A Comparison of Membrane Proteins of Normal and Transformed Cells by Lactoperoxidase Labeling

(iodination/fibroblasts/membranes/gel electrophoresis)

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ABSTRACT The enzyme lactoperoxidase (iodide:hydrogen-peroxide oxidoreductase, EC 1.11.1.8) was used to iodinate (^{125}I) accessible proteins on membranes of intact virally transformed and untransformed cells. A number of labeled bands of proteins were detected by acrylamide gel electrophoresis. A heavily labeled band with a molecular weight of approximately 250,000 daltons was found in all untransformed cells but was absent from transformed cells. When Coomassie-blue-stained membrane preparations were compared, a band was seen in normal cells which comigrated with the lactoperoxidase-labeled band. In transformed cell membranes, three discrete bands were present in the same position. Thus, the expression of this protein may be altered when cells are in the transformed state.

The identification of alterations in membrane proteins and glycoproteins after transformation by DNA or RNA oncogenic viruses has been confusing. In general, there is reported to be an overall decrease in numbers of membrane glycoproteins (1, 2), but there are demonstrations of an increased glycosylation of a restricted number of membrane glycoproteins in transformed cells (3, 4). Conversely, other studies have suggested an association with untransformed cell membranes (5), or their growth media (2), of glycoproteins which were absent from membranes of transformed cells.

It was considered that a fruitful approach to resolving some of these complexities would be to examine directly the membranes of intact cells rather than isolated membrane fragments. The lactoperoxidase (iodide:hydrogen-peroxide oxidoreductase, EC 1.11.1.8) catalyzed iodination procedure (6) is a technique by which tyrosine-containing proteins exposed on the surface of cell membranes can be directly labeled. This technique has been used to examine proteins on the membranes of erythrocytes (6, 7), lymphocytes (8, 9), platelets (10, 11), fibroblasts (12), and viruses (13, 14).

In this study, lactoperoxidase-catalyzed iodination has been used to characterize the membranes of intact normal cell lines and their transformed derivatives. It was hoped that the iodination procedure was sufficiently mild that membrane architecture remained relatively unperturbed.

MATERIALS AND METHODS

Cells. The cell lines used were BALB/C 3T3 (clone A31), Swiss 3T6, and secondary cultures of mouse-embryo fibroblasts from Taylors Own mice (ICRF), referred to as TO

cells. All transformed cells used were derivatives of the 3T3 line A31: Py-3T3 and SV-3T3 were isolated after infection of 3T3 cells by the DNA viruses, polyoma and SV-40 viruses, respectively, and were a gift from Dr. Mike Fried. Transformed cells MSV-3T3 were isolated from a single infection of 3T3 by murine leukemia virus (Moloney pseudotype) which contained both sarcoma and leukemia virus activity. MSV-3T3 cells had a transformed morphology and were releasing sarcoma virus, as detected by the focus-forming assay, and leukemia virus, as detected by the XC plaque assay (15). The LX cells were a kind gift from Dr. Mike Shodell.

All the cell lines, except LX, were maintained in Dulbecco's modification of Eagle's medium (E4) supplemented with 5-10% fetal calf serum (FCS). The LX cells were maintained in serum-free media as described by Shodell (16).

Preparation of Membranes. Scraped fibroblasts were suspended in 25% isotonic phosphate buffered saline (PBS), pH 7.2, which also contained phenylmethylsulphonylfluoride, 100 $\mu\text{g}/\text{ml}$. After 30 min at 0° the cells were homogenized by means of an Ultra Turrax homogenizer (Scientific Instrument Centre, London) operated at 20,000 rpm for $1\frac{1}{2}$ min. The "membrane" fraction of this disrupted cell mixture was collected at the interface of a 1.66-2.30 M sucrose density gradient. This procedure for making membranes gives a restricted number of Coomassie-blue-positive bands on acrylamide gel electrophoresis, including bands comigrating with those identified by lactoperoxidase catalyzed iodination. A whole cell lysate gives a more complex spectrum of banding in which several of the major "membrane" proteins form minor components. Small amounts of membrane material were made by the method of Scher and Barland (17).

Lactoperoxidase Labeling of Cells. The cells were removed from their substrates with a combination of 0.05% trypsin and EDTA (0.15%) and seeded at subconfluent density onto 50-mm plastic petri dishes (NUNC) in E4 plus 0.5-1% FCS. This seeding was done 48 hr before enzyme labeling. The cells were usually at a density of 1×10^6 per 50-mm petri dish at the time of labeling.

The petri dishes to be labeled were washed twice with warm Earle's salt solution (ES) and twice with warm PBS. The conditions for iodination were similar to those already described (7, 18). To one ml of PBS in the petri dish was added 50-70 μg of lactoperoxidase (Calbiochem), 3.0 IU glucose oxidase (Worthington), 500 μg of glucose, and 0.1 mCi of carrier-free sodium ^{125}I (Amersham). The reaction was allowed

Abbreviations: TO, secondary cultures of mouse embryo fibroblasts from Taylors Own mice; PBS, phosphate-buffered saline; E4, Dulbecco's modification of Eagle's medium; FCS, fetal-calf serum; ES, Earle's salt solution.

FIG. 1. Autoradiographs from two 7.5% SDS-acrylamide slab gel electropherograms. Autoradiograph *a* was not from the same electropherogram as *b*, *c*, *d*, and *e* and was aligned according to migration relative to bromphenol blue. (*a*) 3T6 cells maintained in E4 plus 1% FCS before labeling, (*b*) SV-3T3 cells, and (*d*) Py-3T3 cells maintained in E4 plus 0.5% FCS before labeling; (*c*) and (*e*) are SV-3T3 cells and Py-3T3 cells, respectively, which were trypsinised after lactoperoxidase labeling.

to progress for 10 min at room temperature and was halted by the addition of NaI (final concentration 50 mM). After two washes with ES, the labeled cells were scraped from the plates by means of a rubber policeman and given two further washes before acrylamide gel electrophoresis. The deletion of either enzyme from the reaction mixture reduced the incorporation of ^{125}I into membrane protein to insignificant amounts.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was done on an E-C slab gel apparatus (E-C Co., Philadelphia) using the "SDS-disc" system of gels and buffers described by Maizel (19). After electrophoresis was completed, the gels were dried and the location of radioactive proteins was determined by autoradiography. In some cases the gels were stained with Coomassie blue (22).

RESULTS

Comparison of Lactoperoxidase Labeling of Normal and Transformed Cells. The observed labeling patterns were of two types. All untransformed cells (3T3, 3T6, and TO cells) gave identical patterns, although individual proteins did not always label to the same extent. The patterns of transformed cells were essentially similar to the normal cells, with one notable exception. The pattern of 3T6 is compared with those of SV-3T3 and PY-3T3 in Fig. 1(*a*, *b*, and *d*). The most strongly labeled bands in the 3T6 pattern are numbered L1 through L7. The most obvious difference between this pattern and the patterns of the transformed cells is the lack of a labeled band in position L1.

The most prominently labeled bands are L1, in the preparations in which it is found, and L4. L2 is not always present and may be a serum contaminant (see below). The labeling of L3 varied from one cell type to another, but its presence was not dependent on serum concentration. L3 may be a membrane component which is more easily removed by washing

FIG. 2. Autoradiographs from a 7.5% SDS-acrylamide slab gel electropherogram. (*a*) 3T3 cells; (*b*) MSV-3T3 cells maintained in E4 plus 1% FCS before labeling.

or by procedures such as membrane isolation. L5 comigrates with lactoperoxidase, and L5 and, to some extent, L6 are obscured when labeling was done using lactoperoxidase in amounts larger than 50 $\mu\text{g}/\text{ml}$ [see Fig. 1(*b* and *d*)]. L7 was a protein which labeled well in most preparations. Moreover, there were other individual variations in each cell line which did not label as strongly as L1 through L7.

The three transformed cell lines independently isolated from the same infection of 3T3 cells with MSV (Moloney) virus showed similar characteristics of lactoperoxidase labeling. In Fig. 2 the labeling pattern of 3T3 is compared to one of the MSV-3T3 lines and numbered L1 through L7. In this particular 3T3 pattern, L1 is labeled much more heavily than the other bands but is entirely absent from the MSV-3T3 samples. The approximate molecular weights of the seven proteins labeled by lactoperoxidase are given in Table 1.

TABLE 1. Molecular weights of lactoperoxidase-labeled proteins

Lactoperoxidase (L)-labeled band number	Molecular weight (daltons)
1	220–290,000 (~250,000)
2	185–190,000
3	100–105,000
4	90–95,000
5	80–85,000
6	75–78,000
7	65,000

Approximate molecular weights were determined from a semi-logarithmic plot of molecular weights of standards against mobility in 5–10% acrylamide gels. The standards used were: myosin, 200,000 daltons; β -galactosidase, 135,000 daltons; *E. coli* RNA polymerase, 155–165,000, 90,000 and 40,000 daltons; lactoperoxidase, 80,000 daltons; glucose oxidase, 75,000 daltons (20); bovine serum albumin, 68,500 daltons; and ovalbumin, 43,000 daltons.

FIG. 3. Autoradiographs from a 7.5% SDS-acrylamide slab gel electropherogram. (a) 3T6 cells; (b) 3T6 cell membrane sample (17) prepared from lactoperoxidase-labeled cells; (c) LX cells. (a and b) were maintained in E4 plus 1% FCS and (c) was maintained in serum-free medium (16) before labeling.

Are all Labeled Proteins on the Surface of the Membrane? Membranes from lactoperoxidase-labeled 3T6 cells, isolated by the method of Sher and Barland (17), were compared to the intact-cell-labeling pattern of 3T6 in Fig. 3 (a and b). L1 and L4 through L7 are present in the membrane pattern.

SV-3T3 and PY-3T3 cells were labeled with lactoperoxidase before exposure to 0.05% trypsin-EDTA (0.15%) for 3 min. In Fig. 1 (c and e) it can be seen that most of the labeled bands have disappeared. Some label remained in position L5 where the lactoperoxidase migrates and a new band with a molecular weight of approximately 70,000 daltons has appeared. The patterns of lactoperoxidase-labeled 3T3 and 3T6 trypsin-treated cells are identical to the patterns of the trypsin-treated transformed cells shown in Fig. 1. Therefore, the association of the labeled proteins with isolated membranes and the removal of the label by tryptic digestion of intact cells supports the proposal that lactoperoxidase is labeling only proteins exposed externally on the cell membrane.

Are the Labeled Proteins Serum Components Adhering to the Plasma Membranes? It is necessary to demonstrate that the proteins labeled on the cell surface by lactoperoxidase are not firmly adhering serum components which have remained bound in spite of the washing procedure. The labeling pattern of the LX cell which grows in the absence of serum is shown in Fig. 3c. The pattern is similar to that of the cells already described. L1, a double band in the L3 region, and L4 through L7 can be labeled, although with varying intensities. There appears not to be a band in the L2 region. Moreover, the L2 band is missing from the 3T6 membrane sample prepared after lactoperoxidase labelling (Fig. 3b). The appearance of this band in lactoperoxidase-labeled cells is dependent on serum concentration, being observed in cells cultivated in 1% but not in cells cultivated in 0.5% serum [Fig. 1(a, b, and d)]. Furthermore, when cells are grown in the presence of ^{125}I -labeled FCS, a band is found which comigrates with the L2 band. Although L2 may be an absorbed serum protein, it is not excluded that it is a membrane protein whose expression is dependent on serum concentration.

FIG. 4. Coomassie-blue-staining pattern from a 5.0% SDS acrylamide slab gel electropherogram. (a) TO cell membrane sample; (b) SV-3T3 cell membrane sample. The cells were maintained in E4 plus 1% FCS before preparation of membranes by the Ultra-Turrax procedure. The arrows designate the L1 region (see text).

Comparison of Coomassie-Blue-Stained Membranes from Normal and Transformed Cells. An investigation of isolated membranes from normal and transformed cells was undertaken to see whether the difference detected by lactoperoxidase labeling was reflected in membrane proteins. The membranes were made by the Ultra-Turrax homogenization procedure. The Coomassie-blue-staining patterns of two normal cell membranes (from 3T3 to TO cells) were similar to each other as were those of two transformed cell membranes (from PY-3T3 and SV-3T3 cells). The Coomassie-blue-staining patterns of TO and PY-3T3 cell membranes are given in Fig. 4.

The L1 region was identified by electrophoresis of membranes from lactoperoxidase-labeled intact cells followed by Coomassie-blue staining and autoradiography. In TO cell membranes the lactoperoxidase-labeled protein, L1, and a Coomassie-blue-staining protein were indistinguishable in their electrophoretic migration and band width. There was no lactoperoxidase-labeled protein in the corresponding position in the SV-3T3 cell membrane patterns. The Coomassie-blue-staining pattern of SV-3T3 cell membranes consisted of three discrete bands: one band migrated more slowly than the L1 band of TO cell membranes and the other two bands were coincident with but less diffuse than the L1 band of TO cell membranes. Therefore, the major differences between the membrane proteins of normal and transformed cells detected both by surface labeling and by Coomassie-blue staining are found in the L1 region. There appears to be two proteins in transformed cells which migrate with the protein(s) of the L1 region of nontransformed cells and another which migrates more slowly. Whether these proteins are related to L1 will only be demonstrated by isolation and chemical characterization.

DISCUSSION

This study has been undertaken in an effort to characterize the proteins on cell membranes of normal and virally transformed fibroblasts using the enzyme lactoperoxidase to catalyze the iodination (^{125}I) of exposed proteins. Although

five membrane proteins were labeled in common, the major difference between the normal and transformed cell types was the *presence* in normal cells compared to the *absence* in transformed cells of a membrane protein, called L1, with molecular weight of approximately 250,000 daltons. This phenomenon is a common feature of the 3T3 cells transformed by both the DNA (SV-3T3 and Py-3T3, Fig. 1) and the RNA (MSV-3T3, Fig. 2) viruses tested in this study. This protein may be similar to the lactoperoxidase-labeled protein of 230,000 daltons described by Poduslo et al. (12). However, in contrast to this study, the protein labeled well in both trypsinized normal and Rous-sarcoma-virus-transformed fibroblasts from baby hamster kidney.

An additional labeled protein, L2, may be due to an adhering serum component. The labeling of another protein, L3, varied in intensity from one cell type to another, which suggests that it may be a loosely bound molecule which can be easily removed by procedures such as washing or membrane isolation.

The lactoperoxidase-labeled proteins comigrated with the higher-molecular-weight membrane proteins ranging in size from 65,000 daltons to 220–290,000 daltons (Table 1). Preliminary results indicate that membrane bands comigrating with L1 through L5 are glycoproteins.

Before claiming the altered expression of the L1 protein as a universal characteristic of transformed cells, many more independently transformed cell lines of different origins will have to be examined. This characteristic may not apply to every cell type exhibiting a transformed morphology. For example, preliminary evidence indicates surface labeling of the L1 protein by lactoperoxidase at both permissive and nonpermissive temperatures of the SV-3T3 cell mutant of Renger and Basilico (21), which is temperature sensitive for some transformed characteristics. It has also been noted that the L1 protein can be labeled on LX cells which are derivatives of the transformed L cell (Fig. 3c). However, the tumor-forming capacity of the LX cells has never been tested.

Membranes from normal and transformed cells were examined in order to determine whether the difference detected by lactoperoxidase labeling was reflected in membrane proteins. Membranes were made from lactoperoxidase-labeled intact cells so that the L1 position could be exactly localized. In normal cells, the L1 position was found to coincide with a rather diffusely staining Coomassie-blue band (Fig. 4). In contrast, in the transformed cell membrane preparation where there was no lactoperoxidase-labeled band, three discrete Coomassie blue staining bands were evident; one band migrated more slowly than the L1 band and two others were coincident with it but less diffuse. These observations suggest that there may be proteins or a group of closely related proteins which are present in the membranes of normal cells but which are absent from or exist in an altered form in the membranes of transformed cells. It is tempting to speculate that the heterogeneity in molecular weight shown by the diffuse L1 band in normal cells is generated by limited proteolysis at the surface of the membrane. The fact that the bands in this position in transformed cells are more discrete

and are not labeled by lactoperoxidase might indicate that the proteins are sterically blocked or "covered," thus inaccessible to proteolytic degradation. However, the protein(s) present in normal cells might have a more rapid turnover rate in transformed cells or might be too tenuously bound to the membrane to withstand the washing procedures.

Coomassie-blue staining detects a third protein in transformed cells which migrates slightly more slowly than the L1 area of normal cell membranes. Therefore, the major differences detected between proteins of normal and transformed cells both on the surface and in the membranes are to be observed in the proteins (glycoproteins) with molecular weights of approximately 250,000 daltons.

After the completion of this work it was learned that Hynes (23) has obtained similar findings to those reported here for surface labeling using NIL cells and transformed derivatives.

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