Isolation of Plasma Membrane and Analysis of Membrane Glycoproteins from the Slime Mould *Physarum polycephalum*

By TAKASHI KURODA, MASASHI MORITA, KAZUHISA OKAI,
SHOJI OKAMURA AND ARASUKE NISHI*

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani,
Toyama 930-01, Japan

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Plasma membranes were isolated from the acellular slime mould *Physarum polycephalum* by differential centrifugation and an aqueous two-phase polymer method. ATPase activity in the membrane fraction was optimal at pH 6.5 and was severely inhibited by vanadate but resistant to oligomycin. The protein components of the plasma membrane were analysed by polyacrylamide gel electrophoresis. Glycoproteins were located on Western blots by incubation of the sheets with lectin-peroxidase reagents. The results indicated that most of the membrane proteins were glycosylated. Pulse-chase experiments with [³H]glucosamine showed that when the plasmodia were induced to differentiate to macrocysts, turnover of the membrane glycoproteins occurred more rapidly than in growing plasmodia.

INTRODUCTION

Plasma membrane proteins and glycoproteins play important roles in the growth and development of many eukaryotic cells. The plasma membranes of micro-organisms, however, have received limited attention because they are difficult to isolate (Goffeau & Slayman, 1981). Attempts to isolate fungal plasma membranes have sometimes been hampered by the presence of mycelial wall on their surfaces. Plasmodia of *Physarum polycephalum* provide a useful tool in studying the role of plasma membrane proteins since they lack a rigid cell wall.

In previous papers (Okamura *et al.*, 1983; Morita *et al.*, 1986), we presented evidence showing that differentiation of the slime mould to macrocysts is accompanied by drastic changes in the surface structure of the plasmodium and the biosynthesis of *N*-glycosylated proteins was greatly depressed in encysting plasmodia. To obtain a better insight into the physiological events associated with the differentiation of *P. polycephalum*, we studied the changes in the surface proteins during macrocyst formation.

METHODS

Organism and cultivation. Microplasmodia of *P. polycephalum* were grown in a semi-defined medium containing glucose, yeast extract, tryptone and haemin (Daniel & Baldwin, 1964). The cultures were maintained in 100 ml of the medium and agitated on a reciprocal shaker at 26 °C. Macrocyst (spherule) formation was induced by transferring the plasmodia to a nutrient medium containing 0.5 M-mannitol (Chet & Rusch, 1969).

Plasma membrane preparation. Plasma membranes were prepared from growing and encysting plasmodia. Microplasmodia (2 d old) were washed with saline and suspended in Tris/HCl buffer (10 mM, pH 7.5) containing 2 mM-EDTA. The suspension was cooled in an ice bath and the plasmodia were ruptured with 10 strokes in a Potter-type glass/Teflon homogenizer. The homogenate was centrifuged at 5000 *g* for 10 min and the pellet was suspended in the Tris buffer containing 1 mM-MgCl₂. The plasma membrane fraction was sedimented by low-

Abbreviations: CBB, Coomassie brilliant blue; Con A, concanavalin A; endo H, endo- β -*N*-acetylglucosaminidase H; PNA, peanut agglutinin; WGA, wheat germ agglutinin.

speed centrifugation at 100 g for 5 min and washed with the same buffer several times until the supernatant became clear.

The plasma membrane fraction was further purified by an aqueous two-phase polymer system as described by Brunette & Till (1971) with some modifications. Stock solutions for the two-phase method were prepared as follows: 9.68 g Dextran T500 (Pharmacia) in 100 ml Tris/HCl/EDTA buffer (pH 7.5), 7 g polyethylene glycol 6000 (Wako Pure Chemical Company) in 50 ml of the same buffer and 33.32 ml of the buffer were mixed in a separating funnel and allowed to settle at 4 °C. The plasma membrane fraction obtained by the low-speed centrifugations was suspended in 10 ml of the top phase, then mixed with an equal volume of the bottom phase. The mixture was centrifuged at 10000 g for 10 min and the materials at the interphase were collected.

Enzyme assay. ATPases were assayed by the method of Serrano (1978). The reaction mixture contained 2 mM-ATP, 5 mM-MgCl₂, 50 mM-Tris/MES (pH 6.5) and 0.1 ml enzyme preparation in a total volume of 1 ml. The reaction was carried out at 30 °C for 30 min, and terminated by the addition of 2 ml of a solution containing 0.5% (w/v) ammonium molybdate, 0.5% (w/v) SDS and 2% (v/v) H₂SO₄. The released P_i was determined by measuring the absorption at 750 nm after the addition of 20 µl of 10% (w/v) ascorbic acid to the solution. Alkaline and acid phosphatases were assayed according to Loomis (1969) and van Etten & McTigue (1977), respectively. *p*-Nitrophenyl phosphate was used as a substrate. Protein content was determined by the Lowry method.

Nucleic acids. RNA and DNA were extracted by the method of Schmidt & Thannhauser (1945) and determined by measuring the absorption at 260 nm of the appropriate fractions. Yeast RNA and calf thymus DNA were used as standards.

Polyacrylamide gel electrophoresis (PAGE). Electrophoresis on SDS-polyacrylamide gels was done according to the method of Laemmli (1970). Samples containing about 50 µg protein were applied to a polyacrylamide slab gel. The following proteins were used as *M_r* markers: bovine serum albumin (66000), ovalbumin (45000), pepsin (34700), trypsinogen (24000), β -lactoglobulin (18400), and lysozyme (14300). Electrophoresis was done with constant current at 20 mA.

Gels were fixed and stained in a solution of 50% methanol, 10% acetic acid, 40% water and 0.1% Coomassie brilliant blue (CBB) for 1 h, and destained first in a solution consisting of 5% methanol, 7.5% acetic acid and 87.5% water for several hours, then with 5% methanol. In some experiments, proteins were detected by the silver stain method of Merril *et al.* (1981).

Glycoproteins on the SDS-PAGE were detected using lectin-peroxidase reagents. Concanavalin A (Con A) was obtained from Wako Pure Chemical Company, Osaka, and wheat germ agglutinin (WGA), peanut agglutinin (PNA) and horse-radish peroxidase were from Sigma. Staining of glycoproteins with Con A-peroxidase was done according to Faye & Chrispeels (1985). Proteins on a gel were transferred to a nitrocellulose sheet (Bio-Rad) and treated with Con A (25 µg ml⁻¹ in Tris buffered saline, pH 7.4, containing 1% gelatin and 1 mM of both MnCl₂ and CaCl₂) and subsequently with the peroxidase (50 µg ml⁻¹ in the same buffer). The sheet was then immersed in the staining solution [50 mg 3,3'-diaminobenzidine and 20 µl of 30% (v/v) H₂O₂ in 15 mM-phosphate buffer, pH 6.7] to visualize glycoprotein bands.

Detection of glycoproteins with WGA and PNA was carried out according to Kijimoto-Ochiai *et al.* (1985). These lectins were coupled with the peroxidase by the method of Nakane (1975) before the treatment. Glycoproteins immobilized on nitrocellulose sheets were reacted with these reagents and then with diaminobenzidine as above.

Removal of oligosaccharides from glycoproteins on nitrocellulose sheets by endo- β -N-acetylglucosaminidase H (endo H). Endo H was obtained from Seikagaku Kogyo Company, Tokyo. Nitrocellulose sheets blotted with proteins were treated with the glycosidase according to Faye & Chrispeels (1985). The sheets were incubated in a small plastic bag containing the enzyme (0.2 unit) in 2 ml of 0.1 M-citrate buffer (pH 6.0) supplemented with 1% gelatin at 37 °C for 48 h. After incubation, the sheets were washed with the buffer and the glycoproteins were visualized as above.

Isotope labelling of membrane glycoproteins. For pulse-chase experiments, glycoproteins were labelled with [³H]-glucosamine [New England Nuclear, 44.8 Ci mmol⁻¹ (1.66 T Bq mmol⁻¹)]. Two-d-old plasmodia were pre-incubated in the basal medium without glucose for 5 h, and then incubated with [³H]glucosamine (50 µCi ml⁻¹) for 1 h. The plasmodia were washed and divided into three equal parts. One part was immediately homogenized to prepare plasma membrane and the other two parts were cultured in the ordinary growth medium and in the differentiation medium containing 0.5 M-mannitol, respectively, for a further 4 h. Plasma membranes were prepared from these samples and separated by SDS-gel electrophoresis as above. The slab gel was cut into pieces 2 mm long and each piece was solubilized in 0.5 ml Protosol (New England Nuclear). Radioactivity was measured by a liquid scintillation spectrometer using a commercial scintillation liquid, ACS II (Amersham).

Electron microscopy. Plasma membrane preparations were fixed with 1% glutaraldehyde, then post-fixed in 1% osmium tetroxide. The specimens were embedded in 1.5% (w/v) agar and stained with 0.5% (w/v) uranyl acetate as described previously (Okamura *et al.*, 1983). Dehydrated samples were embedded in Epon 812. Thin sections were double stained with 2% uranyl acetate and 80 mM-lead citrate, and examined in a JEOL JEM 7A electron microscope.

Fig. 1. (a) Fraction of 100p viewed under a phase-contrast microscope. Microplasmodia of *P. polycephalum* were homogenized and the precipitate obtained by low-speed-centrifugation (100 g, 5 min) was denoted as the 100p fraction. Bar, 100 μ m. (b) Electron micrograph of plasma membrane fraction. Plasma membranes were prepared as described in Methods. Bar, 1 μ m.

RESULTS

Isolation of plasma membrane

The pellet obtained after low-speed-centrifugation (100 g, 5 min) of plasmodial homogenate in the presence of MgCl_2 contained plasma membrane ghosts (fraction 100p). When viewed under phase contrast microscopy, they appeared as sheets of material of more than 100 μ m in size (Fig. 1a). On the other hand, supernatant of the homogenate contained smaller particles and soluble proteins (fraction 100s). To test the relative enrichment of plasma membrane in the 100p fraction, ATPase activity was determined in the presence of inhibitors. Membrane-associated ATPase in several fungi is inhibited by vanadate but resistant to oligomycin and azide (Goffeau & Slayman, 1981). The results (Fig. 2) indicated that the ATPase in the 100p fraction showed the highest activity at slightly acidic pH and was resistant to oligomycin treatment. On the other hand, in the 100s fraction, the enzyme activity was very high at about pH 9 and markedly decreased when the assay mixture contained oligomycin. These properties of the 100p and 100s ATPases are considered to be characteristic of plasma membrane and mitochondrial enzymes, respectively, in many other organisms.

Besides ATPase, alkaline phosphatase, which was co-purified with the plasma membrane ATPase in *Dictyostelium discoideum* (Serrano *et al.*, 1985), was also enriched about sixfold in the 100p fraction. On the other hand, the specific activity of acid phosphatase (lysosomal enzyme) decreased to about 40% of that of the whole homogenate. The 100p fraction contained approximately 2.2% and 1.4% of total DNA and RNA, respectively, indicating that contamination of cytoplasmic factors was very low. Plasma membranes were further purified by an aqueous two-polymer phase method. ATPase activities in these purification steps are shown in Table 1.

Based on these observations, we devised a purification procedure for the plasma membrane of *P. polycephalum* as described in Methods. Transmission electron microscopy revealed membrane profiles as shown in Fig. 1(b).

Fig. 2

Fig. 3

Fig. 2. ATPase activities in 100s and 100p fractions in the presence (○) or absence (●) of oligomycin ($20 \mu\text{g ml}^{-1}$). Microplasmodia of *P. polycephalum* were homogenized and a membrane-rich fraction (100p fraction) was obtained by low-speed-centrifugation ($100 g$, 5 min). The unsedimented fraction was denoted as the 100s fraction. The reaction was carried out at 30°C for 30 min.

Fig. 3. ATPase activity in the plasma membrane fraction of *P. polycephalum* in the presence [1 mM -azide (○); $50 \mu\text{M}$ -vanadate (Δ)] or absence (●) of inhibitors. Plasma membranes were prepared as described in Methods.

Table 1. *Specific activity of ATPase during purification of plasma membranes.*

Plasma membranes were prepared from 2-d-old plasmodial culture. The identities of the fractions are given in the text. Data from a single representative experiment are shown. Repeated experiments gave similar results.

	ATPase activity [$\mu\text{mol min}^{-1}$ ($\text{mg protein})^{-1}$]		
	pH 6.5	pH 8.0	pH 6.5/pH 9.0
Whole homogenate	0.026	0.202	0.13
100p fraction	0.115	0.059	1.95
Plasma membrane fraction	0.104	0.027	3.86

Fig. 3 shows the ATPase activity in the purified membrane fraction. ATPase in the plasma membrane exhibited the highest activity at pH 6.5 but the activity at pH 9.0 was very low. The enzyme activity was little affected by the presence of oligomycin and the optimal pH of 6.5 was also unchanged. On the other hand, sodium vanadate strongly inhibited the enzyme activity. These results suggest that the preparation was considerably enriched in plasma membrane and the contamination with mitochondria was low.

Gel electrophoresis of plasma membrane proteins

Proteins of the plasma membrane were separated by SDS-PAGE. The CBB stained profiles of membrane and whole-plasmodial proteins are shown in Fig. 4. Among the proteins discernible on the gel, a protein band at a position of M_r 38000 was most prominent. We sometimes observed another intense band at about M_r 42000 when EDTA was omitted from the homogenizing buffer. Occurrence of this band was, however, not reproducible and easily removed by treatments such as with deoxycholate. This protein seems to be very weakly associated with the plasma membrane. A protein at M_r 45000 in whole homogenate was

Fig. 4. Electrophoretograms of proteins in whole homogenate (lane A) and plasma membrane fraction (lane B) in *P. polycephalum*. Plasma membranes were obtained as described in Methods. Each sample contained about 50 μ g of proteins. Following electrophoresis the gel was stained with CBB. Numbers on the right indicate approximate M_r values calculated from the relative mobility of standard proteins (Methods).

Fig. 5. Detection of *N*-glycoproteins by the lectin-peroxidase method. Plasma membrane protein (about 50 μ g each) was separated by SDS-PAGE and transferred to a nitrocellulose sheet which was treated with lectin and peroxidase. Lane A, Protein bands visualized by the silver stain method; lanes B, C and D, glycoproteins detected by Con A, WGA and PNA, respectively.

probably actin because it reacted with a commercial actin antibody (mouse monoclonal antibody, Amersham).

Fig. 5 shows a gel-electrophoretogram of glycoproteins stained with peroxidase-coupled lectins. Many protein bands reacted with these lectin reagents, indicating that most of the plasma membrane proteins were glycosylated. Protein bands at M_r 80000 and 75000 were only lightly stained by CBB or Ag were intensely stained by Con A and WGA reagents. On the other hand, a protein band at M_r 38000 was predominant in a CBB or Ag stained gel but showed a very low affinity for Con A.

The oligosaccharide chains in these glycoproteins were characterized further by treating them with endo H. The enzyme cleaves high-mannose type oligosaccharide chains from glycoproteins. Fig. 6 shows that the treatment removed the oligosaccharide moiety from most of the Con A-reacting glycoproteins. A glycoprotein band of M_r 90000 was unaffected by this enzyme.

Fig. 6

Fig. 7

Fig. 6. Effect of endo- β -*N*-glycosidase H on glycoproteins. Proteins were separated by gel electrophoresis and transferred to a nitrocellulose sheet. The sheet was treated with endo H as described in Methods. Lane A, untreated control; lane B, endo H treated.

Fig. 7. Changes in plasma membrane proteins during macrocyst formation (spherulation). Vegetative plasmodia (2 d old) were transferred to a differentiation medium containing 0.5 M-mannitol. Plasma membranes were prepared at 0 h (lane A), 3 h (lane B), 6 h (lane C) and 12 h (lane D). Proteins (50 μ g each) were separated by gel electrophoresis and detected by the silver staining method.

Changes in membrane glycoproteins during macrocyst formation

When the plasmodia of *P. polycephalum* encounter unfavourable conditions, they differentiate into a dormant form called a macrocyst or spherule. In this experiment, macrocyst formation was induced by transferring the plasmodia to 0.5 M-mannitol medium. Plasma membranes were prepared from the plasmodia undergoing the differentiation to macrocysts and analysed for their glycoprotein composition by gel electrophoresis (Fig. 7). During the first 12 h of development, the overall profile of the electrophoretogram was unchanged.

In the next experiment, these glycoproteins were metabolically labelled with [3 H]glucosamine for 1 h, then chased either in growth medium or in differentiation medium for a further 4 h (Fig. 8). The result indicates that the radioactivity in the glycoproteins was generally decreased during the chase period. In the encysting plasmodia, however, the decrease in the radioactivity was more rapid than in growing plasmodia. The total radioactivity in the membrane fraction decreased to about 70% of the initial level in growing plasmodia and to about 40% in encysting plasmodia, respectively.

Fig. 8. SDS-PAGE of plasma membrane proteins labelled with [^3H]glucosamine. Following electrophoresis, the gel was cut into pieces 2 mm long to determine the radioactivity. (a) Vegetative plasmodia incubated with [^3H]glucosamine for 1 h. (b, c) The radiolabelled plasmodia were chased for 4 h in the growth medium (b) and the differentiation medium, (c) respectively. Repeated experiments gave similar results.

DISCUSSION

Enzymes such as 5'-nucleotidase, phosphodiesterase and alkaline phosphatase have been used widely as marker enzymes for plasma membrane. Aldrich & Reiskind (1982), however, concluded from cytochemical studies that none of these enzymes is suitable as a cytochemical marker in *P. polycephalum* myxamoebae. Studies on yeast cells suggest that chitin synthase is localized in plasma membranes (Hubbard *et al.*, 1986), but the slime mould does not possess a chitin-containing cell wall.

Recent investigations indicated that the plasma membranes of many fungi contain the ATPase which functions as a proton pump (Goffeau & Slayman, 1981). These membrane ATPases are clearly distinct from mitochondrial ATPase (Delhez *et al.*, 1977; Bowman *et al.*, 1981; Hubbard *et al.*, 1986). The mitochondrial ATPase, with optimal activity at pH 9.0, is sensitive to oligomycin while the membrane associated enzyme, with optimal activity at pH 6.0–6.5, is insensitive to this drug but is strongly inhibited by vanadate. We considered that the ATPase is the most reliable marker for the plasma membrane of *P. polycephalum*.

Conventional sucrose density gradient centrifugation using either linear or discontinuous gradients was not effective in separating the plasma membrane of the slime mould (data not shown). The ATPase activity occurred always at the bottom fraction and the pH 9 ATPase overlapped with the pH 6.5 ATPase activity under the various experimental conditions so far tested.

In the case of *P. polycephalum*, we found that gentle homogenization of the plasmodia in the presence of Mg^{2+} yielded relatively large sheets of membranous materials which could be separated from other small vesicles or vacuoles by low-speed centrifugation. The role of Mg^{2+} is not clear but it may stabilize the plasma membrane against fragmentation and vesiculation. The addition of Ca^{2+} or Zn^{2+} in place of Mg^{2+} was not effective in yielding large sheets of membrane.

Barden *et al.* (1983) and Reiskind & Aldrich (1984) have isolated plasma membranes of *P. polycephalum* myxamoebae and analysed membrane proteins by SDS-PAGE. Our present results show that plasmodial membrane proteins of *P. polycephalum* are different both qualitatively and quantitatively from those of myxamoebae. Con A interacts with glycoconju-

gates which have at least two non-substituted or 2-*o*-substituted α -mannosyl residues (Ogata *et al.*, 1975). Treatment of glycoproteins on nitrocellulose sheet by the Con A-peroxidase method, therefore, revealed the existence of high-mannose, hybrid and complex biantennary-type oligosaccharides (Kijimoto-Ochiai, 1985). On the other hand, WGA can react with *N*-acetylglucosamine and sialic acid and, therefore, bind to hybrid- and complex-types of oligosaccharide chains. Glycoprotein bands at M_r 120000, 38000 and 23000 reacted with WGA but barely with Con A suggesting that these bands may contain complex type *N*-linked oligosaccharides. Glycoprotein bands which reacted with PNA possibly contain galactose residues in their oligosaccharide moiety.

Faye & Chrispeels (1985) have shown that the treatment of glycoproteins on a nitrocellulose sheet by endo H removes high-mannose type oligosaccharides but that complex type sugar chains are generally resistant to the enzyme. Most of the *Physarum* glycoproteins that reacted with Con A were sensitive to the endo H treatment but a glycoprotein band at M_r 90000 was resistant. This band, however, was stainable with Con A even in the presence of methyl- α -D-mannose suggesting that non-specific binding of Con A is involved in the reaction.

Bernier *et al.*, (1986) have found that *Physarum* produces spherule specific mRNAs and the major changes in mRNA population occurred after 24 h of starvation. We followed the changes in membrane proteins during the initial 12 h of spherule formation. In this time, the spherules completed cytoplasmic cleavage and commenced cell wall formation, after which the isolation of plasma membrane was difficult. Although no significant alteration in membrane protein was observed during this early stage of differentiation, results of the pulse-chase experiment on membrane glycoproteins suggested that certain changes in glycoprotein metabolism occurred immediately after the induction of spherule formation.

Membrane glycoproteins were rapidly labelled by the addition of [3 H]glucosamine to the medium. Radioactivity distribution on an SDS-PAGE was coincident with a WGA-stained profile of glycoproteins. When these labelled plasmodia were transferred to a non-radioactive growth medium, the radioactivity in the membrane glycoproteins decreased gradually indicating that the glycoproteins in the plasma membrane turn over continuously during growth. On the other hand, in the plasmodia chased in the differentiation medium, the decrease in the radioactivity in the glycoproteins was very rapid. In a previous paper (Morita *et al.*, 1986), we reported that macrocyst formation of *Physarum* was appreciably accelerated by the presence of tunicamycin which is a potent inhibitor of *N*-linked glycoprotein biosynthesis. We also found that the synthesis of the lipid-oligosaccharide intermediate of glycoprotein was greatly depressed when the plasmodia underwent the differentiation process of spherule formation and suggested that most of the glycoproteins in growing plasmodial membrane are specific to the vegetative state and are not required in the spherule. The present experimental results, showing that the surface glycoproteins of *P. polycephalum* were rapidly degraded in encysting plasmodia, are consistent with the previous view. These surface proteins will be gradually replaced by new, spherule-specific membrane proteins during the later stage of differentiation.

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