Proc. Nat. Acad. Sci. USA
Vol. 70, No. 11, pp. 3170–3174, November 1973

Alteration of Cell-Surface Proteins by Viral Transformation and by Proteolysis

(fibroblasts/lactoperoxidase/iodination/polyacrylamide gels)

RICHARD O. HYNES

Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, England

Communicated by Renato Dulbecco, July 9, 1973

ABSTRACT **Putative cell-surface proteins of tissue-culture cells were identified by lactoperoxidase-catalyzed iodination, a technique that attaches label only to proteins outside the cell membrane. Evidence is presented that these proteins are cell derived, not contaminating serum proteins. On "normal" cells, which exhibit density dependence of growth, one protein of high molecular weight was particularly readily iodinated. This protein was easily removed by mild proteolytic digestion, and, in virus-transformed cells, was either absent or unavailable for iodination. The possible relevance of these observations to the control of growth in cell culture is discussed.**

The idea that the surfaces of virus-transformed cells differ from those of their normal counterparts has received support from various lines of research. There is immunological evidence for antigenic differences between normal and transformed cells (1, 2) and biochemical evidence for changes in surface components on transformation (3, 4). The different cell agglutinability by plant lectins, such as concanavalin A and wheat-germ agglutinin (5, 6), also suggests that there are differences in surface architecture between normal and transformed cells.

The fact that growth properties of normal cells can be influenced by mild proteolytic treatments (7, 8) implicates the surface directly in growth control, and the observation that transformed cells in culture produce proteolytic enzymes (9–12) suggests a possible mechanism for alteration of their growth properties. It would be desirable to assay directly for surface changes, in order to investigate further the roles of transformation and proteolytic enzymes and their possible involvement in growth control.

Techniques for identifying and characterizing proteins and glycoproteins on the outsides of cells have recently been developed. I wish to report some studies on normal and transformed cultured cells using lactoperoxidase-catalyzed iodination. When applied to erythrocytes or platelets, this procedure labels only external membrane proteins (13, 14). In the present work, a modification of this technique was used, in which hydrogen peroxide was generated by glucose oxidase plus glucose. The method is similar to that reported recently by Hubbard and Cohn (15).

Using this method I have been able to identify several exterior proteins on cultured animals cells. One of these is particularly heavily iodinated on normal cells and is iodinated weakly or not at all in their virus-transformed derivatives. This protein is also very sensitive to proteolytic digestion.

MATERIALS AND METHODS

Cells used were as follows. Two clones of the hamster fibroblast cell line NIL.2E (16, 17), NIL.1 and NIL.8, which show "normal" behavior in culture, and various clonal derivatives transformed by hamster sarcoma virus (HSV) or polyoma virus (Py) were obtained from Dr. I. A. Macpherson. Eight subclones of NIL.8 were freshly isolated by plating cells at 0.5 cells per well in plastic multiple well trays (Linbro) and growing up those which initially appeared to be single colonies. All eight showed well-orientated growth patterns in culture and a low saturation density similar to that of the parent line. LX cells were kindly provided by Dr. M. Shodell (18).

NIL cells and their transformants were cultured in Dulbecco's modification of Eagle's medium with 10% calf serum. LX cells were grown in Waymouth's medium. Cells were checked periodically for contamination by *Mycoplasma* both by staining and by culture. None of the cells used ever gave a positive result.

Iodination was usually done in monolayer cultures, but in a few experiments cells were labeled in suspension after removal from dishes by trypsin–EDTA or EDTA alone. Cells were washed three times with phosphate-buffered saline (pH 7.2) to remove serum. Phosphate-buffered saline + 5 mM glucose was then added, followed by carrier-free $Na^{125}I$, usually to a final concentration of 400 μCi/ml. The reaction was initiated by addition of lactoperoxidase (Calbiochem; EC 1.11.1.7) and glucose oxidase (Worthington; EC 1.1.3.4) to final concentrations of 20 μg/ml and 0.1 units/ml, respectively. Reaction was allowed to continue for 10 min at room temperature with occasional swirling, and labeling was stopped by addition of phosphate-buffered iodide (phosphate-buffered saline with the NaCl replaced by NaI). This solution usually contained 2 mM phenyl methyl sulfonylfluoride to inhibit proteases. The medium was removed and the cells were washed twice more with phosphate-buffered iodide + phenyl methyl sulfonylfluoride. The cells were then scraped into phosphate-buffered iodide and phenyl methyl sulfonylfluoride, centrifuged, and dissolved in buffer containing 2% Na dodecyl sulfate and phenyl methyl sulfonylfluoride for Na dodecyl sulfate–polyacrylamide electrophoresis.

Na Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis was done by the methods of Laemmli (19) as described by Studier (20). Electrophoresis was usually in slabs where 12 samples could be run in parallel on the same gel. The slabs, after they were stained with Coomassie blue if required, were dried down onto paper, and autoradiographs made on Kodirex x-ray film. Before electrophoresis, samples were reduced by addition of dithiothreitol to 0.1 M and boiling for at least 2 min. Electrophoresis was done at 50 V for about 1 hr, until the bromphenol blue marker entered the separation

gel, and then at 100 V (about 10 V/cm) until the marker reached the bottom.

Enzymes. Lactoperoxidase was from Calbiochem; glucose oxidase was from Worthington Biochemicals; trypsin, twice crystallized, and soyabean trypsin inhibitor were from Sigma, London.

Radiochemicals. [^{125}I]Sodium iodide, carrier-free, and [^{14}C]-leucine 342 Ci/mol, were obtained from the Radiochemical Centre, Amersham, Bucks.

RESULTS

Monolayer cultures of the normal hamster fibroblast cell line NIL.8 were iodinated, washed, harvested by scraping, concentrated, and run on Na dodecyl sulfate–polyacrylamide gels. Fig. 1 shows an autoradiograph of a 7.5% slab gel on which several samples were run in parallel. The iodination is selective (gels *b* and *i*): the major cell proteins are not labeled and only a limited number of proteins is iodinated. Fig. 1*d* shows the result obtained when the enzymes were omitted from the iodination mixture. Omission of either of the enzymes or of glucose eliminates labeling (Table 1). Thus, iodination depends on the presence of both enzymes. Since these are macromolecules, which presumably do not enter the cell, this is suggestive evidence that the labeled proteins are external.

If the cells were removed from the monolayer *before* iodination, lysed with distilled water, and then labeled, the result shown in Fig. 1*g* was obtained. In this case, all the major cell proteins were iodinated (compare Fig. 1*g* with 1*b* and 1*i*). Thus, all or most cell proteins can be iodinated but, in the intact cell, only a few are available to the iodide–lactoperoxidase complex.

Further independent evidence for the external location of most of the iodinated proteins was obtained by tryptic digestion. Fig. 1*e* shows the result of very mild treatment with trypsin after iodination. This trypsin treatment did not cause rounding up or detachment of any cells from the dish. However, all but one of the major iodinated proteins were removed. Removal of labeled proteins was blocked by soyabean trypsin inhibitor (Fig. 1*f*) and was, therefore, due to proteolytic digestion. Identical results were obtained when trypsinization was done *before* iodination (see Fig. 4) or if the trypsin treatment was more extensive and the cells were completely detached from the dish (data not shown). One of the iodinated proteins was therefore resistant to trypsin whereas the others were removed without leaving iodine-labeled cores attached to the cells.

TABLE 1. *Dependence of iodination on enzymes*

Complete mixture	Trichloroacetic acid precipitable cpm
Complete mixture	24,283
Complete mixture	22,408
Omit glucose	102
Omit glucose oxidase	111
Omit lactoperoxidase	418
Omit both enzymes	105

Cells were iodinated in suspension. The reaction was stopped by addition of Na dodecyl sulfate to 1%. Macromolecular material was assayed by trichloracetic acid precipitation.

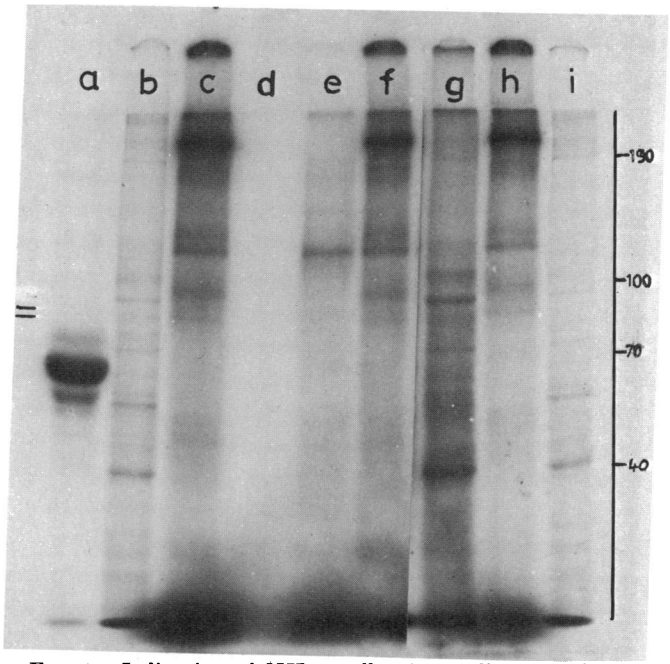

FIG. 1. Iodination of NIL.8 cells. Autoradiogram of 7.5% gel. (*a*) Iodinated calf serum; (*b* and *i*) cells labeled with [^{14}C]-leucine for 72 hr; (*c* and *h*) cells iodinated in monolayer; (*d*) cells iodinated as for *c* except that the enzyme solution was omitted; (*e*) cells iodinated and then treated for 10 min with 10 μg/ml of trypsin in phosphate-buffered saline; (*f*) cells iodinated and then treated with 10 μg/ml of trypsin + 10 μg/ml of soyabean trypsin inhibitor; (*g*) cells lysed with distilled water and then iodinated. All iodinated cell samples contained equal quantities of cell protein. Molecular weight $\times 10^{-3}$ is marked on *right* and positions of lactoperoxidase (*top line*) and glucose oxidase (*bottom line*) on *left*.

Comparison of the pattern of iodination of NIL.8 cells (Fig. 1*c* and *h*) with that of iodinated serum (Fig. 1*a*) shows little or no correspondence between them, suggesting that the labeled proteins are not bound serum proteins. Further, when a dish that had contained only complete medium but no cells, was iodinated, washed, and then rinsed with 2% Na dodecyl sulfate to collect labeled proteins, only major serum proteins could be detected. This finding argues against selective binding of minor serum proteins to the dish. The prominent iodinated bands observed on iodination of cells were not seen (data not shown). Experiments in which prelabeled serum was incubated with cells again failed to detect selective binding of minor serum components; again only the major serum bands could be detected (data not shown). Finally, evidence that the iodinated bands are not due to serum contamination was provided by experiments with a mouse cell line, LX, (18) which grows in the absence of serum. Replicate cultures were grown for 3 days in 0, 1, or 2% fetal-calf serum, iodinated, and run in parallel on Na dodecyl sulfate–polyacrylamide gels (Fig. 2). The three profiles of radioactivity were almost identical; only one labeled band appeared in cells grown in serum and not in the controls (*arrow*, Fig. 2). The iodination pattern for the LX cells grown without serum, while broadly similar to that observed for NIL.8 cells grown in 10% serum, showed reproducible differences in its details. Both cells have the heavily labeled high-molecular-weight band, but these differ slightly in mobility on 5% gels, the one from LX cells being somewhat slower.

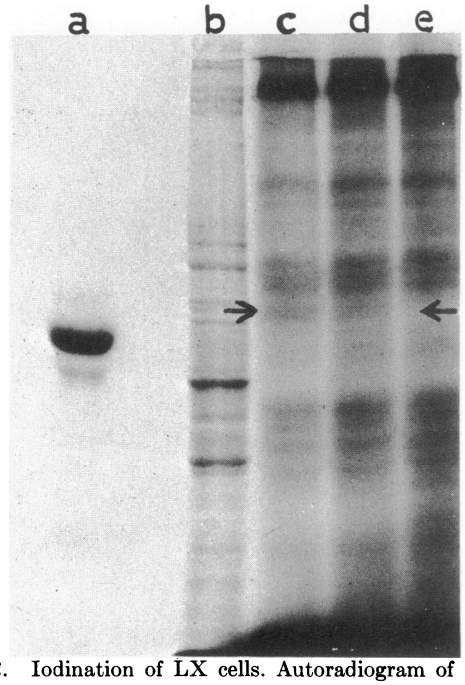

FIG. 2. Iodination of LX cells. Autoradiogram of 7.5% gel. (*a*) Iodinated fetal-calf serum; (*b*) cells labeled with [^{14}C]leucine; (*c*) cells grown in 2% fetal-calf serum and then iodinated; (*d*) cells grown in 1% fetal-calf serum and then iodinated; (*e*) cells grown without serum and then iodinated. Cell samples contained equal quantities of cell protein. *Arrows* mark serum band adhering to cells.

The migration positions of the two enzymes used for labeling are marked on Fig. 1. It was shown in separate experiments that prelabeled enzymes can bind to the cells, although

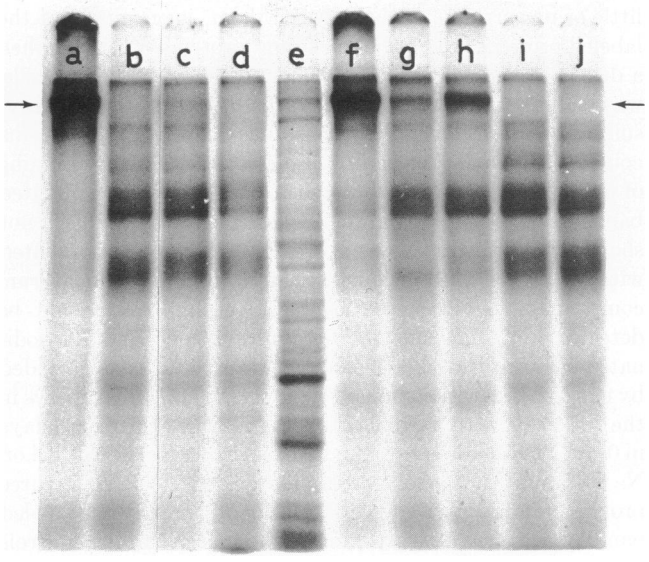

FIG. 3. Iodination of normal and virus-transformed NIL cells. Autoradiogram of 7.5% gel. (*a*) NIL.8; (*b*) NIL.8.HSV6; (*c*) NIL.8.HSV9; (*d*) NIL.8.HSV11; (*e*) NIL.8 labeled with [^{14}C]leucine; (*f*) NIL.1; (*g*) NIL.1.Py1; (*h*) NIL.1.Py8; (*i*) NIL.1.HSV1; (*j*) NIL.1.HSV3. Iodinated samples contained equal amounts of radioactivity. *Arrow*, location of band 1.

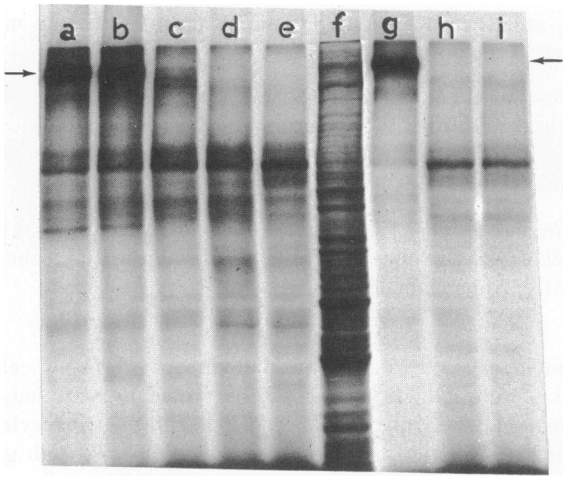

FIG. 4. Effects of tryptic digestion on the pattern of iodination of NIL.8 cells. Autoradiogram of 7.5% gel. (*a–e*) NIL.8 cells iodinated and subsequently treated before harvest with: (*a*) 10 μg/ml of trypsin plus 10 μg/ml of soyabean trypsin inhibitor, 10 min; (*b*) 1 μg/ml of trypsin for 1 min; (*c*) 1 μg/ml of trypsin for 5 min; (*d*) 1 μg/ml of trypsin for 10 min; (*e*) 10 μg/ml of trypsin for 10 min. All were at room temperature in phosphate-buffered saline; (*f*) [^{14}C]Leucine-labeled NIL.8 cells; (*g*) cells iodinated and harvested without further treatment; (*h* and *i*) cells treated with trypsin (10 μg/ml for 10 min) before (*h*) or after (*i*) iodination. Iodinated samples contained equal amounts of radioactivity. *Arrow*, location of band 1.

generally at low levels relative to the other iodinated bands observed on labeling cells. However, this fact may complicate interpretation of this region of the gels (molecular weight 75–85 × 10^3).

Another clone of NIL cells, NIL.1, gave results very similar to those obtained with NIL.8. Eight subclones of NIL.8 all showed similar iodination profiles. Thus, there was no evidence for clonal variations. In addition to LX cells, several other fibroblastic cell lines were examined (BHK, 3T3, 3T6) and all showed similar patterns of iodination with one prominent labeled band and several minor ones. There were reproducible differences in detail between the various cell lines (unpublished data).

When virus-transformed cells were examined, a different result was obtained (Fig. 3). Cloned lines of NIL cells trans-

TABLE 2. *Iodination of normal and virus-transformed cells*

Cell line	Specific activity (acid-precipitable ^{125}I cpm per μg of protein)
NIL.8	2210
NIL.8.HSV6	632
NIL.8.HSV9	786
NIL.8.HSV11	729
NIL.1	1350
NIL.1.Py1	430
NIL.1.Py8	599
NIL.1.HSV1	738
NIL.1.HSV3	619

Cells were iodinated at confluence in 5-cm petri dishes, and processed as described in *Methods*.

formed by hamster sarcoma virus (HSV), initially isolated by their ability to grow in suspension in agar, showed a typical transformed growth pattern on dishes, i.e., they reached higher densities than their normal parents and showed irregular cell orientation. In the iodination pattern of all these transformed cells, the iodinated band with the highest molecular weight (band 1) was absent or much reduced (Fig. 3). A similar result was observed with polyoma virus-transformed NIL.1 cells (Fig. 3), although the disappearance of band 1 was not always complete. In the experiment of Fig. 3, equal amounts of radioactivity were applied to the gels. Therefore, more material from transformed cells was used, since they were less heavily labeled (Table 2), presumably because of absence of label in band 1. If the labeling of band 1 of the polyoma-transformed cells is considered relative to that of the other bands on the same track, it is clearly reduced. When equal amounts of cell protein were applied to gels, the iodinated bands of lower molecular weights seen on normal cells were unaffected by transformation. A group of fainter bands of variable intensity ahead of band 1, which appeared to be increased in certain transformed lines, could conceivably be breakdown products of band 1.

Similar results were obtained in a comparison of chicken-embryo fibroblasts and chicken-embryo fibroblasts transformed by Rous sarcoma virus Prague strain A (unpublished data). As with polyoma-transformed NIL cells, Rous sarcoma virus-transformed chicken-embryo fibroblasts generally showed a trace amount of band 1.

In view of the increased proteolytic activity observed in transformed cells (9–12), and the evidence that mild proteolytic digestion alters the surfaces of normal cells (21–25) and stimulates growth (7, 8), I tested the sensitivity of the iodinated proteins to very mild trypsin treatments (Fig. 4). 1 μg/ml of crystalline trypsin degrades band-1 protein partially within 1 min, leading to a band running ahead of band 1 (not clearly seen in Fig. 4). Band 1 was largely removed by 5 min and removed completely by 10 min of digestion. The other iodinated bands were less sensitive than band 1 but were progressively removed by increasing trypsin treatments, with the exception of one major trypsin-resistant band and several minor ones. After very light digestion, new bands running ahead of band 1 could be seen (e.g., Fig. 4) similar to those observed in transformed cells (Fig. 3).

The experiments described so far were done with approximately confluent cell cultures. The possibility arises that the differences observed between normal and transformed cells were due to the fact that the latter were dividing more rapidly. However, in other experiments with normal cells, band 1 was detected whether the cells were in exponential growth or had stopped dividing after forming a confluent sheet (unpublished data).

DISCUSSION

A major exterior cell protein (band 1) of normal fibroblasts, which is detected by lactoperoxidase-catalyzed iodination, is detected in small quantities or not at all on virus-transformed derivatives. This is true for hamster cells transformed by hamster sarcoma or polyoma virus and for chicken-embryo cells transformed by Rous sarcoma virus and is not a clonal variation unrelated to transformation.

Several possible explanations for this observation exist: either transformed cells synthesize band-1 polypeptide in reduced quantities or not at all, or else they synthesize it at normal rates but it is not available for iodination, either because it is masked or because it is continually removed from the cell surface. It is not possible to decide which of these explanations applies.

The observations that transformed cells produce proteases (9–12) and that mild trypsin treatment removes band 1 from normal cells (Fig. 4) are consistent with the idea that failure to iodinate band-1 polypeptide on transformed cells is due to its removal by proteolytic digestion, but do not prove it. The level of proteolytic digestion that brings about removal of band 1 is of the same order as that which causes normal cells to react with lectins like transformed cells (21, 23, 25). Mild treatments with proteases also stimulate quiescent normal cells into growth (7, 8) and reduce their cyclic AMP levels to those found in transformed cells (26, 27). These similarities may well be coincidental, but they encourage the speculation that transformation leads to production of proteolytic enzymes which, in turn, lead to the changes mentioned and allow transformed cells to escape normal growth controls. If this sequence of events were a necessary part of transformation, treatment of transformed cells with inhibitors of proteolytic enzymes should tend to render their surface characteristics and growth patterns normal. This has been shown to be so for density-dependent growth inhibition (28), morphology (12), and ability to grow in agar (12).

It should be noted that although the evidence presented demonstrates that the iodinatable proteins are located outside the cell membrane and are cell-derived rather than serum contaminants, it has not been shown whether they are part of the cell membrane or laid down as extracellular materials. This distinction may be a semantic one. Several of the iodinatable proteins, including band 1, appear to be glycoproteins, and evidence suggests that they are not collagen nor do they contain sulfated mucopolysaccharides (unpublished data).

After completion of the work described, I learned that similar results were obtained independently using chicken cells transformed by RSV (29) and 3T3 and its viral transformants (N. M. Hogg, personal communication).

I thank Jacqueline Bye for her very competent and conscientious technical assistance and Drs. I. A. Macpherson and R. Dulbecco for their helpful comments on the manuscript.

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