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Topography of the *Dictyostelium discoideum* Plasma Membrane: Analysis of Membrane Asymmetry and Intermolecular Disulfide Bonds†

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ABSTRACT: Through the application of a unique method for isolating plasma membranes, it was possible to specifically iodinate cytoplasm-exposed plasma membrane proteins in vegetative cells of the cellular slime mold *Dictyostelium discoideum*. The original procedure [Chaney, L. K., & Jacobson, B. S. (1983) *J. Biol. Chem. 258*, 10062] which involved coating cells with colloidal silica has been modified to yield a more pure preparation. The presence of the continuous and dense silica pellicle on the outside surface of the isolated plasma membrane permitted the specific labeling of cytoplasm-exposed membrane proteins. Lactoperoxidase-catalyzed iodination was employed to label cell-surface and cytoplasm-exposed membrane proteins. The isolated and radioiodinated membranes were then compared and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The cell-surface and cytoplasmic face labeling patterns were distinct. A total of 65 proteins were found to be accessible to at least one surface of the membrane. Sixteen intermolecular disulfide bond complexes were observed in the plasma membrane of *Dictyostelium*; most of these complexes involved glycoproteins and, hence, were exposed to the cell surface.

For the elucidation of the function of membrane components at the molecular level, a comprehensive analysis of the composition and topography of the membrane in question is essential. The composition of purified membranes can be determined by one-dimensional sodium dodecyl sulfate (Na-

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[1] Abbreviations: bis(acrylamide), *N,N'*-methylenebis(acrylamide); BSA, bovine serum albumin, fraction V; CCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Con A, concanavalin A; Con A buffer, 50 mM Tris-HCl, 0.1 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.0; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; NaDodSO_4, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PM, plasma membrane(s); PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; kDa, kilodalton(s).

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$DodSO_4$)[1] gel electrophoresis or two-dimensional isoelectric focusing/$NaDodSO_4$ gel electrophoresis. However, assessing the distribution, orientation, and physical relationship of membrane components to one another is a much more difficult problem. The current methodology for analyzing these aspects of membrane structure has been summarized and evaluated (Carraway, 1975; Etemadi, 1980a,b; Ji, 1979).

Using membrane vesicles (right-side out, inside out, or leaky) to study or to identify transmembrane proteins by immunological, chemical, or isotopic labeling has, in some cases, led to confusing or contradictory results. Several studies, employing different techniques, have suggested that protein conformational changes occurred as a product of cell lysis, membrane isolation, and leakiness (Carraway, 1975). Differences in membrane labeling in the intact cell and in isolated right-side out vesicles have been observed [see Carraway (1975) and Etemadi (1980a)]. Conformation changes in proteins and the concomitant difficulties it can cause in membrane topographic studies should be much reduced when the proteins are immobilized before cellular disruption occurs. In a plasma membrane (PM) isolation procedure recently developed in our laboratory (Chaney & Jacobson, 1983), cells were coated with colloidal silica and polyacrylic acid which form a dense continuous pellicle around the cell. Surface proteins were immobilized and simultaneously shielded from modification by chemical and isotopic probes. Thus, this PM isolation procedure offered two distinct advantages: (1) surface protein reorientation is prevented, and (2) the cytoplasmic face of the membrane can be specifically labeled or otherwise analyzed.

This paper describes the application of this membrane isolation procedure to topographic studies on the PM of *Dictyostelium discoideum* vegetative cells. Cell-surface, cytoplasm-exposed, glycoproteins, and possible transmembrane proteins have been identified. In addition, protein complexes formed by intermolecular disulfide bonds have been identified and their subunit proteins and stoichiometries determined.

Materials and Methods

Cells. *Dictyostelium discoideum* strain AX-3 cells (obtained from the American Type Culture Collection) were grown to a density of (6–8) $\times 10^6$ cells/mL in 1-L culture flasks containing 400 mL of HL/5 medium (Franke & Kessin, 1977). New cells were started from spores every 4 weeks.

Plasma Membrane Isolation. Plasma membranes were prepared by either of two methods. Plasma membranes from surface-labeled cells were isolated by using the method of Prem Das and Henderson (1983a). Prior to iodination, the cells were suspended in 50 mM sodium phosphate/50 mM NaCl, pH 6.5, and treated with 2 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (CCP) for 15 min at room temperature. The cells were then surface iodinated as described in a following section. After iodination, the washed and labeled cells were lysed, and the crude PM was pelleted (Prem Das & Henderson, 1983a). The continuous gradient was replaced by a step gradient consisting of equal volumes of 20%, 35%, 45%, 55%, and 60% (w/w) sucrose in 50 mM glycine–NaOH, pH 8.5. The membranes forming the upper band in the 35% sucrose layer were used.

Membranes to be labeled on the cytoplasmic face were isolated from cells coated with colloidal silica (Chaney & Jacobson, 1983). Cells were coated twice and then suspended in 20 mM glycine–NaOH, 2.5% sucrose, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF), pH 8.5, for lysis by nitrogen cavitation (1500 psi in a Parr bomb). The lysate was diluted with an equal volume of the glycine buffer, and the membranes were pelleted by differential centrifugation at 400*g* for 5 min. The membranes were washed twice more with the glycine buffer and once each with 20 mM Tris-HCl/70 mM NaCl, pH 7.6, and 10 mM Tris-HCl, pH 7.6. Silica-coated membranes prepared with the glycine buffer had specific activities of the plasma membrane marker, alkaline phosphatase, twice that of membranes prepared by the original procedure. Prior to iodination, the PM were treated with 2.5 mM *N*-ethylmaleimide (NEM) in 50 mM sodium phosphate/50 mM NaCl, pH 6.5.

Cells treated with concanavalin A (Con A) prior to plasma membrane isolation were first washed and resuspended to 10^7 cells/mL in cold sodium phosphate, 50 mM NaCl, and 0.5 mM PMSF, pH 6.5, or 70 mM NaCl, 20 mM Mes, and 0.5 mM PMSF, pH 6.5. Con A (1 mg/mL in 1 M NaCl, 50 mM Tris-HCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 8.0) was added to a final concentration of 20 μg/mL. The cells were shaken on a gyratory shaker set at 150 rpm and incubated for 5 min. The cells were repelleted, washed once, and then processed as usual for PM isolation.

Enzyme and Protein Assays. Protein content in samples was determined by the method of Lowry et al. (1951). Samples were solubilized in $NaDodSO_4$ (2–5% final concentration) and heated for 20 min at 65 °C. Silica bead containing samples were centrifuged to pellet the silica and the supernatants assayed.

The specific activity of alkaline phosphatase [same as 5′-AMP-specific nucleotidase; see MacLeod and Loomis (1979) and Armant and Rutherford (1981)] was routinely determined and was used to judge the relative purity of plasma membrane preparations. The following procedure was adapted from Green and Newell (1974). All samples to be tested, except the isolated silica bead coated PM, were diluted to 0.3–0.5 mg of protein/mL with 10 mM Tris-HCl, pH 7.6. Ten percent Triton X-100 was added to a final concentration of 1%. Silica bead coated PM were diluted to 0.6–1.0 mg of protein/mL with 10 mM Tris, and an equal volume of 4% Triton X-100, 3% sodium deoxycholate, and 10 mM Tris, pH 7.6, was added. Duplicate samples containing 15–75 μg of protein were assayed. After the sample volumes were adjusted to 0.2 mL, 2 mL of 0.5 M diethanolamine, 5 mM $MgCl_2$, and 2 mM KF, pH 9.8 was added to each sample. The samples were incubated at 21 °C for 30 min after the addition of 0.2 mL of *p*-nitrophenyl phosphate (37 mg/mL in the above buffer). Parallel samples were incubated in the buffer above except that $MgCl_2$ was replaced with 10 mM EDTA to determine the residual Mg^{2+}-independent alkaline phosphatase activity.

Lactoperoxidase-Catalyzed Iodination of Cell-Surface and Cytoplasm-Exposed Plasma Membrane Proteins. The iodination procedure described here was based upon that of Siu et al. (1976).

For cell-surface labeling, 2×10^8 washed and CCP-treated *Dictyostelium* cells were suspended to 1.3 mL in 50 mM sodium phosphate/50 mM NaCl, pH 6.5. The cells were treated with CCP to prevent pinocytotic uptake of iodide and, consequently, reduce the amount of internal labeling which occurred during cellular iodination (Siu et al., 1976). One hundred microliters of 200 μM NaI and 100 μL of lactoperoxidase (0.4 mg/mL water) were added to the cells. In an iodination hood, 1 mCi of $Na^{125}I$ was added and the reaction initiated by the addition of 235 μL of 176 mM H_2O_2. After 2.5 min, an additional 235-μL aliquot of H_2O_2 was added to the cells. After 5-min total reaction time, the reaction was stopped by the addition of 100 μL of 100 mM NaN_3. The cells were washed 3 times by centrifugation in 160 mL of 50 mM sodium phosphate/50 mM NaCl, pH 7.0. The cells were

then incubated in 2.5 mM NEM in the pH 7.0 phosphate buffer for 15 min at room temperature. Cell viability as judged by trypan blue exclusion was 97–98%. The plasma membranes from these labeled cells were usually isolated by the modified method of Prem Das and Henderson as described above.

For iodination of the internal face of the *Dictyostelium* PM, 0.5 mg of freshly isolated, NEM-treated silica-coated PM protein was resuspended to 1.7 mL in 50 mM sodium phosphate/50 mM NaCl, pH 6.5. Fifty microliters of 200 μM NaI and 50 μL of lactoperoxidase (0.4 mg/mL water) were added to the membranes. In an iodination hood, 0.25 mCi of $Na^{125}I$ followed by 117 μL of 176 mM H_2O_2 was then added to the mixture. After 2.5 min, a second aliquot of H_2O_2 was added. The reaction was terminated after 5 min total time by the addition of 50 μL of 100 mM NaN_3. The membranes were washed 3 times with 100 mL of 50 mM sodium phosphate/50 mM NaCl, pH 7.0, buffer.

Preparation of the Iodinated PM for PAG Electrophoretic Analysis. To remove many of the unbound counts remaining on the membranes even after extensive washing, the PM were solubilized in 2.5% $NaDodSO_4$/10 mM Tris-HCl, pH 7.6, heated at 75 °C for 15 min, and chromatographed in a 1.5-mL Sephadex G-25 column equilibrated in the above buffer. For iodinated colloidal silica PM, the silica was first removed by sedimentation before the supernatant was applied to the Sephadex column. The protein-containing fraction was pooled and the protein content assayed. Just prior to electrophoresis, the PM samples were diluted to 1 mg of protein/mL with 125 mM Tris-HCl, 1 mM EDTA, 20% glycerol, and 2.5% $NaDodSO_4$, pH 6.8, in the presence or absence of dithioerythritol (DTE). A small amount of bromphenol blue was added as a tracker dye. If the protein concentration was >1 mg/mL, 0.5 volume of the pH 6.8 buffer was added.

Approximate specific activities of the preparations were determined after gel electrophoresis using the following formula: (cpm applied to the gel − cpm in lower gel reservoir)/micrograms of protein applied to the gel. The specific activities for surface-labeled PM were between 400 and 600 cpm/μg of protein and that of cytoplasm-labeled PM was between 2000 and 3000 cpm/μg of protein.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. All gels described in this paper were 1.6 mm thick and 14 cm wide. The separation gels were 12.5 cm long and the stacking gels 1.5 cm tall. The buffer system of Laemmli (1970) was used except for the following modifications: all buffers contained 1 mM EDTA, and the amount of glycine in the running buffer was doubled. In addition, polyacrylamide [Aldrich Chemical Co., M_r (5–6) × 10^6] was added to the gel solutions to a final concentration of 0.5% except where noted.

For regular one-dimensional gel electrophoresis, a separation gel composed of a linear 5–18% gradient of acrylamide with a stacking gel of 4.7% acrylamide was used.

Two-Dimensional Nonreduced/Reduced $NaDodSO_4$–PAGE. The first-dimensional gel consisted of a 2.5% [acrylamide stock solution, 19% acrylamide/1% *N,N'*-methylenebis(acrylamide)] to 15% [acrylamide stock solution, 30% acrylamide/0.8% bis(acrylamide)] linear gradient. The stacking gel was composed of 2.0% acrylamide (using the former acrylamide stock solution). Polyacrylamide was not added to either the stacking gel or the 2.5% separation gel solutions. The sample wells were 1 cm wide and 2 cm deep; 75–150 μg of protein was applied per well. The gels were electrophoresed at 8–10 mA until the bromphenol blue dye front was 10 cm into the separation gel (about 14 h). The lanes were cut apart, the stacking gel was removed, and the separation gel below the dye line was discarded. The gel lanes either were used immediately or were wrapped in aluminum foil, stored at −20 °C, and used within 10 days.

The second-dimension gel was poured in the same manner as the regular gel described two paragraphs previously except that only one sample well was made to one side of the gel (for molecular weight standard proteins). Agarose (1.5%) was dissolved in 62.5 mM Tris-HCl, 15 mM DTE, 1 mM EDTA, and 0.2% $NaDodSO_4$, pH 6.8, and poured onto the top of the polymerized stacking gel. The first-dimension gel lane was quickly laid on top of the still liquid agarose. More agarose was pipetted onto the gel strip so that the strip would remain affixed to the gel plate when the agarose solidified. The agarose layer between the gel strip and the stacking gel of the second-dimension gel was about 1 cm tall. Bromphenol blue was added to the upper reservoir. The gel was electrophoresed at 12 mA until the bromphenol blue had migrated to the stacking/separation gel border (about 4 h). At this point, the first-dimension gel strip and agarose were removed; we found that this reduced the amount of vertical smearing of the proteins. Standard proteins were also applied to the premade well at this time. Electrophoresis was continued at 10 mA until the bromphenol blue just migrated out of the bottom of the gel (about 16 h). Information on the analysis of two-dimensional gels is given in Wang and Richards (1974) and Hynes and Destree (1977).

Glycoprotein Detection after $NaDodSO_4$–PAGE. Polyacrylamide gels, immediately after the completion of the electrophoretic run, were gently shaken in electroblotting buffer (50 mM Tris, 95 mM glycine, and 0.005% $NaDodSO_4$) for 30–45 min. The proteins in the gel were electroblotted to cellulose nitrate paper (Schleicher & Schuell, 0.2-μm pore size) for 65 min at 11 V/cm. The omission of methanol and the inclusion of the very small amount of $NaDodSO_4$ in the electroblotting buffer resulted in the most even transfer of proteins over a broad molecular weight range [10000–250000; see also Gershoni and Palade (1983)]. We also found that many glycoproteins below M_r 45000 bound poorly to the standard 0.45-μm-pored paper; thus, the smaller pored paper is recommended.

After electroblotting, the paper was incubated with gentle shaking for 1 h at room temperature in 50 mM Tris-HCl, 0.1 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.0 (Con A buffer), containing 1% BSA. For a 13 cm^2 piece of cellulose nitrate paper, 150 mL of buffer was used for all washes and incubations. Next, Con A (1 mg/mL in Con A buffer) was added directly to the BSA-containing buffer to a final concentration of 50 μg/mL. The paper was incubated for 30 min or longer. The paper was then washed twice for 10 min each with 0.1% BSA in Con A buffer and then incubated for 30 min or longer at room temperature in Con A buffer containing 0.1% BSA and 13 μg/mL horseradish peroxidase (250 units/mg of protein). This was followed by two washes with 0.1% BSA in Con A buffer for 10 min each and then once with Con A buffer for 5 min. The substrate solution was made up directly before use. For 60 mL of substrate solution, 30 mg of 4-chloronaphthol was dissolved in 10 mL of methanol and chilled. Just prior to use, 10 μL of 30% H_2O_2 was added to 50 mL of cold Con A buffer followed by the chloronaphthol solution. The solution was quickly mixed and poured onto a small, flat container, and the cellulose nitrate paper was laid in the solution. The reaction was allowed to proceed for 10–30 min in the dark with no agitation until the desired band intensities were achieved. The water-rinsed and air-dried paper was sprayed with clear matte finish to retard fading; the developed electroblots were also photographed immediately with Kodak Technical Pan 2415 film.

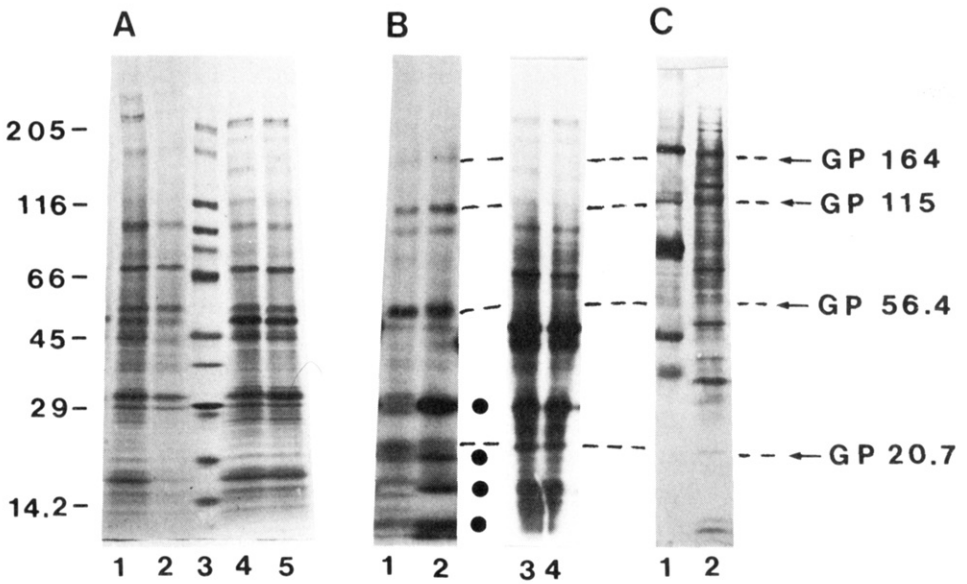

FIGURE 1: (A) Coomassie blue stained proteins in PM in *Dictyostelium*. Lanes 1 and 2 are PM prepared by the modified Das and Henderson method. The cells used for the preparation in lane 2 were treated with Con A before cell lysis. Lane 3 is molecular weight standards. Molecular weights are shown to the left of the gel. Lanes 4 and 5 are PM isolated by the colloidal silica method. Cells in lane 5 were treated with Con A before the cells were coated with the silica; 30–50 μg of PM protein was applied per lane. (B) Autoradiogram of the gel in (A). Lanes 1 and 2 show the radiolabel pattern obtained after cell-surface labeling and correspond to lanes 1 and 2 in (A), respectively. About 20 000 bound cpm were applied per lane. Lanes 3 and 4 show the radiolabel pattern obtained after iodination of the cytoplasmic face of the PM. The preparations were the same as those shown in lanes 4 and 5 in (A), respectively, except that about 10 μg of protein (about 40 000 bound cpm) was applied per lane for the autoradiogram. The dots between lanes 2 and 3 indicate the positions of the protein bands found in Con A. Since most of the radiodinated proteins with molecular weights greater than 150K were only weakly labeled, these bands were photographically enhanced during reproduction. Other weakly labeled bands of lower molecular weight were not always reproducible. (C) Glycoprotein staining of PM proteins transferred from polyacrylamide gels to cellulose nitrate paper. The staining procedure is described under Materials and Methods. Lane 1, glycoprotein standards (α-macroglobulin, lactoperoxidase, and ovalbumin). Lane 2, the staining pattern obtained from isolated PM (20 μg of protein). Some of the proteins listed in Table I are denoted with dotted lines across the Figure.

Ten to twenty micrograms of PM protein for one-dimensional gels and 100–150 μg of PM protein for resolving off-diagonal spots on two-dimensional gels were required for glycoprotein detection.

Electroblots processed in the above manner except for the presence of 0.1 M methyl α-mannoside during the Con A incubation and the two washes immediately following yielded no stained bands [see also Clegg (1982)]. Glycoprotein detection in these studies was based upon Con A affinity since the vast majority of glycoproteins in *Dictyostelium* PM have been shown to bind Con A (Hoffman & McMahon, 1978).

Autoradiography. Polyacrylamide gels were stained and fixed in Coomassie blue R/2-propanol/acetic acid. After being destained, the gels were equilibrated with 2.5% glycerol in water for at least 3 h and then dried down between dialysis sheets (Hoefer Scientific). The dried gels and cellulose nitrate papers were exposed to Kodak X-OMAT-AR film and intensifying screens at −80 °C.

Molecular Weight Standard Proteins. Standard proteins were purchased as a kit from Sigma Chemical Co. The molecular weights listed in the company catalog were used for the calculations in this paper. Glycoprotein standards included α_2-macroglobulin (M_r 180 000), lactoperoxidase (M_r 77 500), and ovalbumin (M_r 45 000). Rabbit muscle actin used for chloramine T iodination was isolated from acetone protein powder (Seraydarian et al., 1967) according to Spudich and Watt (1971). A final purification step by chromatography on Sephadex G-150 in actin depolymerization buffer was included.

RESULTS

Radiolabeled Cell-Surface and Cytoplasm-Exposed Proteins in Dictyostelium Plasma Membrane. The Coomassie blue stained protein profiles of PM prepared by the modified Prem Das and Henderson (1983a) and the modified Chaney and Jacobson (1983) methods are shown in Figure 1A. The protein compositions of the two preparations were very similar although the relative content of some of the proteins did differ. Some of these differences were probably due to the fact that the former preparation was about 2–3 times as enriched as the latter preparation (based upon alkaline phosphatase specific activity). About 80 proteins could be resolved on the gradient gel system employed in this paper.

The membrane proteins labeled by iodination of whole cells can be seen in Figure 1B, lanes 3 and 4. Figure 1B, lane 3, shows the cell-surface labeling pattern obtained from normal *Dictyostelium* amoeba while lane 4 shows the pattern obtained from the Con A treated amoeba. For both of these labeling experiments, the PM were isolated by the Prem Das and Henderson technique as described under Materials and Methods. The same labeling pattern was obtained if the PM were isolated by the colloidal silica method; however, to minimize the chance of exposure to radioactive aerosol during the lysis of silica-coated cells by nitrogen cavitation, cell-surface-labeled PM were isolated by the former technique. Of the 80 membrane proteins, 27 became radiolabeled by cell-surface iodination (refer to Table I). No radioactive actin could be detected in surface-labeled membranes (Figure 1) or in the Triton-insoluble pellets of such labeled membranes (data not shown); thus, very little iodination of proteins on the internal face of the PM seemed to have occurred.

Proteins labeled after isolation of PM by the colloidal silica method resulted in a very different pattern (Figure 1B, lanes 1 and 2) compared to that of cell-surface-labeled PM (Figure 1B, lanes 3 and 4). Also noteworthy was that the ^{125}I specific activities of the two types of labeled membranes were very different. The specific activities of the cytoplasmic face labeled PM were 5–8 times greater than that of the surface-labeled

Table I: Glycoproteins and Radiolabeled Plasma Membrane Proteins in *Dictyostelium discoideum*

descriptive[a] name	relative intensity: labeling outside	labeling inside	glycoprotein staining	possible function
GP_S 245	–	–	++	
GP_{TM} 221	–	+	++	
GP_{TM} 198	–	++	++	
GP_{TM} 182	–	+	++	actin binding protein[b]
GP_S 176	–	–	++	
GP_{TM} 164	++	+	+++	
P_C 155	–	+	–	
GP_S 153	–	–	+	
GP_{TM} 142	+	+	++	actin binding protein[b]
P_S 136	+	–	–	
GP_S 134	–	–	+	
GP_S 130	–	–	+++	
P_S 128	+	–	–	
P_C 127	–	+	–	
GP_S 121	–	–	+	
GP_{TM} 115	+++	++	++++	actin binding protein[b] and contact site B[c]
GP_S 110	–	–	+++	5′-nucleotidase[d]
GP_S 108	–	–	+	
P_C 106	–	++	–	
GP_S 100	–	–	+	
GP_{TM} 96.6	++	++	+++	
GP_S 92.8	–	–	+++	
P_{TM} 89.6	+	+	–	
GP_S 86.8	–	–	++	
GP_{TM} 83.6	+	++	+++	actin binding protein[b]
GP_S 80.6	–	–	+	
P_S 79.5	+	–	–	
P_C 78.4	–	++	–	
GP_S 76.3	–	–	+	
P_S 74.5	+	–	–	
GP_S 73.7	–	–	+++	
GP_{TM} 71.5	–	+++	++	actin binding protein[b]
GP_S 68.6	+	–	+	
P_C 66.7	–	++	–	
GP_S 65.3	–	–	++	
GP_S 61.5	–	–	+	
GP_{TM} 56.4	++++	++	++	
GP_S 54.2	–	–	++	
P_S 50.9	+	–	–	
P_C 50.2	–	++++	–	
GP_S 47.4	+	–	+++	
GP_{TM} 45.6	–	+++	++	
P_C 43.2	–	+++	–	actin
GP_S 43.1	+	–	+	
P_C 39.9	–	++	–	
P_S 39.6	++	–	–	
GP_{TM} 38.2	–	++	++	
GP_S 36.4	+	–	++	
P_S 33.4	++	–	–	
GP_S 33.3	–	–	++	
GP_{TM} 32.2	–	+	+	actin binding protein[e]
GP_S 29.8	+++	–	+++	
P_{TM} 28.4	+	++++	–	actin binding protein[e]
GP_S 26.6	+++	–	+++	
P_C 24.6	–	+++	–	actin binding protein[f]
P_C 23.4	–	+++	–	actin binding protein[f]
GP_{TM} 20.7	+++	+++	++	
P_S 19.6	+++	–	–	
P_C 18.2	–	+++	–	actin binding protein[e]
P_C 16.6	–	+++	–	actin binding protein[e]
P_S 16.1	+	–	–	
P_C 14.5	–	++	–	
P_S 14.3	+	–	–	
GP_{TM} 12.8	+	++	+++	
GP_{TM} 11.5	+	+	+++	

[a] Abbreviations: GP, glycoprotein; P, protein; S, surface exposed; C, cytoplasm exposed; TM, transmembrane. [b] Luna et al. (1984). [c] Beug et al. (1973), Vogel et al. (1980), and Chadwick et al. (1984). [d] Armant & Rutherford (1981). [e] Schleicher et al. (1984). [f] Stratford & Brown (1985).

PM. Thirty-one proteins were labeled; some of these coincided with surface-labeled proteins (see Table I). The pretreatment of cells with Con A did not alter the extent or the distribution of iodinatable cytoplasm-exposed protein compared to non-treated cells (Figure 1B, lanes 1 and 2); Con A itself did not appear to become labeled (compare lanes 3 and 4, non Con A and Con A treated PM, Figure 1B). In contrast, cell-surface labeling of Con A treated cells clearly showed the labeled lectin (Figure 1B, lanes 1 and 2; Con A bands indicated by the dots) even though the lectin was not seen in the Coomassie blue stained gels (Figure 1A, lanes 1 and 2).

Although Con A is reported to be a homotetramer having a subunit molecular weight of about 30000, we have always observed multiple bands on $NaDodSO_4$ gels of commercial preparations of the lectin.

The molecular weights presented in Table I are the averages of 12 measurements for cell-surface-labeled PM and of 6 measurements for cytoplasm face-labeled PM. Only those proteins which were consistently labeled were tabulated.

Concanavalin A Binding Proteins in Dictyostelium PM. In addition to analyzing which membrane proteins could be radiolabeled under two distinct conditions, the glycoprotein content (Con A binding proteins) and the extent to which these proteins became iodinated under the two labeling conditions were also studied. The bands appearing after the reaction of chloronaphthol with horseradish peroxidase are shown in Figure 1C. Forty glycoproteins could be detected in the PM by this method (see Table I; data based upon 10 measurements). The glycoprotein contents of the two membrane preparations were identical and resembled the staining patterns observed by Hoffman and McMahon (1978), Gilkes et al. (1979), and Toda et al. (1980). Although glycoproteins are generally believed to be surface-exposed proteins, not all were iodinated (see Table I). Steric hindrance by the carbohydrate moiety and a lack of accessible tyrosine residues are probably the primary causes for the absence of label in these proteins.

Transmembrane Proteins in Dictyostelium PM. Identification of potential transmembrane proteins was accomplished by analyzing the relative mobilities of radiolabeled proteins in cell-surface-labeled and cytoplasmic face labeled PM electrophoresed on the same gel. Additional transmembrane proteins were identified by comparing the glycoprotein staining pattern of iodinated PM with the resulting autoradiogram. Proteins which were labeled on the internal face of the PM and not on the cell surface, but were glycoproteins, were concluded to be transmembrane proteins.

The assignment of the PM (glyco)proteins to the cell-surface, transmembrane, or cytoplasm-exposed categories was based upon six comparative electrophoretic runs of surface and cytoplasm face-labeled membranes and comparisons of chloronaphthol-stained electroblots and the respective autoradiograms of four internal face-labeled PM.

Analysis of Intermolecular Disulfide Bonds in Dictyostelium PM. The presence of intermolecular disulfide bonds in isolated PM was analyzed by two-dimensional electrophoresis in which the disulfide bonds were left intact during the first electrophoretic separation (nonreducing conditions) and were reduced as the proteins were electrophoresed from the first gel through a DTE-containing agarose layer and into a second gel. The mobilities of protein complexes lacking intermolecular disulfide bonds were unchanged by the electrophoretic conditions and formed a diagonal in the second-dimension gel. However, disulfide bonds occurring between the subunits of a protein complex were reduced by DTE in the second-dimension gel. The resultant polypeptides migrated faster than

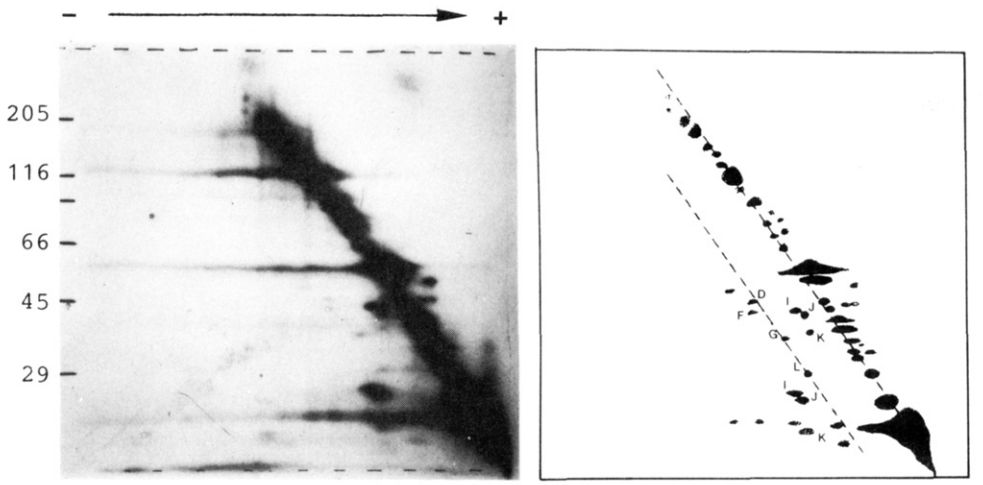

FIGURE 2: Autoradiogram and diagrammatic representation of the two-dimensional nonreduced/reduced gel obtained from cell-surface-labeled PM. Approximately 1×10^5 cpm were applied to the first-dimension gel. The arrow indicates the direction of electrophoresis for the first-dimension gel. The shapes of the spots were traced as they appeared on the X-ray film. The diagonal dotted line to the right represents the "true" diagonal, and the line to its left represents the imaginary line on which proteins which formed homodimers would fall. Labeled off-diagonal spots were referred to in Table II.

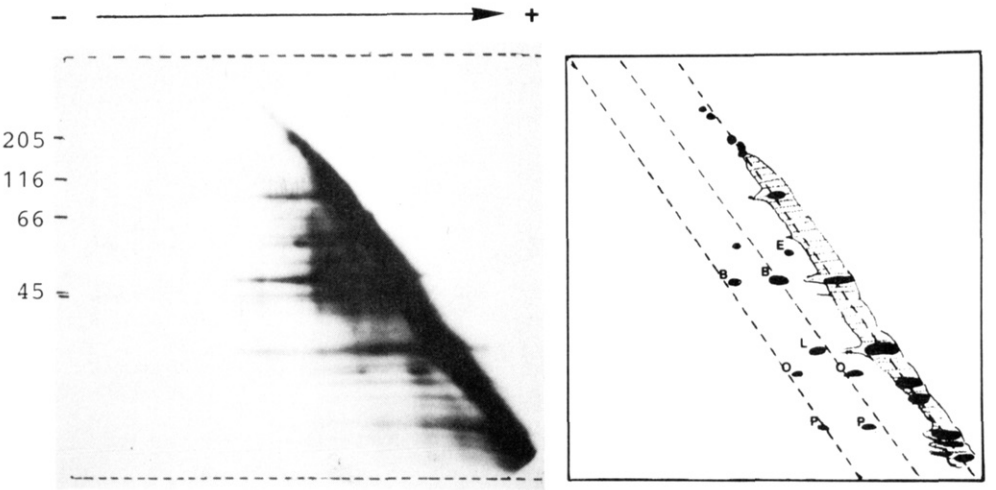

FIGURE 3: Autoradiogram and diagrammatic representation of the two-dimensional gel obtained from PM labeled on the cytoplasmic face. Approximately 2×10^5 cpm were applied to the first-dimension gel. The dotted line to the extreme left represents the imaginary line on which proteins forming homotetramers would fall. See Figure 2 for remaining explanations.

the intact complex and yielded one spot, in the case of homodimers, and two spots, in the case of heterodimers, below the diagonal. Extrapolation vertically to the diagonal yielded the molecular weight of the disulfide bond containing complex.

For the two-dimensional gel system described here, gradient gels for both dimensions were used. The resolution of off-diagonal spots was much better in the gradient gel system than that obtained on single percent composition gels [see Hynes and Destree (1977), Philips and Agin (1977), and Goding and Harris (1981)].

In order that the molecular weights of the disulfide bond containing complexes and their respective subunits could be determined as accurately as possible, the time at which the molecular weight standards should be applied to the second-dimension gel was determined. Chloramine T iodinated rabbit muscle actin was electrophoresed on a first-dimension gel. This gel lane was attached to a second-dimension gel as described under Materials and Methods. At various times after the start of the second electrophoretic run, iodinated actin was applied to premade wells in the second-dimension gel. The two actin samples migrated parallel to each other if the actin was applied to the well of the second-dimension gel when the bromphenol dye front had reached the border of the stacking and separation gels.

All membranes to be analyzed by two-dimensional nonreduced/reduced electrophoresis were treated with NEM to prevent disulfide bond–sulfhydryl group interchange. Control gels (nonreduced/nonreduced) were run of both types of labeled membranes; no off-diagonal spots were observed.

Figures 2 and 3 are representative autoradiograms of two-dimensional gels obtained from PM labeled on the outer surface and inner face, respectively. Figure 4 is an electroblot of PM proteins separated by two-dimensional electrophoresis and stained for Con A binding proteins. Next to the autoradiograms and electroblot are schematic diagrams. The main or true diagonal is drawn in; proteins containing no disulfide bonds fall on this line. A dotted line showing where proteins form homodimers is also shown. Many of the off-diagonal proteins fell on this line. Some off-diagonal proteins were apparently homotetramers since they fell on the dotted line drawn to indicate where such proteins would migrate.

Only those spots which appeared consistently were labeled on the diagrams. Proteins which have been identified with the same letter belong to the same disulfide bond containing

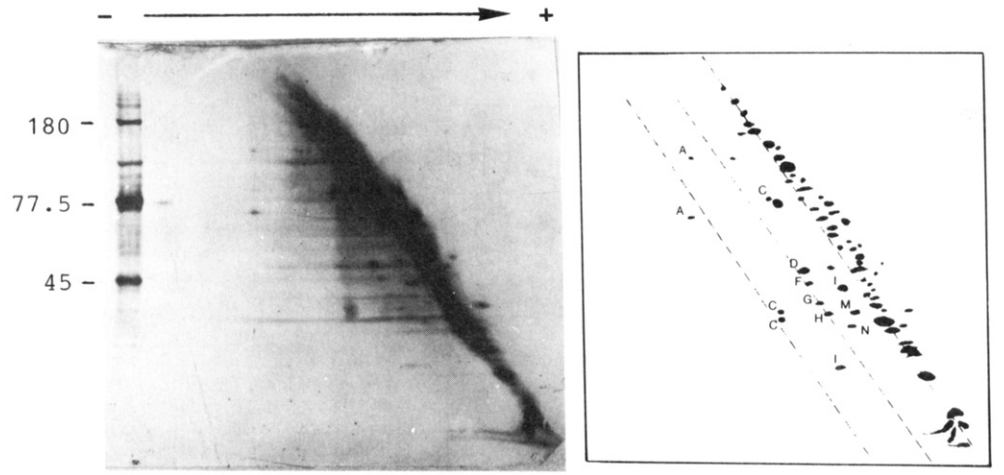

FIGURE 4: Electroblot and diagrammatic representation of the glycoprotein staining pattern after two-dimensional electrophoresis of isolated *Dictyostelium* PM. Approximately 120 μg of protein was applied to the first-dimension gel. See Figures 2 and 3 for remaining explanations.

complex, or if the same letter was used for a spot occurring in differently labeled or stained gels, it was the identical protein in both preparations. For example, in the case of spots D, F, G, and I, the autoradiograms of the chloronaphthol-stained two-dimensional electroblots of surface-labeled PM revealed that these glycoproteins were also radiolabeled. Spot L was concluded to be the same protein in surface- and internal face-labeled PM on the basis of the molecular weights of the intact complex and the subunit.

Two-dimensional gels were also run of labeled Con A treated membranes (data not shown). The presence of the lectin did not lead to any changes in the status of intermolecular disulfide bonding in either outside- or inside-labeled PM. The only change observed was the presence of a very thick band, attributable to the Con A subunit, extending to both sides of the true diagonal in cell-surface-labeled PM.

In preliminary chemical cross-linking experiments using the cleavable bis(imido ester) dimethyl 3,3′-dithiobis(propionimidate) for nearest-neighbor analysis (Wang & Richards, 1974; Ji, 1979), only two more off-diagonal glycoproteins were observed in PM isolated from both chemically cross-linked Con A treated and nontreated cells. Glycoproteins of M_r 115K and 68.8K formed cross-linked complexes of M_r 160K and 123K, respectively; the other subunit(s) in the complexes were not observed. This observation implied that the extent of aggregation of glycoproteins in *Dictyostelium* PM was not significantly altered by Con A treatment.

A summary of the intermolecular disulfide bonds occurring in *Dictyostelium* PM is presented in Table II.

Table II: *Dictyostelium* Plasma Membrane Proteins Containing Intermolecular Disulfide Bonds[a]

ID letter	M_r of complex ($\times 10^{-3}$)	M_r of subunits and labeling	stoichiometry and protein constituent from Table I
A	>322	135, GP NR	×2 GP_S 130
		74.0, GP NR	×2 GP_S 73.7
B	199	50.1, R_C	×4 P_C 50.2
	99.0	48.9, R_C	×2 P_C 50.2
C	141	80.8, GP NR	×1 GP_S 80.6
		32.0, GP NR	×1 GP ?
		29.2, GP NR	×1 GP ?
D	101	48.6, GP R_S	×2 GP_S 47.4
E	94.2	61.6, R_C	×1 P_C 66.7, X 27.5
F	88.3	42.9, GP R_S	×2 GP_S 43.1
G	76.6	35.7, GP R_S	×2 GP_S 36.4
H	63.9	32.3, GP NR	×2 GP ?
I	61.7	40.9, GP R_S	×1 GP ?
		18.7, GP R_SR_C	×1 GP_{TM} 20.7
J	59.5	39.5, R_S	×1 P_S 39.6
		18.0, R_S	×1 P ?
K	53.4	37.0, R_S	×1 P ?
		17.4, R_S	×1 P ?
L	51.6	27.2, R_SR_C	×2 P_{TM} 28.4
M	49.3	35.2, GP NR	×1 GP ?, X 14.0
N	48.2	30.4, GP NR	×1 GP ?, X 18.0
O	40.0	21.9, R_C	×2 P_C 23.4
	78.5	21.9, R_C	×4 P_C 23.4
P	26.1	13.0, R_C	×2 P_C 14.5
	58.9	13.0, R_C	×4 P_C 14.5

[a] Abbreviations: P, protein; GP, glycoprotein; NR, not radiolabeled; R_S, cell surface labeled; R_C, cytoplasm face labeled; ?, cannot determine corresponding protein in Table I; X, subunit not observed [approximate molecular weight ($\times 10^{-3}$) given].

Discussion

The general membrane labeling pattern obtained from cell-surface iodination of *Dictyostelium* amoeba resembled that reported by other works (Siu et al., 1976; Prem Das & Henderson, 1983b), but because a gradient separation gel was used, more labeled proteins could be discerned. Most of the heavily iodinated proteins on the cell surface were not major proteins of the PM on the basis of Coomassie blue staining; this had also been observed by Siu et al. (1976) and Prem Das and Henderson (1983b). The labeling pattern of the cytoplasmic face of the PM was distinct from that of cell-surface-labeled PM. As seen in Figure 1B, it appeared that cell-surface-bound Con A in cytoplasmic face labeled PM did not become iodinated. This observation indicated that the colloidal silica pellicle covering the outer membrane surface prevented the iodination of surface-exposed proteins. However, this point has been convincingly demonstrated by Chaney & Jacobson (1983; see Figure 10).

A summary and compilation of the *Dictyostelium* PM proteins observed as radiolabeled or as glycoproteins are presented in Table I. The proteins are listed in decreasing molecular weight with a prefix P for protein and GP for glycoprotein. The subscript TM indicates that the protein has been tentatively identified as a transmembrane protein. The subscripts S and C indicate, that on the basis of labeling and chloronaphthol-staining, these proteins have been identified as surface or cytoplasm-exposed proteins, respectively. Also, the relative labeling and glycoprotein staining intensities have been included to aid in the identification of these proteins in future studies. The most strongly labeled or stained polypeptides are indicated by four positive signs. Three, two, and one positive signs indicate successively weaker radiolabeling

or staining; a minus sign means that no labeling or staining was detected. The molecular weights reported here are not absolute since it has been observed that the molecular weights of glycoproteins and membrane proteins varied depending upon the gel composition and buffers used (Banker & Cotman, 1972; Segrest & Jackson, 1972; Chua et al., 1975; Frank & Robard, 1975).

Sixteen glycoproteins and two proteins have been identified as potential transmembrane proteins. Of the remaining radiolabeled proteins observed, 34 appeared to be cell-surface proteins, and 13 appeared to be cytoplasm-exposed proteins. Prem Das and Henderson (1983b) have analyzed their *Dictyostelium* PM preparations by two-dimensional isoelectric focusing/NaDodSO_4–PAGE; they observed 135 proteins in [^{35}S]methionine-labeled PM of early interphase cells. Sixty-five proteins or almost half of the total PM proteins seemed to be accessible to at least one of the membrane surfaces.

The degree of accuracy to which cytoplasm-exposed proteins could be identified was dependent upon the purity of the colloidal silica PM preparation. Contaminant proteins could become radioiodinated and, thus, be interpreted as being labeled PM proteins. Such contaminants were difficult to identify especially if they occurred consistently. In addition, some "cytoplasmic" proteins such as actin and myosin were known to be associated with the PM and were commonly coisolated with the membrane even though they were not truly a membrane component. To minimize the problem of nonspecific adsorption of proteins, the membranes were washed extensively, first at alkaline pH, then at moderate pH, and, finally at low ionic strengths. Furthermore, no change in the Coomassie blue staining pattern was observed after low ionic strength, EDTA extraction (Luna et al., 1981) of the colloidal silica PM (data not shown).

As already noted under Results, less than half of the glycoproteins were iodinated in PM isolated from labeled whole cells. Thus other proteins on both surfaces of the PM may also not have been labeled. Labeling cells and isolated PM with hydrophilic reagents such as the sulfonated Bolton–Hunter reagent may reveal other exposed proteins. Additionally, some of the putative transmembrane proteins may actually be two differently labeled proteins with very similar molecular weights. This ambiguity could be resolved by analysis of two-dimensional isoelectric focusing/NaDodSO_4–PAGE of the two types of labeled PM. As with all iodination procedures, the extent of labeling nonsurface integral membrane proteins could not be determined.

The function of most plasma membrane components of *Dictyostelium* was not known. On the basis of published labeling properties as well as relative molecular weights of known *Dictyostelium* PM proteins, a tentative assignment of functions to some proteins appearing in Table I has been made. It should be kept in mind that these assignments could only be confirmed by analysis with monospecific antibodies. The protein GP_{TM} 115 which was relatively heavily labeled from both sides of the PM and was a major glycoprotein may be contact site B (Beug et al., 1973). It has been proposed to be a phagocytotic receptor as well as a cell cohesion protein (Vogel et al., 1980; Chadwick et al., 1984) and has been found to be a glycoprotein and accessible to cell-surface labeling (Chadwick & Garrod, 1983; Chadwick et al., 1984). Plasma membrane of *Dictyostelium* also contains a 5′-AMP-specific nucleotidase/alkaline phosphatase which reportedly has a molecular weight of 120 000 and is also a glycoprotein (Armant & Rutherford, 1981). In our gel system, this protein would have a molecular weight of 110 000 since the molecular weights used for the standard proteins differed slightly. Thus, GP_S 110 may be this enzyme. P_C 43.2 which was observed only in cytoplasmic face labeled PM is probably radiolabeled actin. Sievers et al. (1978) have reported glycosyl transferase activities in the PM of vegetative *Dictyostelium* cells; nothing is known about the enzyme(s) itself.

Radiolabeled discoidin has also been immunologically identified on cell surfaces of axenically grown *Dictyostelium* cells (Siu et al., 1976). A surface-labeled protein of this molecular weight (approximately 26 000) was not observed in the PM preparations described here. Employment of more selective detection methods such as antibodies specific for the lectin or by elution from the labeled membranes (Springer et al., 1980) may resolve this discrepancy.

Several proteins have been suggested to be PM-associated actin binding proteins. Four of these (M_r 180K, 130K, 86K, and 77K) were probably transmembrane glycoproteins (Luna et al., 1984). A 24-kDa protein which required relatively rigorous treatment before becoming solubilized from the membrane (Stratford & Brown, 1985) and proteins having molecular weights of 31K and 17K (Schleicher et al., 1984) have also been proposed to be membrane-associated actin binding proteins. In Table I, the proteins having labeling characteristics and molecular weights similar to those of the above-mentioned PM actin binding proteins have been indicated.

The amount of intermolecular disulfide bonding in *Dictyostelium* PM (Table II) was comparable and, perhaps, even slightly higher than that observed in mammalian cells (Hynes & Destree, 1977; Phillips & Agin, 1977; Goding & Harris, 1981). Like the mammalian cell PM, the majority of the intermolecular disulfide bonds in *Dictyostelium* PM occurred between glycoproteins. Half of the complexes observed were homodimers; three of these complexes also formed homotetramers. The other complexes were primarily heterodimers. Only two of the intermolecular disulfide bond complexes could be positively identified as being transmembrane complexes (I and L).

On the basis of labeling and chloronaphthol-staining characteristics, the intermolecular disulfide bond forming polypeptides (Table II) were correlated to the proteins listed in Table I. In most cases, a match-up was feasible. Attempts to identify the unobserved subunits in some of these complexes by two-dimensional nonreduced/reduced gel analysis of totally iodinated PM were unsuccessful. PM solubilized with NaDodSO_4 and then iodinated with Iodobeads (Markwell, 1982) still contained unlabeled glycoproteins.

In Figures 2 and 4, some proteins can be seen that were just above the main diagonal. These spots have been attributed to proteins possessing intrachain disulfide bonds; most, if not all, of these proteins appeared to be glycoproteins. Wang and Richards (1974) and Philips and Agin (1977) have made similar observations in erythrocyte ghosts and platelet membranes, respectively.

The function of the intermolecular disulfide bond containing protein complexes is not known. The two transmembrane complexes could possibly function in transmembrane signalling for cytoskeletal interaction with the plasma membrane [see Brandts and Jacobson (1983)]. In fact, one of the proteins forming a transmembrane disulfide complex, P_{TM} 28.4 (complex L), is a possible actin binding protein (Schleicher et al., 1984; see Table I).

Preliminary data have already been published demonstrating the possibility of using plasma membrane isolated by the colloidal silica method for studying membrane asymmetry (Chaney & Jacobson, 1983). A more detailed analysis of the topography of *Dictyostelium discoideum* plasma membrane

has been undertaken. The level of purification of the original procedure has been increased 2-fold by changing the buffer in which the silica-coated cells were lysed and in which the crude plasma membrane was initially washed. Taking advantage of the shielding afforded by the silica/polymer pellicle surrounding the cell exterior, cytoplasm-exposed plasma membrane proteins were labeled by lactoperoxidase-catalyzed iodination and analyzed by $NaDodSO_4$–PAGE. In addition, glycoproteins and cell-surface and potential transmembrane proteins were identified, and the molecular weights were calculated. Another aspect of membrane topography was also investigated. Numerous glycoproteins were found to form intermolecular disulfide bonds; homodimers and homotetramers, heterodimers, and also a heterotrimer and heterotetramer were observed. Nearest-neighbor analysis using chemical cross-linkers on membranes isolated by the colloidal silica method has also been undertaken. The protein and lipid components on specific sides of the membrane can also be studied through labeling with fluorescent, radioisotopic, photoaffinity, and immunological probes, chemical modification, or proteolytic cleavage before or after isolation of the plasma membrane by this technique.

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