Analysis of Transmembrane Proteins From Eukaryotic Cells

R.M. Evans, F.G. Grillo, and L.M. Fink

Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado 80262

The topography and properties of plasma membrane proteins from mouse L-929 cells are studied by comparing their availability for enzymatic labeling on the external and internal surfaces of the membrane. In order to study the internal surface, phagolysosomes are prepared from cells after they ingest latex particles. The plasma membrane surrounding these seems to have an "inside-out" orientation. The sugars of the membrane glycoproteins in intact phagolysosomes are not available for interaction with lectins or available for periodate-borotritide labeling. A comparison of the lectin-binding proteins labeled by lactoperoxidase-catalyzed iodination on the external cell surface with those labeled on the internal cell surface suggests that a variety of plasma membrane glycoproteins span the lipid bilayer.

Using two-dimensional gel electrophoresis it has been shown that selected proteins are labeled at both the internal and external faces of the plasma membrane. Analysis of the 2-D gel electrophoregrams reveals that there are two distinct prominent proteins at 60,000 and 100,000 daltons which are enzymatically iodinated from both sides of the membrane. The partial hydrolysis of the 100,-000 dalton protein reveals that different peptides are iodinated when the iodination is performed on intact cells or on the phagolysosomes. These proteins are extensively phosphorylated in cells incubated with inorganic ^{32}P. We conclude that the phagolysosome is probably oriented in an "inside-out" configuration and that this membrane preparation can be used to study the topographic organization of membrane proteins.

The use of oriented membranes, selective labeling of proteins, and affinity separation of proteins in combination with gel electrophoresis to define the position and properties of proteins is discussed.

Key words: mouse L-929 cells, "inside-out" configuration, gel electrophoresis, lectin-binding proteins

Changes in the composition and structure of the plasma membrane seem to be critical in determining how cells interact with their environment. These changes are probably intimately connected with some of the altered phenotypic characteristics associated with neoplastic transformation. Much of the information on the structure and organization of the plasma membrane comes from studies on red blood cells. However, the anucleate RBC may not be useful as a paradigm for plasma membrane structure in other cells. Because of the complexity of physiologic responses which occur in nucleated replicating cells but not in erythrocytes, we have begun to analyze the protein structure and topography of plasma membrane proteins in fibroblastic mouse cells in culture.

The studies presented are designed to assess the location of proteins in the plasma membrane. A vectoral analysis designates which proteins are available for enzymatic modification a) on the internal surface, b) on the external surface, and c) on both sides of the plasma membrane; the latter thus are candidates likely to have a transmembrane (TMB) configuration. These studies depend upon the selective labeling of the internal and external surfaces of the membranes. One approach has been to saturate all the external labeling sites with an impenetrable probe and then either to permeabilize the cell so that internal labeling can occur [1, 2], or use a permeable probe for internal labeling [3]. Another approach, which is the major method used in the present studies, is to label either "inside-out" or "right-side-out" membranes with an impenetrant probe. Some cells can ingest latex particles to form latex-filled phagolysosomes that appear to have an "inside-out" orientation and a protein composition similar to the plasma membrane from which they are derived.

Hubbard and Cohn [4] iodinated L cells using lactoperoxidase and subsequently prepared phagolysosomes. They showed that the phagolysosomes prepared in this manner contained most of the same iodinated proteins found at the cell surface. Hunt and Brown [5] used the susceptibility of a high-molecular-weight protein in intact cells and phagolysosomes to trypsin hydrolysis to suggest a transmembrane configuration for this protein.

We have used "inside-out" oriented phagolysosomes from mouse cells in conjunction with nonpenetrant enzymatic labeling to orient specific plasma membrane proteins. Our experiments suggest that there are plasma membrane proteins which bridge through the lipid bilayer of the plasma membrane as single polypeptides. Additional experiments show that there are lectin-binding proteins whose carbohydrate moieties are not exposed on the cytoplasmic face of the plasma membrane, that appear to have a transmembrane configuration. Several of the TMB proteins can be identified in high-resolution 2-D gels. Certain TMB proteins are metabolically labeled by $^{32}PO_4 =$ and ^{35}S-methionine and are glysolated. One of the predominant TMB plasma membrane proteins that we have studied has a molecular weight of approximately 100,000 daltons. Some properties of this protein will be described.

METHODS

Cell Culture, Metabolic Labeling of Cell Proteins, Preparation of Phagolysosomes, and Plasma Membranes

Mouse L-929 cells were grown in suspension culture in minimal essential medium (MEM) containing 5% fetal calf serum and 0.15% methylcellulose (15 cps). For analysis of the metabolically labeled proteins the cells are placed in MEM (without methionine) with 5% fetal calf serum and 10 μCi of ^{35}S-methioine per milliliter. After 4 h an equal volume of medium containing 0.2 mM methionine is added and the cells are incubated for 6 h prior

to preparation of the plasma membranes. For labeling of the phosphoproteins the cells are incubated for 4 h in phosphate-free MEM containing 1% dialyzed serum and 100 μCi/ml of ^{32}P orthophosphate.

Incubation of cells with 1-μ latex particles and preparation of latex-filled phagolysosomes from these cells was performed as previously described [6].

Plasma membrane was prepared as described by Brunette and Till [7].

Labeling of Cell Surfaces and Phagolysosomes

Cells were rinsed in phosphate buffered saline (PBS) and ^{131}I- or ^{125}I-lactoperoxidase labeled as described by Hynes [8]. After a 10-min labeling period the cells were rinsed in PBS containing 0.5 mM tyrosine. Phagolysosomes were prepared from unlabeled cells and iodinated under similar conditions except that the concentrations of lactoperoxidase and glucose oxidase were reduced to 2.5 and 0.25 μg/ml, respectively.

Phagolysosomes were labeled with dansyl cadaverine using transglutaminase purified from guinea pig liver as previously described [6].

Cells and phagolysosomes were labeled with a periodate-NaB^{3}H$_4$ technique described by Gahmberg and Andersson [9].

Membrane Sample Preparation and Isolation of Specific Membrane Proteins by Affinity Chromatography

The isolation of dansyl cadaverine-labeled proteins on antidansyl IgG affinity columns was performed on phagolysosomes solubilized in Triton X-100 as previously described [6].

Isolation of the iodinatable lectin-binding proteins from intact cells and phagolysosomes was performed by lysing the preparations in 60 mM borate 1% Triton X-100, 1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 2 mM N-ethylmaleimide, 2 mM iodoacetamide, and 2 mM dithiothreitol at pH 7.8. The lysate was clarified at 10,000g for 10 min and chromatographed on a Bio-Rad P-10 column. The excluded proteins were applied to columns containing wheat germ agglutinin (WGA)-Sepharose 6B, Con A-Sepharose 6B, or Lens culinaris-Sepharose 6B. The columns were washed with 0.05 M Tris 1% Triton X-100, pH 7.5, until the effluent radioactivity was essentially at background level. The retained material was eluted with Tris-Triton X-100 containing 0.3 M of the appropriate sugar. The iodinated protein recovered from WGA-Sepharose affinity columns in the presence of N-acetyl-D-glucosamine was dialyzed. The samples from the retained column fractions were precipitated in acidified acetone, washed with ethanol:ether (1:1 v/v), dried under N$_2$, and resuspended in 0.14 M Tris, 22.3% glycerol, 6% sodium dodecylsulfate, 2 mM dithiothreitol, and 0.001% bromphenol blue (pH 6.8). Samples to be analyzed by two-dimensional electrophoresis were resuspended in 9.5 M urea, 2% Triton X-100, 1.6% carrier ampholine (pH 5–7), 0.4% carrier ampholine (pH 3–10), and 2 mM dithiothreitol.

Polyacrylamide Gel Electrophoresis

Discontinuous sodium dodecysulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described [6]. Two-dimensional electrophoresis was performed essentially as described by O'Farrell [10]. The gels were fixed, stained, and dried, and were either directly autoradiographed or prepared for fluorography as described elsewhere [11].

Studies on the limited proteolysis of membrane proteins recovered from two-dimensional gels were performed essentially as described by Cleveland et al [12].

RESULTS

Studies on the Orientation of the Plasma Membrane Proteins in Phagolysosomes Using Periodate NaB^{3}H$_4$ Labeling and Lectin Affinity Columns

When cells were labeled with periodate-borotritide at 4°C, 4 × 10^5 cpm of ^{3}H pre-cipitable by 10% cold trichloracetic acid was incorporated per 10^6 cells. In contrast, fewer than 1,000 cpm of acid-precipitable counts were recovered from labeling of phagolysosomes recovered from 10^7 cells.

Both Con A-Sepharose 6MB and WGA-Sepharose 6MB were found to retain intact L cells effectively. This is shown in Table I. The retention of the cells was reduced by running the columns in the appropriate competing sugar. Phagolysosomes containing approximately an equal amount of membrane to the cells showed almost no binding to the lectin affinity columns. The amount of membrane was equalized by matching the iodinatable counts of intact cells with those in the phagolysosomes obtained from cells that had been lactoperoxidase-iodinated.

Double Labeling of Phagolysosomal Proteins

The details of labeling of the external surfaces of cells with lactoperoxidase-catalyzed iodination, preparation of phagolysosomes, and labeling by a transglutaminase-mediated dansyl cadaverine reaction of the inside-out phagolysosomes has been reported [6]. The protocol for these experiments is diagrammed in Figure 1. The enzymatically dansyl ca-daverine-labeled proteins from the phagolysosome, which appear to be on the cytoplasmic face, were solubilized and separated from the other iodinatable proteins by affinity chroma-tography on antidansyl IgG-affinity columns. From these types of analyses and those previously reported [6], we conclude that the plasma membranes in the phagolysosomes have predominantly, if not exclusively, an "inside-out" orientation. The selective vectoral orientation of the membrane in these preparations provides a simple procedure for determining the presence of transmembrane proteins. Figure 2 shows the results of these studies. It can be seen in Figure 2E that there are three major polypeptides of 55,000, 80,000, and 110,000 daltons containing both the dansyl cadaverine and radioactive iodine.

TABLE I. Affinity Chromatography of Lactoperoxidase-Iodinated L Cells and Phagolysosomes on Lectin-Sepharose 6MB

	% Acid-precipitable ^{125}I retained[a]			
	Con A-Sepharose		WGA-Sepharose	
	– Mannose	+ Mannose[b]	– N-AcGlucosamine	+ N-AcGlucosamine[c]
Cells	92	35	98	22
Phagolysosomes	9	11	18	16

[a]Approximately 10^6 cpm of ^{125}I-labeled cells or phagolysosomes prepared from labeled cells were applied to 0.9 × 8-cm lectin-Sepharose 6MB columns in PBS. Values represent percentage of total trichloroacetic acid-precipitable ^{125}I retained after a 100-ml volume was eluted from the column.

[b]Column run in the presence of 0.1 M mannose.

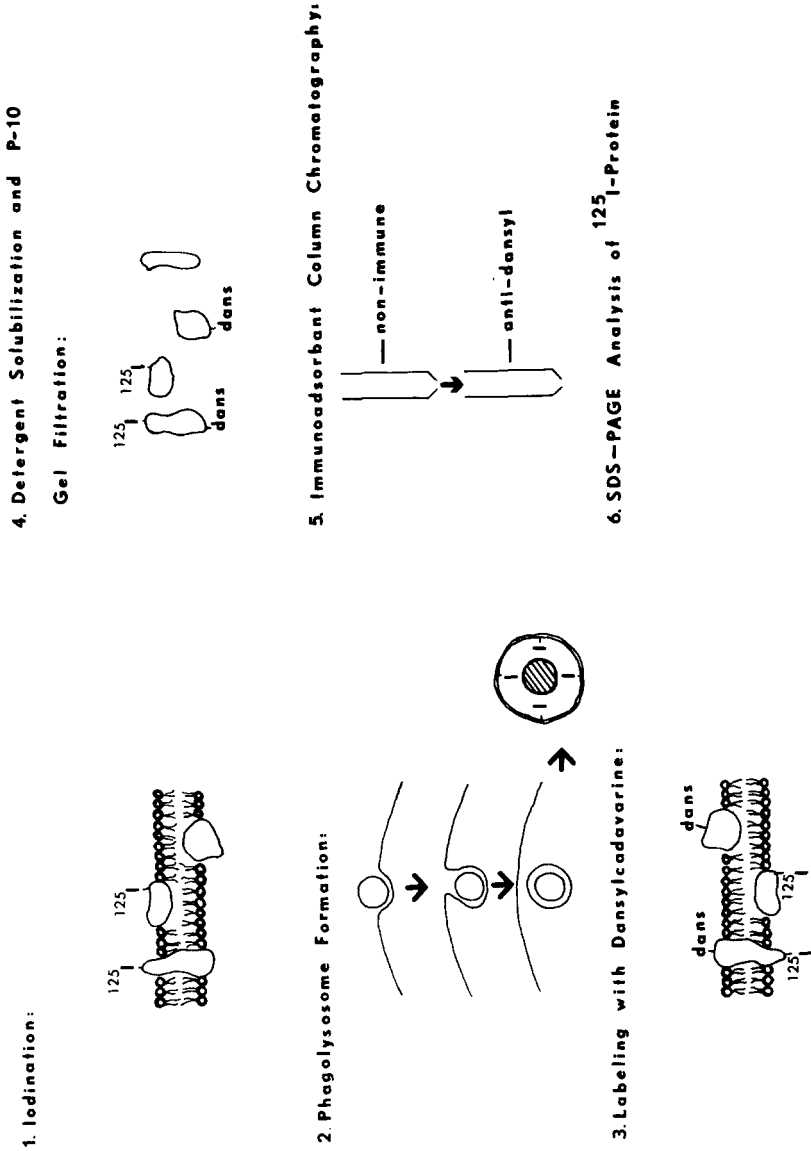[c]Column run in the presence of 0.2 M N-acetyl-D-glucosamine.

Fig. 1. Diagrammatic representation of labeling and preparation of phagolysosomal proteins. The methods for surface iodination, preparation of phagolysosomes, dansyl cadaverine labeling of proteins using transglutaminase, immune affinity chromatography with antidansyl IgG and SDS-PAGE are given in [6].
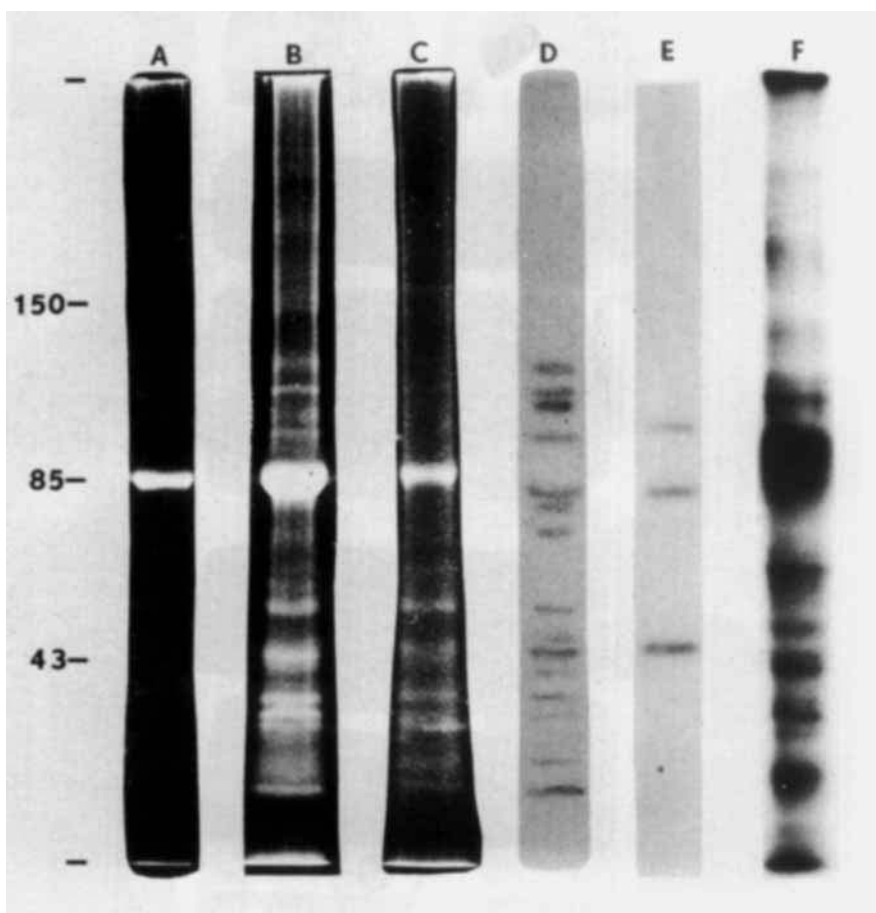
Fig. 2. Sodium dodecylsulfate (5–15%) polyacrylamide gel electrophoresis of ^{125}I-labeled and dansyl cadaverine-labeled cell proteins. Molecular weights ($\times$ 10^{-3}) are shown at the left. Lanes A–C are the fluorescent proteins and lanes D–F are autoradiographs of iodinated cell surface proteins. A) Guinea pig liver transglutaminase labeled with dansyl cadaverine. B) Fluorescent proteins solubilized from phagolysosomes labeled with dansyl cadaverine by a transglutaminase-catalyzed reaction after isolation from the cells. C) The fluorescent proteins labeled as described in B that were retained on an antidansyl IgG affinity column. D) Autoradiographic pattern of the cell surface proteins, phagolysosomes; this is an autoradiograph of the proteins in column B. E) Autoradiograph of column C showing the iodinated proteins retained by an antidansyl IgG affinity column. F) Lactoperoxidase-iodinated cell surface proteins from intact L-929 cells.

Lectin Affinity Chromatography of Iodinated Proteins From Cells and Phagolysosomes

The Triton X-100-soluble proteins from iodinated cells, phagolysosomes prepared from labeled cells, and phagolysosomes iodinated directly were chromatographed on Con A and WGA affinity columns. The labeled material was analyzed by SDS-PAGE and the results are shown in Figures 3 and 4.

Figure 3 shows the densitometric tracings of autoradiographs of the SDS-PAGE of ^{125}I-labeled proteins retained by Con A columns. The intact cells have four major protein bands, of 20,000, 100,000, 150,000, and 230,000 daltons. These proteins were also present in the Con A-binding fraction of proteins from phagolysosomes prepared from iodinated

cells. The phagolysosomes that were directly iodinated showed Con A-binding proteins of 100,000, 150,000, and 230,000 daltons. A protein of 20,000 daltons was found in the phagolysosomes prepared from iodinated cells but was not present in the phagolysosomes directly iodinated.

The SDS-PAGE analysis of these iodinated preparations retained by WGA affinity columns is shown in Figure 4. The major iodinatable protein of 100,000 daltons found in iodinated intact cells and in phagolysosomes prepared from these cells is not found in directly iodinated phagolysosomes. A 150,000-dalton protein was heavily iodinated in the phagolysosomes that were directly iodinated.

A selected region of the two-dimensional gel analysis of ^{131}I-labeled L cell proteins prepared from lactoperoxidase-iodinated cells and the fraction of this material retained by a WGA affinity column is shown in Figure 5. At least three externally iodinatable proteins which are found in the total phagolysosomal preparation (Fig. 5A) are not detectable in the fraction retained by the WGA affinity column (Fig. 5B). These three proteins are of similar molecular weight to other ^{131}I-labeled proteins retained by the WGA affinity column and are difficult to resolve in one-dimensional SDS-PAGE.

The gels show a string of spots of 85,000 and 115,000 daltons, which form a beads-on-a-string pattern. The mobility of these proteins is markedly affected by affinity purified neuraminidase treatment and probably is related to the glycosylation of these proteins (data not shown).
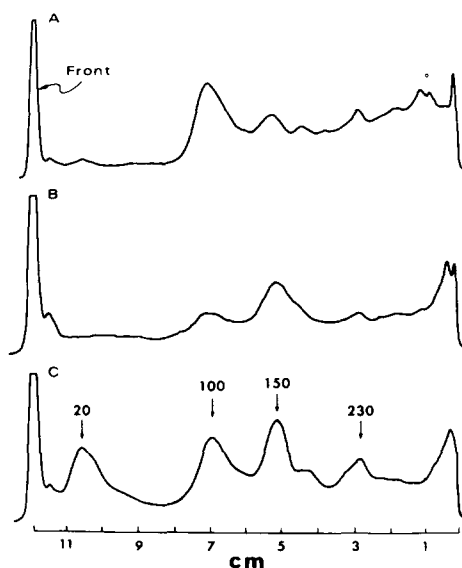

Fig. 3. Sodium dodecylsulfate gel electrophoresis analysis of lactoperoxidase ^{125}I-labeled L-929 proteins eluted from Con A affinity columns. The samples analyzed on 7.5% acrylamide gels were obtained from A) phagolysosomes prepared from iodinated cells, B) directly iodinated phagolysosomes, C) intact cells. The direction of migration is from right to left. The numbers indicated above the densimetric tracings represent approximate molecular weight × 10^{-3}.

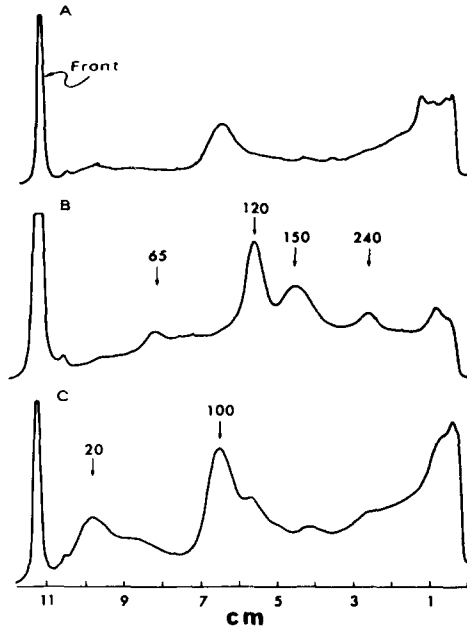Fig. 4. Sodium dodecylsulfate gel electrophoresis analysis of lactoperoxidase ^{125}I-labeled L-929 proteins eluted from WGA affinity columns. The samples analyzed on 7.5% acrylamide gels were obtained from A) phagolysosomes prepared from iodinated cells, B) directly iodinated phagolysosomes, C) intact cells. For further details see legend to Figure 1.
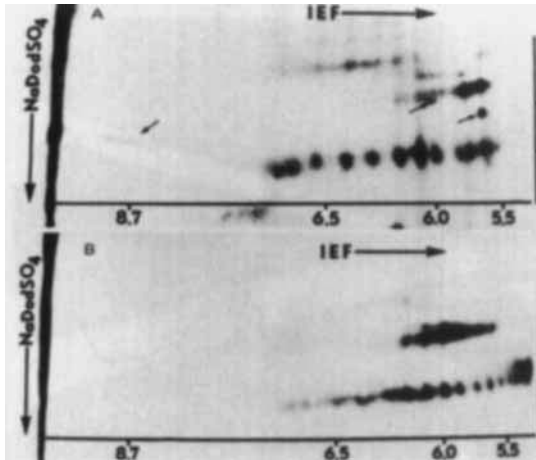

Fig. 5. Two-dimensional gel electrophoresis of lactoperoxidase ^{131}I-labeled L-929 proteins from A) phagolysosomes prepared from iodinated cells and B) phagolysosomes prepared from iodinated cells and retained by a WGA affinity column. The abcissa gives pH values for the first dimension; this figure shows a selected region of a 2-D gel. The arrows denote cell surface iodinatable proteins present in phagolysosomes which were not retained by WGA affinity columns.
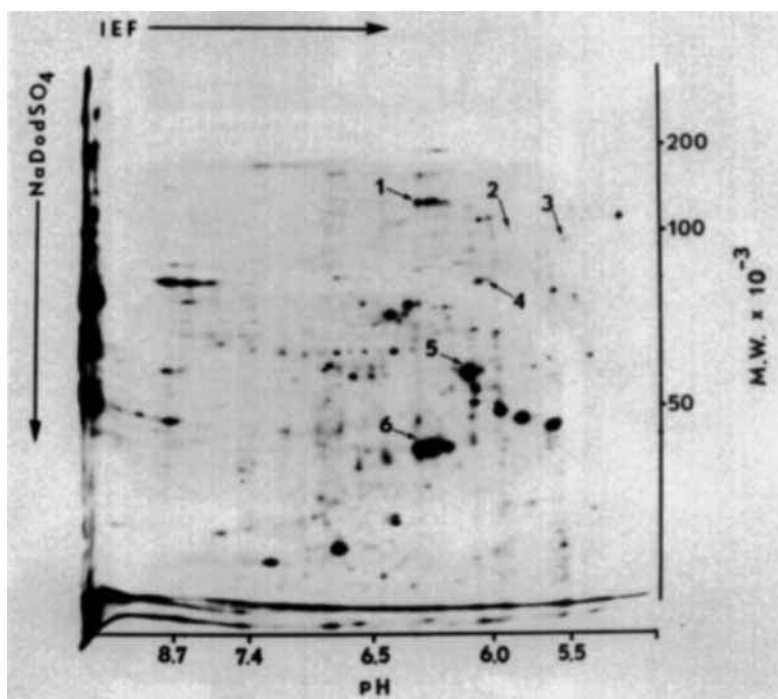
Fig. 6. Two-dimensional gel electrophoresis of phagolysosomal proteins from cells labeled with ^{35}S-methionine. The proteins labeled 1, 3, and 5 are phosphoproteins as shown in Figure 10. The protein 2, 3, and 5 are iodinatable from the outside surface of cells and from the inside surface of isolated phagolysosomes. The protein labeled 6 is actin, which is not iodinatable from the outside surface, as shown in Figure 8.

Two-dimensional Electrophoretic Analysis of the Proteins From Cells and Phagolysosomes

The pattern of phagolysosomal proteins which are metabolically labeled in cells by incubation of the cells with ^{35}S-methionine is shown in Figure 6. Approximately 250 labeled proteins can be detected. The major protein seen at 42,000 daltons is actin. We have prepared membrane from cells by several methods including those described by Allan and Crumpton [13] and Brunette and Till [7]. There are many similarities in the protein composition of these preparations observed in analysis of 2-D gel electrophoresis (data not shown). Therefore, unlabeled membrane from Brunette and Till membrane preparations was added to locate the position of specific proteins in Coomassie blue-stained gels where the mass of the radiolabeled phagolysosomal preparation is too small to be visualized.

The 2-D pattern of the ^{131}I-labeled proteins in phagolysosomes prepared from previously iodinated intact cells is shown in Figure 7. It should be noted that there is extensive labeling of surface proteins which "string out" during isoelectric focusing. Many of these iodinatable cell surface proteins are probably glycoproteins because neuraminidase treatment or endoglycosidase treatment of the cells after the iodination markedly alters the electrophoretic mobility of these proteins following isoelectric focusing (data not shown). The 2-D gels show that there are proteins iodinated on the cell surface to which we cannot find an exact counterpart in the patterns of ^{35}S-methionine-labeled proteins from plasma membranes (Figs. 6 and 7). There is iodination of proteins which exhibit mobility similar to actin, but these do not comigrate with actin as determined by a comparison of the Coomassie blue-stained proteins and the images produced by autoradiography of the iodinated proteins.
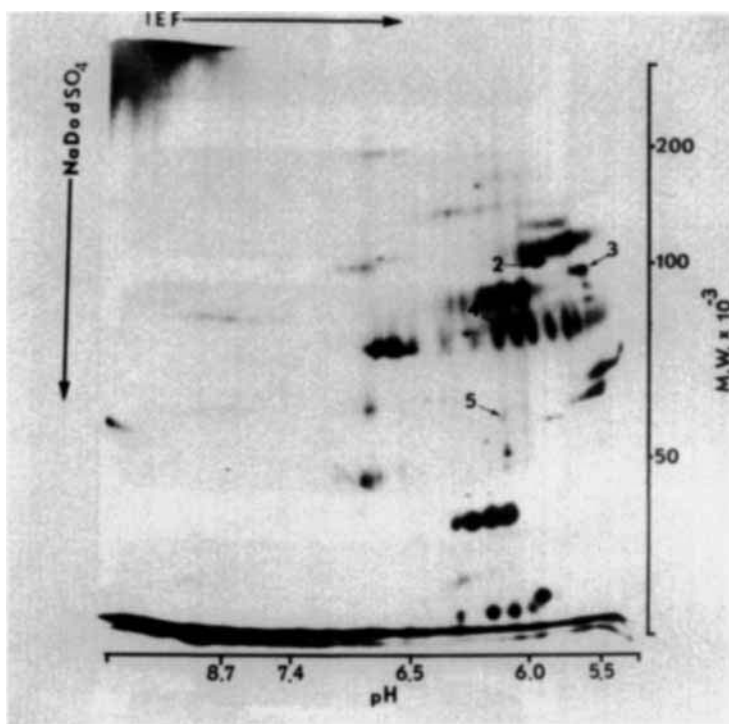
Fig. 7. Two-dimensional gel electrophoresis of the phagolysosomal proteins derived from lactoperoxidase iodinated intact cells. The proteins labeled 2, 3, and 5 are also iodinatable in intact phagolysosomes as shown in Figure 9.

Two-dimensional electrophoresis of phagolysosomes which are iodinated after they are isolated from cells reveals that there are several proteins which have mobilities in isoelectric focusing and SDS-PAGE identical to those of iodinated proteins from the cell surface (Fig. 8). It should be noted that there are marked differences in the proteins labeled by lactoperoxidase when the internal and external surfaces are selectively iodinated (see Figs. 7, 8).

We have selected for further analysis certain specific proteins which are labeled from both the external and the internal side of the plasma membrane. Using 2-D gels we were able to locate an iodinated protein at 100,000 daltons (pI approximately 5.5). The spot corresponding to this protein was punched out of the 2-D gel and was treated separately with papain, chymotrypsin, and Staphylococcal V-8 protease. Limited proteolysis and SDS-PAGE as described by Cleveland et al [12] and shown in Figure 9 reveals that the iodinated peptides are different from the externally labeled and internally labeled protein.

When preparations of plasma membrane from cells metabolically labeled with ^{35}S-methionine and $^{32}PO_4$ = are mixed and coelectrophoresed, selective autoradiography with and without a plastic screen reveals that the 100,000 dalton protein is the most heavily phosphorylated protein resolved by 2-D gel electrophoresis of this membrane preparation (Fig. 10).
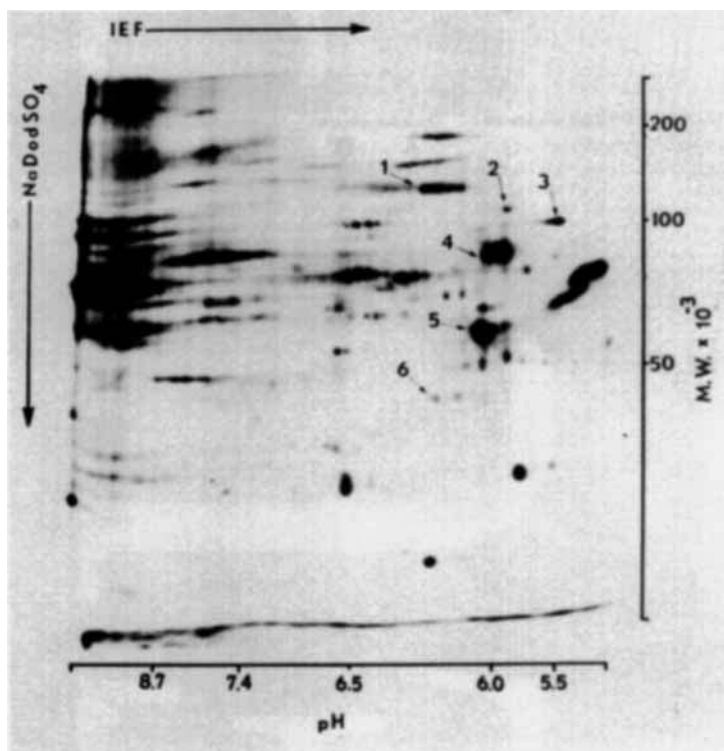
Fig. 8. Two-dimensional gel electrophoresis of the proteins from phagolysosomes which were iodinated after isolation from the cells.

DISCUSSION

Information about the relationships between membrane proteins, and between peripheral membrane proteins and integral proteins, will be important in understanding cellular responses to environmental changes. Because the transduction of signals may be mediated by changes in the distribution or conformation of proteins which span through the lipid bilayer, it is important to determine whether there are a small number of protiens or a wide variety of proteins which have transmembrane properties.

There is extensive indirect evidence suggesting that there are integral membrane proteins in nucleated cells, which are exposed at both the external and internal faces of the plasma membrane [14]. However, only several proteins have been directly characterized as having a TMB orientation [15–18]. A number of different approaches have been used to directly analyze the topographic location of membrane proteins. Walsh and Crumpton [19] have prepared "inside-out" vesicles from lymphocytes and have combined lactoperoxidase iodination of this preparation and immune precipitation with antibodies against cell surface antigens to demonstrate a TMB configuration for HLA antigens. We have used phagolysosomes formed by L cells to obtain plasma membranes in an "inside-out" conformation. This membrane contains most of the cell surface iodinatable proteins, as previously shown by Hubbard and Cohn in 1-D SDS-PAGE [4]. The phagolysosomal membrane
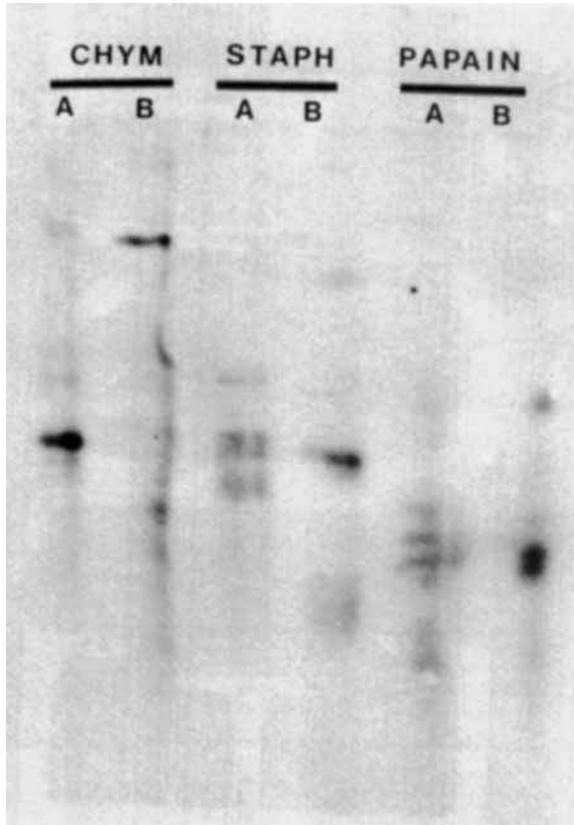
Fig. 9. Sodium dodecylsulfate gel electrophoresis of iodinated peptides from partial proteolytic hydrolysis of the protein designated number 3 in Figures 7 and 8. A) Protein 3 from phagolysosomes labeled after isolation; B) Protein 3 from phagolysosomes derived from iodinated cells.

is also very similar to plasma membrane obtained by other methods [7, 13], with respect to protein composition as analyzed by 2-D gel electrophoresis of either the phagolysosomal proteins from ^{35}S-methionine-labeled cells or the iodinated cell surface proteins in phagolysosomes prepared from previously iodinated intact cells [20].

Several chemical and enzymatic methods have been used to confirm the "inside-out" orientation and the asymmetry of the phagolysosomal membrane preparation. The trypsin insensitivity of lactoperoxidase-iodinated cell surface proteins incorporated into the phagolysosomes has been previously demonstrated [5]. Sandra and Pagano [21] have shown that the asymmetrical distribution of phospholipids in phagolysosomes is consistent with what would be expected in an "inside-out" membrane if there were no major rearrangements during formation and isolation of this membrane.

In the present studies the differential labeling of intact cells and phagolysosomes with periodate-NaB^{3}H$_4$ at 4°C, and the demonstration that intact cells are efficiently retained on lectin affinity columns while phagolysosomes are not, indicate that there is asymmetry of the carbohydrate in phagolysosomes. The results are consistent with evidence showing that glycosylated regions of membrane proteins are asymmetrically distributed and are found only at the exterior cell surface [22—27] if the phagolysosome is "inside-out."
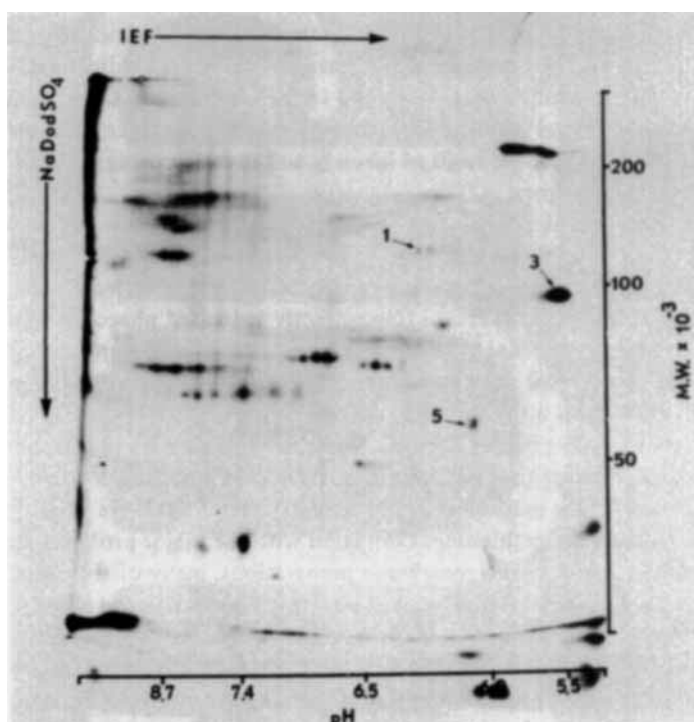
Fig. 10. Two-dimensional gel electrophoresis of the phagolysosomal proteins derived from cells labeled with inorganic $^{32}PO_4 =$.

Because of the asymmetry of the carbohydrates on glycoproteins it is possible to define a glycoprotein as TMB if it is iodinatable on the cytoplasmic surface and reacts with the lectin tested. The experiments show that most of the Con A-binding proteins in phagolysosomes are iodinatable from both sides of the membrane. However, there are WGA-binding proteins which were selectively iodinated at the external or internal cell surface. It is possible that some of these proteins, which are iodinated when isolated phagolysosomes are iodinated, are derived from lysosomes. Another less likely possibility is that these proteins are retained on the WGA lectin affinity column in association with a lectin-binding protein rather than by direct interaction with the WGA. Proteins were reduced and alkylated during the membrane solubilization to decrease the possibility of a protein-protein-lectin interaction. The 2-D gels of WGA proteins show the complexity of proteins at given molecular sizes and illustrate the hazards of suggesting that proteins are identical because they have the same mobility in SDS-PAGE. Even with this caveat, the data are consistent with a TMB configuration for a variety of lectin-binding cell surface proteins.

Another approach to identification of TMB proteins involved iodination of cell surface proteins, internalization of plasma membrane on latex particles by cells, disruption of cells, preparation of phagolysosomes, and labeling of the proteins on the cytoplasmic face of phagolysosomes using transglutaminase to covalently modify the available proteins with the hapten dansyl cadaverine. Using immune affinity chromatography with antidansyl IgG, we can isolate the proteins solubilized from phagolysosomes. We can show that some of these proteins are labeled with iodine. The predominant plasma membrane proteins labeled both externally and with LP iodination and internally with dansyl cadaverine by transglutaminase have molecular weights of 55,000, 80,000, and 100,000. These studies do not

prove that these are the only TMB proteins, since the labeling is restrictive in the sense that we can detect only those TMB proteins which have an amino acid iodinatable on their external segment and a glutamine available for the transglutaminase-catalyzed linking of dansyl cadaverine on the internal segment. Because we wanted to analyze TMB proteins further, we have developed other methods of labeling and isolating proteins from the plasma membrane and phagolysosomes.

Another method developed to analyze the proteins available for enzymatic modification on the internal or external face of the plasma membrane is to electrophorese, on O'Farrell 2-D gels, the lactoperoxidase-catalyzed iodinated proteins from phagolysosomes prepared from iodinated intact cells and from directly iodinated phagolysosomes. Using these 2-D gels we have shown that there is marked difference in the proteins exposed to lactoperoxidase at the internal and external surfaces (Figs. 8 and 9) of the plasma membrane. For example, actin is not iodinated at the surface of intact L-929 cells. Cells grown in the absence of serum have similar proteins iodinated as cells washed after growth in the presence of serum, indicating that the pattern is not due to sequestration and labeling of serum proteins. Many of the major iodinatable cell surface proteins are poorly labeled by incubation with radioactive methionine. Compared with the major protein species isolated in phagolysosomes or conventional membrane preparations, many of the most extensively iodinated cell surface proteins give a "beads-on-a-string" pattern in the isoelectric focusing dimension. The mobility of these proteins is markedly altered by neuraminidase or endoglycosidase treatments.

Several proteins which can be identified in 2-D gels are available for lactoperoxidase iodination on both the internal and external surface of the plasma membrane. In Figures 7 and 8 it can be seen that a protein of 100,000 daltons, focusing at approximately pH 5.5, is available for iodination from both sides of the plasma membrane. This protein is very likely a TMB protein. The protein spot was cut out of the 2-D gels and subjected to partial proteolysis. The fragmentation pattern of this protein as shown in Figure 10 suggests that different portions of the same protein are available for iodination at the internal and external faces of the plasma membrane. Since unlabeled carrier membrane proteins are used to localize the iodinated 100,000 dalton protein in the 2-D gels, there is still the unlikely possibility that the discrete spot contains more than one iodinatable plasma membrane protein.

The ability to determine exactly the coordinates for certain proteins in the 2-D gel system allows a comparison of various types of protein labeling. For example, a 2-D analysis of the plasma membrane proteins from cells incubated with $^{32}PO_4^=$ shows that the 100,000 dalton TMB protein is also one of the most phosphorylated proteins in the plasma membrane. Another protein available for iodination at both membrane surfaces has a molecular weight of 60,000 and is extensively phosphorylated. At present the function of these phosphoproteins and the role of the phosphorylation are not known. Experiments are in progress to characterize further the phosphorylation using both proteins from cells labeled with $^{32}PO_4^=$ and proteins from membranes phosphorylated with γ-^{32}P-ATP.

The 100,000 dalton TMB protein is found in membrane preparations from a variety of mouse cell lines, mouse peritoneal macrophages, rat liver cells, and HeLa cells [20]. We speculate that some of the TMB proteins which we are analyzing are structural components of the plasma membrane of a variety of cells in culture.

The studies presented show a variety of approaches to studying the topology of membrane proteins, and they should provide a framework for the future analysis of protein-protein interactions between membrane proteins and for studies of the possible alterations in the orientation of membrane proteins in response to specific stimuli.

ACKNOWLEDGMENTS

REFERENCES

1. Bretscher M: Nature New Biol 231:229, 1971.
2. Schmidt-Ullrich R, Mikelsen RB, Wallach DFH: J Biol Chem 253:6973, 1978.
3. Whiteley NM, Berg HC: J Mol Biol 87:541, 1974.
4. Hubbard AL, Cohn ZA: J Cell Biol 64:461, 1975.
5. Hunt RC, Brown JC: J Mol Biol 97:413, 1975.
6. Evans RM, Fink LM: Proc Natl Acad Sci USA 74:5341, 1977.
7. Brunette DM, Till JE: J Membrane Biol 5:215, 1971.
8. Hynes RO: Proc Natl Acad Sci USA 70:3170, 1973.
9. Gahmberg CG, Andersson LC: J Biol Chem 252:5888, 1977.
10. O'Farrell PH: J Biol Chem 250:4007, 1975.
11. Lasky RA, Mills AD: Eur J Biochem 56:335, 1975.
12. Cleveland DW, Fisher SG, Kirschner MW, Laemmli UK: J Biol Chem 252:1102, 1977.
13. Allan D, Crumpton MJ: Biochem J 120:133, 1970.
14. Ash JF, Louvard D, Singer SJ: Proc Natl Acad Sci USA 74:5584, 1977.
15. Kyte JJ: J Biol Chem 249:3652, 1974.
16. Louvard D, Semeriva M, Maroux S: J Mol Biol 106:1023, 1976.
17. Henning R, Milner R, Reske K, Cunningham BA, Edelman GM: Proc Natl Acad Sci USA 73:118, 1976.
18. Springer TA, Strominger JL: Proc Natl Acad Sci USA 73:2481, 1976.
19. Walsh FS, Crumpton MJ: Nature 269:306, 1977.
20. Evans RM, Fink LM: Unpublished data.
21. Sandra A, Pagano RE: Biochemistry 17:332, 1978.
22. Hirano H, Parkhouse B, Nicolson GL, Lennox ES, Singer SJ: Proc Natl Acad Sci USA 69:2945. 1972.
23. Nicolson GL, Singer JJ: J Cell Biol 60:236, 1974.
24. Pinto da Silva P, Nicolson GL: Biochim Biophys Acta 363:311, 1974.
25. Roos E, Temmick JHM: Exp Cell Res 94:140, 1975.
26. Walsh FS, Barber BH, Crumpton MJ: Biochemistry 15:3557, 1976.
27. Nigam VM, Brailovsky CA: Biochim Biophys Acta 468:472, 1977.