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IODINATION OF PLASMA MEMBRANE PROTEINS OF BHK CELLS IN DIFFERENT GROWTH STATES

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SUMMARY

The surfaces of BHK cells in confluent monolayers, immediately after mechanical dispersal and in logarithmically growing suspension cultures have been iodinated with ^{125}I using the lactoperoxidase technique. Electrophoretic resolution of the labeled proteins revealed that the representation of plasma membrane proteins varies with the growth state. Trypsinization of the cells produced a drastic revision of the surfaces leaving behind root fragments of membrane components and exposing additional proteins for iodination. The rapid turnover of membrane proteins in growing BHK cells restored the plasma membrane to a state characteristic of the replicating cell within 10 h.

INTRODUCTION

There is increasing evidence that the peripheral membranes in eucaryotic cells play an important role in the control of cell replication. With respect to the growth of normal cells in organized tissues and in confluent monolayers, it appears that the interaction of membranes leads in some way to a reduced probability for the expression of genes for nuclear replication and the restraint of the cells in the G_1 or G_0 state [1]. Accepting the concept of a dynamic mosaic structure for membranes [2], an explanation for the restriction of cell replication under these conditions might be found in the stable associations of certain membrane components; associations which in turn might restrict the activity of a component necessary for the activation or expression of the replication genes. In accord with this general view it has been shown in many systems that membrane perturbation of cells in the restricted state, either specifically with mitogens or hormones or generally with enzymes, nutrients or mechanical dislodging, activates cell replication in a matter of hours (for reviews see Pardee [3], Burger [4] and Herschman [5]). In addition, the particular growth states have been correlated with the presence in the membrane of certain glycolipids [6, 7], glycoproteins [8] and the activity of certain membrane associated enzymes [9–11].

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To inquire further into the role of membrane complexes in the control of cell replication the representation of surface proteins has been studied in BHK cells in different growth states. For this study the lactoperoxidase iodination technique with ^{125}I [12, 13] has been used to label the surface proteins which are subsequently resolved by electrophoresis in dodecylsulfate-polyacrylamide gels. The representation of surface proteins of cells in confluent growth state, after mechanical dispersal, and during recovery from trypsinization has been studied. Within the limits of the resolution of these experiments a rapid turnover of surface proteins in living cells growing in suspension has been documented. On trypsinization the pattern of representative proteins is grossly altered but rapidly returns to normal. Evidence for the existence of surface protein patterns characteristic of particular growth states is presented.

MATERIALS AND METHODS

Cell cultures

Baby hamster kidney fibroblasts, (BHK 21/13), were routinely cultured in 75 cm^2 Falcon flasks or occasionally in 32 oz glass pharmacy bottles with Eagle's medium containing 10% bovine serum [14] at 37 °C with an air–CO_2 (95 : 5, v/v) atmosphere. Cells, planted at about $1 \cdot 10^5$ cells per ml of serum-containing Eagle's medium, 30 ml per flask, were allowed to attach and to grow for 3 days before the first medium change. The medium was changed every 24 h thereafter. Post-confluent-inhibited cultures were used 7 or 8 days after planting when cell density had leveled off at about $8 \cdot 10^5$ per cm^2. For some experiments cells were grown before use for three or four generations in suspension cultures as described previously for HeLa cells [14].

Trypsinization procedure

Culture flasks were drained and washed one time with 10 ml of Saline A [15] at 37 °C. 10 ml of 0.05% trypsin (Nutritional Biochemicals Corp., 1-300) in Saline A were added and the flask returned to a 37 °C incubator for 7 min. To stop the trypsin reaction, 20 ml of serum-containing Eagle's medium were added and the cells drained from the flask, centrifuged and were either resuspended in serum-containing Eagle's medium for culture or prepared for iodination. In some experiments soybean trypsin inhibitor (Sigma, 1 mg inhibits 1.85 mg trypsin) at 2 mg/mg trypsin was used to stop the reaction.

Trypsinization of dislodged or suspension grown cells was carried out by sedimentation and resuspension of cells in 0.05% trypsin, 10 ml per $50 \cdot 10^6$ cells, and followed by incubation at 37 °C for 7 min.

Iodination procedure

The iodination was carried out using a modification of the lactoperoxidase method described by Phillips and Morrison [12] and modified by Hubbard and Cohn [13]. Glucose oxidase (D-glucose:O_2 oxidoreductase, EC 1.1.3.4) and glucose were used to generate H_2O_2 for the catalysis of iodination by lactoperoxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7.).

Monolayers of cells to be iodinated in situ were gently washed one time with

10 ml of Saline A and two times with 10 ml of Medium A (Dulbecco's phosphate-buffered saline minus Ca^{2+} and Mg^{2+} (pH 7.3)) at room temperature, before being covered with 10 ml Medium A containing 5 mM glucose. To the flasks were added in order and to the final concentrations indicated: lactoperoxidase (Calbiochem, B grade, 412/280 nm ratio = 0.7), 10–20 μg/ml, $Na^{125}I$ carrier-free, 10–20 μCi/ml, and glucose oxidase (Calbiochem, A grade) 0.1 μg/ml. The $Na^{125}I$ was diluted with 0.1 M NaOH prior to use. Sterile stock solutions of the enzymes were prepared in Medium A at 1 mg/ml and stored at 4 °C.

The flasks were gently agitated for 20 min at room temperature. The iodination reaction was terminated by draining off the reaction mixtures and adding 50 ml of Medium A. The cells were shaken loose from the flask and poured into 50 ml Falcon conical plastic tubes.

The iodination of cells already removed from the flask by mechanical dislodging or by trypsin digestion was carried out essentially as described above. The cells, in plastic tubes, were washed twice with Medium A, resuspended to $10 \cdot 10^6$ cells per ml in Medium A containing 5 mM glucose and iodinated for 20 min at room temperature. The reaction mixture was diluted with several volumes of Medium A. When the iodinated cells were to be recultured they were washed once in Saline A and once in culture medium prior to resuspension in Spinner medium [14] for further incubation at 37 °C.

For electrophoretic analysis of the iodinated proteins the cells were washed three more times in Medium A and the final cell pellet resuspended in 10% dodecylsulfate (Pierce Chemical Co.), 0.01 M phosphate buffer, sodium salts (pH 7.2) and 6 M urea (Schwarz/Mann, ultrapure). The lysate was heated at 100 °C until the cells were solubilized, about 1 h. The samples were dialyzed overnight at room temperature against several changes of dialysis buffer (0.01 M phosphate buffer, sodium salts (pH 7.2), 0.1% dodecylsulfate, 0.5 M urea, 5 mM EDTA, 5 mM 2-mercaptoethanol). The samples were collected and aliquots taken for determination of the protein concentration by the method of Lowry et al. [16] using bovine serum albumin, fraction V, as a standard.

In a control experiment the cell residue was washed successively with chloroform–methanol (1 : 1, v/v), chloroform–methanol (2 : 1, v/v) and with ether prior to dissolution and preparation for electrophoresis. This sample from which the lipids had been extracted gave the same staining and radioactivity pattern on electrophoresis as cells which had been dissolved directly in dodecylsulfate in the usual procedure.

Gel electrophoresis

Duplicate dialyzed samples containing 200–300 μg protein, 20% sucrose and bromphenol blue as a tracking dye were applied to 11.0 cm × 0.6 cm gels made with 8.5% polyacrylamide and cross-linked with 0.19% (v/v) ethylene diacrylate in 0.1% dodecylsulfate and 6 M urea. The gels were electrophoresed at 4–6 mA/gel for 20–16 h against 0.1 M phosphate buffer, sodium salts (pH 7.2) containing 0.1% dodecylsulfate. After electrophoresis, one gel of each set was fixed overnight in 50% methanol, 10% acetic acid, stained by soaking several hours in a solution of 0.1% amido black, 50% ethanol, 10% acetic acid and destained with 7.5% acetic acid.

Duplicate gels were frozen quickly on solid CO_2 and stored at −70 °C (up to 5 days) prior to cutting into 1-mm slices with a Macrotome-GTS (Yeda Scientific

Instruments). The slices were digested with 1.0 ml 0.1 M NaOH before addition of 10 ml Scintisol-complete liquid scintillation fluid (ISOLAB Incorporated, Akron, Ohio). Samples were counted either in a Packard Tri-carb liquid scintillation spectrometer model 3003 or in a Computerized Nuclear Chicago Isocap/300 liquid scintillation system with an external standard, and with a counting efficiency of about 65%.

The approximate molecular weights of protein bands were determined from the linear relationship between electrophoretic mobility and the logarithm of molecular weights of known proteins [17]. The standard proteins used were: myosin, collagenase, β-galactosidase, phosphorylase, pyruvate kinase, bovine serum albumin, ovalbumin, aldolase, DNAase, chymotrypsinogen, myoglobin, hemoglobin, cytochrome *c* and insulin.

RESULTS

Variation of cell surface proteins during changes in growth state

When cultures of BHK cells are allowed to grow to confluence on plastic or glass surfaces with daily renewal of the medium, the majority of cells stop replicating after about 5 days. Mechanical dislodging of the confluent cells or dispersal by a light trypsinization, followed by subculture in fresh medium, reactivates cell replication. With mechanical dispersal the response is rather asynchronous with some cells initiating DNA synthesis as early as 5 h and others requiring up to 24 h. With trypsinization the response is highly synchronous with approx. 90% of the cells initiating DNA synthesis after 9–10 h. In attempts to correlate membrane changes with changes in the growth state, the cell surfaces have been iodinated using the lactoperoxidase technique and the representation of ^{125}I-labeled surface proteins deter-

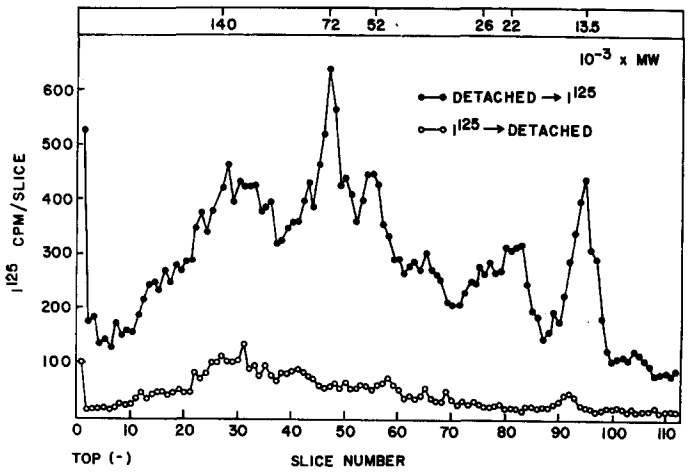

Fig. 1. Gel electrophoresis of protein residues from confluent cultures. BHK cells grown to confluence in monolayers were iodinated by the lactoperoxidase method either directly (○–○) or after mechanical dispersal from the culture flask (●–●). Both samples were treated alike except for the order of iodination and removal from the flask. 300 μg of protein were subjected to electrophoresis in 8.5% polyacrylamide gels containing 0.1% dodecylsulfate and 6 M urea. The gels were frozen and cut into 1-mm slices for ^{125}I assay.

mined by electrophoresis in 8.5% polyacrylamide gels in the presence of 0.1% dodecylsulfate and 6 M urea. The system resolves the representative surface proteins on the basis of molecular weight (i.e. 10 000–> 200 000 mol. wt). With it we have been able to characterize and to follow changes in cell plasma membrane proteins under various growth conditions.

To establish the representation of surface proteins of cells grown to the confluent state, such cultures of BHK cells were iodinated directly in the culture flask. After iodination the cells were shaken from the culture flask and analyzed electrophoretically as described under Materials and Methods. In this procedure the cells retained their protein-bound ^{125}I during the suspension procedure. A typical electrophoretic pattern of the iodinated proteins is shown in Fig. 1; it is characterized by a relatively uniform distribution of the ^{125}I throughout the spectrum of protein sizes. The electrophoretic system resolved cell proteins ranging in molecular weights of 14 000–> 140 000 into more than 40 stainable bands. Whereas there was a general predominance of label in the higher molecular weight components, the concentration of labeling in proteins of distinct sizes seen in other growth states was depressed. It was found that after cells were iodinated in situ and removed, some labeled proteinaceous material remained attached to the flask. Electrophoresis of this material failed to exhibit characteristic peaks and the low level of label, was again distributed rather evenly throughout the gel.

The changes in surface components following mechanical dislodging of cells from confluent monolayers was studied next. This operation was carried out at 37 °C with no attempt to inhibit any of the cells' endogenous enzyme activities. In these experiments the medium was drained from confluent cultures of BHK cells, replaced with 10 ml Saline A at 37 °C and the cells released from the plastic surface by a few vigorous shakes of the flask. After centrifugation the cells were washed in Medium A and iodinated immediately. Electrophoresis of the cell residues was carried out in 8.5% polyacrylamide gels as described under Materials and Methods. The distribution of the radioactivity in a typical gel is plotted in Fig. 1. In contrast to the results obtained with monolayers the iodination of mechanically dislodged cells yielded electropherograms with characteristic peaks of radioactivity in proteins corresponding to molecular weights of approx. 140 000, 72 000, 52 000 and 14 000. From experiment to experiment, (i.e. eight separate experiments) there were slight differences in the labeling of certain peaks, but the overall pattern was highly reproducible. Electrophoresis of the samples on 13% polyacrylamide gels gave essentially the same pattern and did not result in further resolution of the low molecular weight material. It is of considerable interest that these peaks corresponded fairly closely to the peaks of labeled components obtained on direct iodination of BHK cells growing logarithmically in suspension culture (Fig. 3, upper panel).

A second aspect of the iodination of mechanically dislodged confluent cells is that there appear to be many more sites exposed for labeling per cell and that this exposure of sites affects certain size proteins disproportionately. As shown in Table I mechanical removal of confluent cells increased the iodination under standard conditions 5.5–8.8-fold; near-confluent cultures were less affected. Within the limits of our experimental protocol this change attends the simple mechanical removal of the cells from the confluent monolayer state. Whether the increased and selective iodination of cell surface components following this treatment reflects the exposure

TABLE I

CHANGES IN SURFACE IODINATION ON MECHANICAL DISPERSAL OF CONFLUENT CULTURES

BHK cells were cultured to confluent or near confluent state and iodinated either directly or after mechanical suspension of the cells. Results are expressed as the level of ^{125}I incorporated (cpm/μg protein).

Experiment	Culture	Removed (A)	In situ (B)	A/B
I	confluent	148	27	5.5
II	confluent	821	83	8.8
III	near-confluent	499	176	2.8

of components otherwise confined to the cell membranes which are concealed in the borders of confluent cells or to a remarkable relocalization of intracellular components in the course of the dispersing process is a subject for discussion. In any extent the exposure of these components for iodination is of interest since it raises the possibility that certain membrane components may have been exposed for interaction with mitogens, hormones or nutrients in the medium which promote reactivation of cell replication in the dispersed cells.

Changes in the character of the surface proteins following trypsinization of confluent cell cultures

As mentioned earlier, cultures of BHK cells which are allowed to grow to confluence with daily renewal of the medium show very little replicating activity by about 5 days. A light trypsinization of such cultures disperses the cells and causes them to engage synchronously in cell replication. The next series of experiments describes the nature of the membrane changes associated with this transition. In the preliminary studies the immediate effects of trypsin on the membrane were investigated. For this purpose cultures of BHK cells exhibiting the confluent growth inhibition were treated with 0.05% trypsin for 7 min at 37 °C to release the cells from the culture flask surface. After dispersal and subculture of the cells as either monolayer or suspension cultures, DNA synthesis as measured by [^{3}H]thymidine incorporation, characteristically began after 9 h. The cell number increased 8 h later, i.e. approx. 17 h after the trypsinization. When the cells were iodinated at various times following trypsinization and subculture, strikingly different patterns of radioactivity were seen in the electropherograms of the cell proteins (Fig. 2). For example, iodination immediately following trypsinization yielded a pattern in which almost all the radioactive peaks of high molecular weight characteristic of non-treated cells were abolished; only one of the original peaks corresponding to proteins of molecular weights about 140 000 remained (Slices 21–23). This peak appears to be due to protein which is relatively resistant to trypsin action. Two new peaks of radioactive protein appeared in positions corresponding to approximate molecular weights of 16 000 and 9 000; these accounted for 50% of the radioactivity in the gel. This pattern persisted when aliquots of the same sample were electrophoresed on 13% polyacrylamide gels. In trypsinized samples more than 75% of the radioactivity was found in peaks of low molecular weight; these peaks did not correspond to stained proteins suggesting

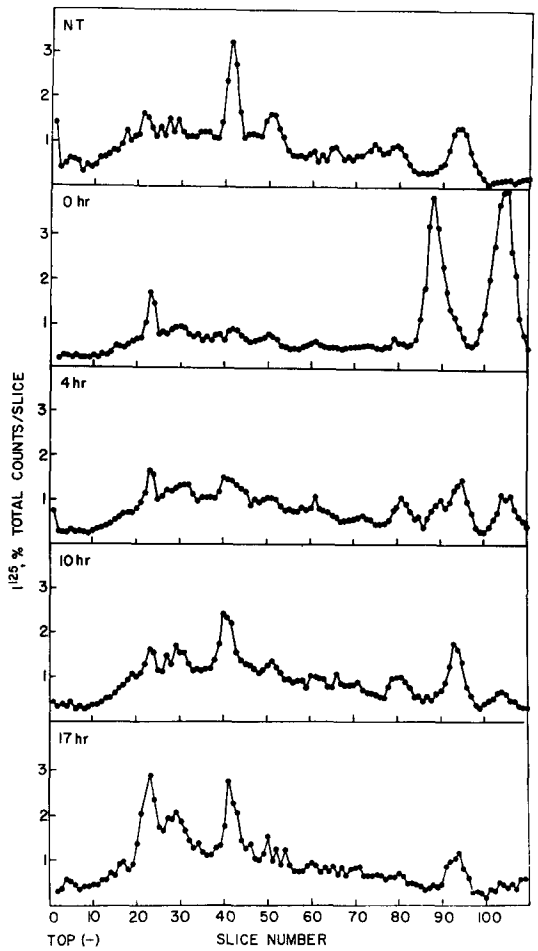

Fig. 2. Effect of trypsinization on the surface of confluent cells. BHK cells, grown to confluence in monolayers, were mechanically dislodged or dispersed by trypsinization. Cells were iodinated by the lactoperoxidase technique after being mechanically dispersed (n.t.) and at 0, 4, 10 and 17 h after trypsinization. 250 μg of cell protein were electrophoresed as described in Fig. 1 and under Materials and Methods.

the presence of highly radioactive fragments which are present in relatively low amounts. Since these low molecular weight components could be hydrolyzed by pronase to a dialyzable size it was concluded that they are small proteins or large peptides.

Iodination and electrophoretic analysis of the cell surfaces at 4, 10, and 17 h after trypsinization revealed a progressive return of the pattern of labeled surface proteins towards that characteristic of the non-trypsinized cells in suspension culture (Fig. 2). At 4 h the pattern contained peaks characteristic of both the untreated and trypsinized cells; the two low molecular weight peaks had largely disappeared and the peaks seen normally in the non-trypsinized cells were beginning to emerge. By 10 and 17 h the pattern was generally similar to that of non-trypsinized cells grown in suspension culture.

Changes in the surface proteins following trypsinization of BHK cells growing in suspension

BHK cells in suspension culture grow exponentially if provided with adequate fresh medium. As stated earlier, the gel electrophoresis pattern of the surface proteins of these cells as determined by iodination was quite similar to that of mechanically dislodged non-trypsinized monolayer cells (Fig. 1 and Fig. 3; n.t.). Trypsinization of the suspension grown cells, while not affecting the growth rate, produced a striking change in cell surface proteins. In fact, treatment of suspension grown cells with trypsin resulted in a similar exposure of proteins for iodination as seen with the trypsinized monolayers (Fig. 4). Again some trypsin resistant protein with a molecular weight of approx. 140 000 was evident; however, more than 76% of the counts on the electropherogram were found with the two peaks of lower molecular weight material. Amido black staining of gels from samples of trypsinized and non-trypsinized cells failed to show any differences in the pattern of bands visible to the eye or in a densitometer scan. This finding is taken as evidence that the trypsin action affects only a small fraction of the total cell proteins, primarily those exposed in the cell surface.

Cells which were trypsinized and then iodinated after 4, 10 and 17 h of subculturing showed again that the pattern of iodinatable surface proteins returns rapidly to the pre-trypsin state (Fig. 3). By 4 h many of the protein classes charac-

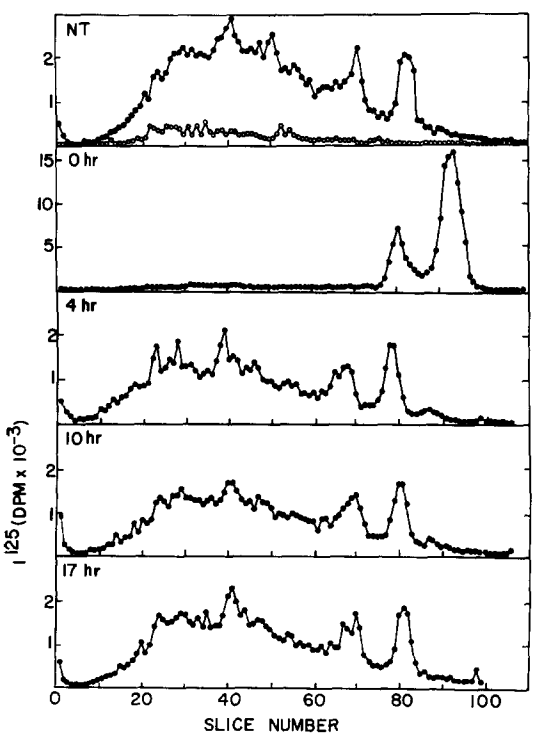

Fig. 3. Effect of trypsinization on the surface of cells grown in suspension. Cells from logarithmically growing suspension cultures were first trypsinized and then iodinated at intervals after 0, 4, 10 and 17 h of subculturing. The cell residues were analyzed electrophoretically for distribution of the label among proteins of different sizes as under Materials and Methods.

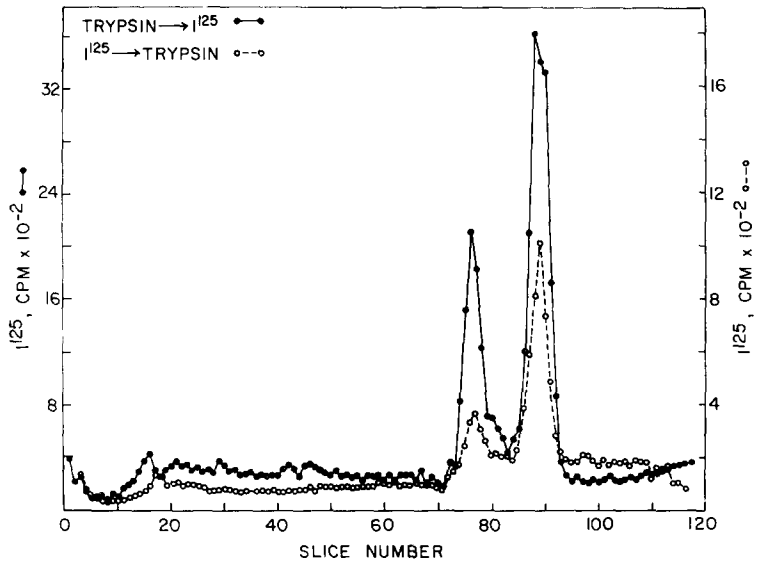

Fig. 4. A study of the surface proteins surviving trypsinization. BHK cells from suspension cultures were either iodinated and then trypsinized (○- - -○) or trypsinized and then iodinated (●–●). Samples of cell residues containing 250 μg of protein were subjected to electrophoresis in 13% polyacrylamide gels containing 0.1 % dodecylsulfate and 6 M urea.

teristic of untrypsinized cells were once again accessible for iodination.

In a second set of experiments monolayer cells were mechanically detached from the flask, iodinated and then subjected to trypsinization. About 50% of the radioactivity was released from the cells. The iodinated material which was retained by the cells ran in the same positions on the gels as the material which had been iodinated after trypsinization (Fig. 4). However, the cells which were iodinated after

TABLE II

THE EFFECTS OF TRYPSINIZATION ON THE IODINATION OF SURFACE PROTEINS OF BHK CELLS

Dispersed cells were iodinated directly or following treatment with 0.05 % trypsin at 37 °C for 7 min. Aliquots of the directly iodinated cells were also subjected to trypsin treatment for removal of the iodinated surface components. Cells which were used in these experiments were either grown for several generations in suspension culture or to confluence in monolayers before being mechanically dispersed. The results are expressed as ^{125}I incorporated (cpm/μg protein) in the protein residues after dialysis against dodecylsulfate–urea buffer as described under Materials and Methods for the preparation of samples for electrophoresis.

Culture conditions	Treatment		
	Iodination directly	Iodination followed by trypsinization	Trypsinization followed by iodination
Monolayer	188	112	313
Suspension	102	20	346
Suspension	90	37	211

trypsinization were labeled to a greater degree (313 cpm/μg protein) than cells iodinated without being trypsinized (188 cpm/μg protein) (Table II), a result similar to that reported by Phillips and Morrison [18] with red blood cells. There was no significant change in the protein content of the various samples following trypsinization.

These results suggested that trypsin acted on the cell surface to cleave and release certain exposed protein fragments while allowing root fragments to remain anchored in the membrane. Acting in this manner it appears that the trypsin also exposed or made accessible for iodination some previously unavailable proteins as well as the partially degraded fragments.

The turnover of cell membrane fragments following trypsinization

Most of the cells which had been removed from a confluent monolayer by trypsin and then iodinated remained viable. In fact, greater than 92% of the cells excluded trypan blue throughout the experiment. Furthermore,the iodinated cells con-

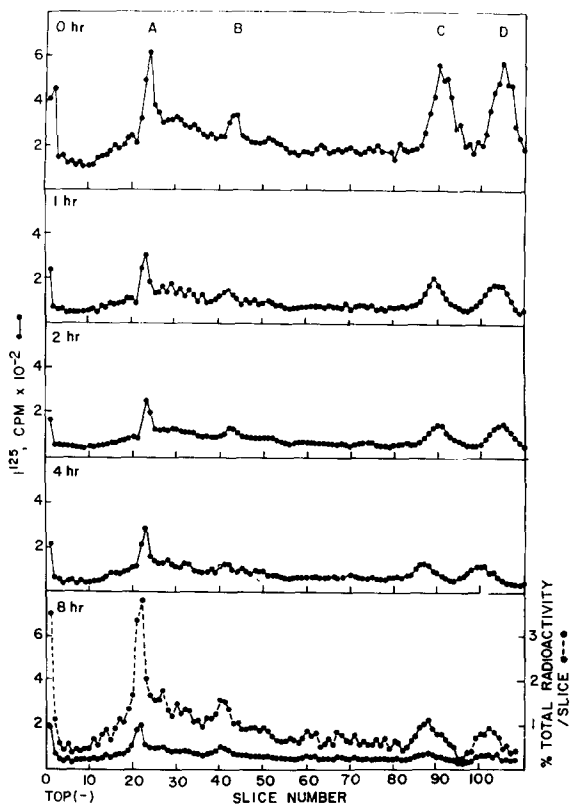

Fig. 5. The turnover of the iodinated surface proteins of trypsinized cells. BHK cells which had been trypsinized were iodinated and returned to culture medium. At the indicated times (0, 1, 2, 4 and 8 h) after reculturing, samples of $50 \cdot 10^6$ cells were taken for electrophoretic analysis as described under Materials and Methods; 250 μg protein were applied to 8.5 % polyacrylamide gels containing 0.1 % dodecylsulfate and 6 M urea. The dotted line at 8 h (●- - -●) is a plot of percentage total counts per slice in order to compare the radioactivity pattern at 8 h with that at 0 h.

tinued to replicate upon subculture and the growth curve was essentially identical to that of trypsinized cells which had not been iodinated. To study the replacement and fate of their surface components, cells from confluent cultures were trypsinized, iodinated, and then reincubated in suspension cultures. At 0, 1, 2, 4 and 8 h after reculture, samples of $50 \cdot 10^6$ cells were taken and prepared for dodecylsulfate gel electrophoresis.

Immediately following trypsinization the gel electrophoresis pattern of iodinated protein (Fig. 5) was similar to that already described in an independent experiment (Fig. 2, 0 h). After 1 h incubation the iodinated cells had lost 54% of the label present at the start of the incubation (Fig. 5): after 8 h only 26% of the original radioactivity remained. During this interval the pattern of radioactivity in the electropherogram (Fig. 5; 8 h, dotted line) remained qualitatively the same; however, a summation of the number of counts associated with the four major peaks visible at time 0, revealed that the labeled low molecular weight material left the cells more rapidly than the higher molecular weight material. By 8 h, Peaks A and B retained approx. 31% of their original (0 h) radioactivity while Peaks C and D retained only 19 and 10%, respectively.

Retention of iodinated surface proteins by non-trypsinized cells

As a control for the above studies the turnover of iodinated surface components in logarithmically growing cells was studied. For this purpose BHK cells were grown for four generations in suspension culture prior to iodination. After iodination, the cells (> 97% excluded trypan blue) were returned to fresh suspension culture medium where they resumed growth on the same schedule as non-iodinated cells handled in an otherwise similar way. The observed lag period was probably due to handling related to the iodination procedure. Samples containing $50 \cdot 10^6$ cells, were taken for analysis at 0, 1, 2, 4 and 8 h. The greatest loss of radioactivity

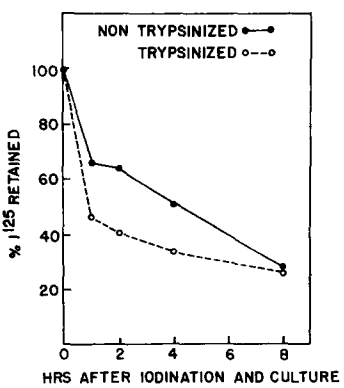

Fig. 6. Retention of ^{125}I by trypsinized and non-trypsinized BHK cells. Cells ($30 \cdot 10^7$) from suspension cultures were iodinated with and without prior trypsinization and returned to culture. Aliquots of $50 \cdot 10^6$ cells were taken at the indicated times and prepared for electrophoresis as described in Materials and Methods. The ^{125}I retained by the cells (cpm/μg protein) was determined for each sample and calculated as the percentage of the ^{125}I in the cells zero time. The specific activity of the non-trypsinized (●-●) and trypsinized (○- - -○) cells at 0 h was 120 cpm/μg and 146 cpm/μg protein, respectively.

(34%) occurred within the first hour (Fig. 6). After 8 h about 28% of the label remained. Electrophoretic analysis showed that the pattern of labeled material retained by the cells after 8 h was very similar to that at zero time. This suggests that surviving components may be normal representatives of proteins which exist in sizable pools in the cells. The surprising observation in these studies is that the turnover of labeled components in the membranes of normal cells growing logarithmically is nearly as rapid as the turnover of surface components in trypsinized cells (Fig. 6). In both cases, however, there is initially a rapid loss of iodinated components from the cell at a rate which approximates an average initial half-life of 1–1.5 h followed by a much slower release of labeled surface components.

Additional control studies

With respect to the localization of the iodinated components, a preparation of iodinated cells was homogenized in ice-cold Medium A and nuclei and remaining whole cells removed by low speed centrifugation ($700 \times g$) for 10 min. The supernatant was centrifuged at $30\,000 \times g$ for 10 min to obtain a pellet containing membrane fragments and large cytoplasmic particulates. This pellet had a specific activity (527 cpm/μg protein) which was eight times greater than the soluble supernatant proteins (66 cpm/μg protein).

BHK cells incubated with ^{125}I in the absence of glucose oxidase and lactoperoxidase contained only about 2% of the counts found in cells incubated in the complete iodination mixture. The result rules out non-specific binding of ^{125}I to the cells.

To check if lactoperoxidase or glucose oxidase which might also have been iodinated during the reaction was remaining with the cells, the iodination reaction was allowed to proceed in the absence of cells for 20 min before it was stopped with cold Medium A containing 10^{-5} M $Na_2S_2O_3$. Cells were then added and the incubation was continued for 20 min. The cells incubated with enzymes "pre-iodinated" in this way, bound less than 4% of the ^{125}I bound by cells incubated with the iodination reaction mixtures in the usual manner for 20 min.

To determine if trypsin itself was adsorbed on the cells, the latter were trypsinized with [^{125}I]trypsin which had been prepared by a modification of the chloramine-T method of McConahey and Dixon [19]. The proteins from these cells contained less than 0.25% of [^{125}I]trypsin. When the cell proteins were resolved by gel electrophoresis the ^{125}I was concentrated in a single peak corresponding to the molecular weight of trypsin (approx. 24 000). In control experiments following trypsinization, this region of the electropherogram was very low in label and was easily distinguished from the two low molecular weight peaks (approx. 16 000 and 9000) resulting from the trypsinization of the cell surface. Since in our experiments involving trypsinization of living cells there was no peak of radioactivity corresponding to trypsin, it was concluded that the iodination of trypsin and the level of adsorption was not significant.

DISCUSSION

The peripheral membrane, like other membranes of the cell, appears to be the product of a self-assembly process in which complementary components of both intracellular and extracellular origin associate non-randomly in a hydrophobic

lipid phase of the cell. These associations have relevance for the cell when one or more of such constellations plays a role in regulating the genetic or enzymatic performance of the cell. As proposed earlier [1], the interaction of membrane components may contribute to the efficiency of some local catalytic process or may modify the availability of a factor which in turn operates in the nucleus to regulate gene expression. The present studies are designed to obtain information on the character of the peripheral cell membrane of the fibroblast in several growth states in the hope of correlating changes in membrane activity with the control of cell replication. In confluent monolayer cultures the BHK cells can be viewed as operating in a complex environmental setting in which they interact with adjacent cells, with the plastic (or glass) surface and with the serum proteins of the medium through extracellular matrices arising from the cells themselves [20–22]. Of significance to the present studies is the fact that these interactions in some way restrict the triggering of genes for nuclear replication. Using the lactoperoxidase–iodination technique it appears that cells in the restricted state have only 11–18% as many sites exposed for iodination as do cells which have been mechanically dislodged from monolayers or are growing logarithmically in suspension. In addition the pattern of iodination seen in the electropherograms suggests that the exposed surfaces of monolayers constitutes a non-random sample of the total surface components. Since it is known from other studies [22] that attached cells do have a polarity of surface topography, it seems quite likely that certain cell surface components are buried in the membranes of the cell to cell to plastic interactions. Accordingly the observation that mechanically dislodging cells immediately exposes both more (Table I) and different sites (Fig. 1) for iodination raises the possibility that the restriction or isolation of one or more membrane components in the confluent state may underlie the failure of such cells to activate the genes for nuclear replication. Whether it is the interaction of such a component with a mitogenic factor in the medium, its release to the medium or increased freedom for interaction in the nuclear scene which activates nuclear replication is the subject of continued study. In this connection it is of interest, that in another system (i.e. bovine lymphocytes) we have observed the migration of some surface components to the nucleus during mitogenic activation.

In addition to releasing the cells from the confluent state, trypsinization appears to produce a rapid revision of the cell membrane, both exposing new sites and leaving multiple small 100t fragments for iodination. This damage appears to be quickly repaired by a process which has similar kinetics to that of the replacement of iodinated surface components on non-trypsinized cells (Figs 2, 3 and 5). Other investigators [23–26] have reported, with a variety of assay systems, that a similar time period is required for the membranes of trypsinized fibroblasts to return to the pre-trypsin state. However, it is of interest in the present studies that cell replication resumes in confluent cells which have been trypsinized when the membranes have attained the character of cells in logarithmically growing suspension cultures (i.e. 9–10 h after trypsinization). The activation as well as the synchronization of nuclear replication achieved in these experiments (i.e. the delay of some cells and the acceleration of others) suggests that the cell membrane may play the role of a clock in timing the expression of the genes for nuclear replication for the particular environment in which the cell resides. Further studies on the turnover of membrane components in growth restricted states are indicated.

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