieved with time (middle curve 6B). This effect is not lytic on the cells because the cell population maintains itself for at least 48 h. We do not know at present what significance to attribute to the drastic decrease in cell number which occurs when the cells are exposed to antiserum prepared against membranes (bottom curve, Fig. 6A); this phenomenon is at present under active investigation. Yang and Vas [9] have shown that heat-inactivated antisera prepared against whole L5178Y cells, inhibited the growth of the cells, their colony formation in soft agar, and the uptake into protein, RNA and DNA of radioactive precursors. However, the exact mechanism of this reaction is unknown.

ACKNOWLEDGMENTS

This research was supported by a U.S.P.H.S. Research Career Award to E.S.C. (C-71-2339); the American Cancer Society (BC 75) and a U.S.P.H.S. grant (CA-04823).

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