Evidence for a Cytosol Counterpart of the Major Plasma Membrane Protein in *Acanthamoeba castellanii*

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SUMMARY

Isolated plasma membranes from *Acanthamoeba castellanii* incorporated radioactivity when incubated in a corresponding labelled cytosol fraction. The incorporation increased linearly with time and reached saturation at high cytosol: membrane protein ratios in the incubation mixture. Polyacrylamide gel electrophoresis of treated membranes showed that the major protein, which comprised 40 to 50 % of the total, was labelled, suggesting that it had exchanged with a radioactive cytosol counterpart. The same protein was discernible in polypeptide profiles of labelled cytosol and showed a higher specific radioactivity in the cytosol than in treated membranes. Inasmuch as it was not detached from the membrane by solutions of high ionic strength or chelation, this protein appears to be integral rather than peripheral.

INTRODUCTION

The plasma membrane of *Acanthamoeba castellanii* apparently lacks specialized transport sites since the amoebae depend solely on phagocytosis and pinocytosis to accumulate nutrients (Bowers & Olszewski, 1972). A major consequence of this nutritional dependence on endocytosis is the expenditure of large amounts of surface membrane which must ultimately be replaced. This appears to involve turnover of plasma membrane at two levels: cycling of membrane which enables replacement of expended surface membrane without *de novo* synthesis; and turnover at the molecular level involving input of newly synthesized material (Goodall, Lai & Thompson, 1972). In this study we have investigated the nature of plasma membrane turnover in *A. castellanii* by examining *in vitro* the molecular interaction between plasmalemma and cytosol.

METHODS

Plasma membrane isolation. Purified plasma membranes were isolated as described by Schultz & Thompson (1969). This procedure has since been shown to compare favourably with an alternative method developed by Ulsamer *et al.* (1971) for *Acanthamoeba*. Cultures to be used for plasma membrane isolation were grown in 2 l Erlenmeyer flasks containing 1 l optimal growth medium; 6 to 8 l were used for a single preparation.

Treatment of membranes with labelled cytosol. Isolated plasma membranes were treated with ^{14}C -labelled or ^{125}I -labelled cytosol to determine whether some of the membrane proteins have soluble cytoplasmic counterparts with which they can exchange. To obtain ^{14}C -labelled cytosol, amoebae were grown for 48 h in two 250 ml Erlenmeyer flasks containing 100 ml optimal growth medium and 50 μCi of a ^{14}C -labelled algal hydrolysate amino-acid mixture (specific activity 57 to 60 mCi per matom carbon; Amersham/Searle,

Arlington Heights, Illinois, U.S.A.). The amoebae were harvested by centrifuging at 800 *g* for 10 min, washed in 0.1 M-phosphate buffer pH 7.5 and homogenized in 5 ml 1 mM-NaHCO₃ pH 7.4 with 50 strokes of a Potter-Elvehjem homogenizer (0.15 to 0.23 mm clearance, rotating at 1700 rev. min⁻¹). The homogenate was diluted to 10 ml with the NaHCO₃ solution and centrifuged at 140 000 *g* for 3 h to yield cytosol and a pellet. The cytosol was collected with a syringe.

In other experiments, the cytosol was labelled with ¹²⁵I after isolation using lactoperoxidase (Marchalonis, 1969; Marchalonis, Cone & Santer, 1971). The iodination reaction mixture comprised (in a total volume of 7.3 ml): 6 ml cytosol fraction; 720 nmol KI; 9 μmol H₂O₂; 55 i.u. lactoperoxidase (Calbiochem); and 780 μCi ¹²⁵I (specific activity > 14 mCi per μg I; Amersham/Searle). The reaction reached equilibrium after about 5 min, and was stopped after 20 min by adding 0.15 ml 83 mM-cysteine solution. Parallel controls were also run in which cysteine was present throughout to inhibit any iodination of protein.

Isolated plasma membranes were treated with either ¹⁴C-labelled or ¹²⁵I-labelled cytosol for 15 min at 30 °C in 0.05 M-Tris/HCl pH 7.5. The cytosol fraction and a mixture of Tris and plasma membranes were equilibrated separately for 10 min at 30 °C before being combined. Total reaction volumes and concentrations of membrane and cytosol for each experiment are specified in the figure legends. After incubation the mixture was diluted with an equal volume of ice-cold 1 mM-NaHCO₃ pH 7.4 and centrifuged at 48 000 *g* for 30 min. The pellet was resuspended in an equal volume of NaHCO₃, centrifuged at 48 000 *g* for 15 min and resuspended again in the same volume of NaHCO₃. This suspension was twice frozen in dry ice/acetone and thawed before a final centrifugation at 48 000 *g* for 15 min.

Radioactivity measurements and gel electrophoresis. Membranes treated with ¹⁴C-labelled cytosol were counted directly. The final pellet of washed membranes was resuspended in 1 to 2 ml 1 mM-NaHCO₃ pH 7.4 and 100 μl of this suspension was mixed with 0.5 ml of NCS tissue solubilizer in a scintillation vial. Ten ml scintillation cocktail (7 g PPO per l toluene) and 50 μl ascorbic acid (4 %, w/v) were added. The samples were stored in the dark for 24 to 48 h and then counted with a Beckman LS-3 scintillation counter. In some experiments, membranes which had been treated with ¹⁴C-labelled cytosol were lipid-extracted with chloroform/methanol (2:1) as previously described (Korn & Wright, 1973) to determine the distribution of ¹⁴C between lipid and non-lipid components.

Membranes treated with ¹²⁵I-labelled cytosol were solubilized and electrophoresed on 10 % gels as previously described (Wilkins & Thompson, 1974) except that 90 to 100 μg protein was applied to the gels. After staining with Coomassie blue and scanning at 570 nm, the gels were sliced into 1 mm sections and counted in a Nuclear Chicago gamma counter. The protein stain did not interfere with the gamma counting so it was possible to get a protein scan and a radioactivity profile for the same gel. Polypeptide and radioactivity profiles were obtained for ¹²⁵I-labelled cytosol in the same manner.

Treatment of membranes with KCl and EDTA/mercaptoethanol. The isolated membranes were treated with either 3 M-KCl in 5 mM-Tris/HCl pH 7.4 or with a solution of 5 mM-EDTA and 1 mM-β-mercaptoethanol, pH 7.4, in an attempt to detach peripheral proteins. The treatment procedure described by Nicolson, Marchesi & Singer (1971) was used, and the effects of the treatment were determined by electrophoresis of the membranes.

Enzyme and protein assays. Lactate dehydrogenase (EC 1.1.1.27) was assayed as described by Kolb, Fleischer & Larner (1970) except that 0.15 % (w/v) cholic acid was included in the assay mixture. Enzyme, buffer and cholic acid were pre-incubated for 30 min at room temperature before the reaction was started by adding NADH and pyruvate. This treatment makes membrane vesicles leaky (Archakov *et al.*, 1974), and thus eliminates latent

Fig. 1. Changes in the specific radioactivity of plasma membrane incubated in mixtures containing different ratios of ^{14}C -labelled cytosol protein to membrane protein. The main profile and the inset illustrate separate experiments. Each point is an average of duplicates in which $400\text{ }\mu\text{g}$ membrane protein was treated for 15 min with sufficient cytosol to give the specified membrane protein:cytosol protein ratio in 5 ml total vol.

enzyme activity which might otherwise not be detected. Measurements of lactate dehydrogenase in treated and untreated cytosol fraction where latency is not a problem showed that cholic acid has no effect on the activity of the enzyme *per se*.

Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Plasma membranes incubated for 15 min in ^{14}C -labelled cytosol incorporated increasing amounts of radioactivity as the ratio of cytosol protein to membrane protein in the incubation mixture increased (Fig. 1). Radioactivity profiles consistently showed a saturation plateau at values for the ratio ranging from 10 to 12, but the specific radioactivities of the treated membrane at saturation varied among experiments. This variation presumably reflects differences in the specific radioactivity of the isolated cytosol fraction from experiment to experiment. The incorporation of ^{14}C radioactivity into the membrane fragments also increased linearly as a function of time over a period of at least 80 min under conditions in which the cytosol protein:membrane protein ratio was 18:1.

The ^{14}C -labelled cytosol fraction was obtained by administering ^{14}C -labelled amino acids to the growing amoebae, a procedure which labels protein primarily, but also other macromolecules including lipid. A major proportion (62 to 92 %), but not all, of the radioactivity incorporated into plasma membranes during their incubation in ^{14}C -labelled cytosol was associated with non-lipid material. The variation in the proportion derives from adhesion of particulate material to the surfaces of the vessels used during the extraction procedure, which could not be removed by repeated rinsing with chloroform/methanol. Although the vessels were scraped, recovery was still incomplete, and this resulted in variation and presumably an underestimation of the radioactivity associated with non-lipid membrane components.

Fig. 2. Polyacrylamide gel electrophoresis scans and radioactivity profiles for plasma membrane and cytosol. (a) Membrane treated with ^{125}I -labelled cytosol: 1.28 mg membrane protein was treated for 15 min with 20.4 mg cytosol protein in 13.5 ml total vol. —, E_{570} ; ●—●, ^{125}I radioactivity (c.p.m.) per gel slice; ×, ^{125}I radioactivity (c.p.m.) per gel slice for a control in which the membrane was treated with cytosol containing cysteine throughout the iodination reaction. (b) Iodinated cytosol. —, E_{570} ; ●—●, ^{125}I -radioactivity (c.p.m.) per gel slice. ····, E_{570} for untreated plasma membrane, included for comparison.

The saturation effect at high cytosol: membrane protein ratios, and the time dependence of the radioactivity incorporation, suggest some form of specific interaction between plasma membrane and the cytosol. The nature of this specificity with respect to protein was examined by electrophoresing cytosol-treated membrane and comparing patterns of radioactivity and protein distribution along the length of the gels. The membranes were incubated in iodinated cytosol rather than ^{14}C -labelled cytosol to obtain higher specific activities for determination of radioactivity in the gel slices. The protein banding patterns for treated and untreated membrane were similar in that at least 50 % of the total membrane protein ran as a single low molecular weight polypeptide with a R_F value of 0.77 to 0.79 (Fig. 2a). The labelling of this protein in membranes treated with iodinated cytosol (Fig. 2a), implied that it had exchanged with a radioactive cytosol counterpart. In fact all the protein bands visible in the spectrophotometric scans for treated membranes appeared to be labelled. However, these

Table 1. *Lactate dehydrogenase activity of homogenates, cytosol and plasma membrane fractions*

The results of three experiments are given. Activities are expressed as nmol NADH oxidized h^{-1} (mg protein) $^{-1}$.

Homogenate	Cytosol	Plasma membrane	
		Unwashed	Washed
1140	2160	68.4	13.2
1200	—	23.4	21.6
—	1860	57.6	18.0

other proteins were present in much lower relative proportions, and the correspondence between protein and radioactivity was definitive only for the major polypeptide. This major protein was not detached by 3 M-KCl or 5 mM-EDTA/1 mM- β -mercaptoethanol, indicating that it is an integral rather than peripheral protein. Treated membranes were washed thoroughly to remove cytosol contaminants by a procedure that included two successive freeze-thaw treatments. This washing reduced the specific activity of lactate dehydrogenase, a marker for cytosol, to less than 2 % of that in homogenates and cytosol fractions (Table 1). Thus, contamination of the treated membrane by cytosol components presumably accounted for only minimal radioactivity.

Notwithstanding its integral character, the predominant protein of *Acanthamoeba* plasmalemma appears to have a soluble counterpart with which it can exchange, since it becomes labelled when isolated membrane is incubated in ^{125}I -labelled cytosol. This interpretation is strengthened by the observation that the same protein (R_f 0.77) is present in the polypeptide profile of iodinated cytosol and appears to have a higher specific radioactivity in the cytosol than in treated membrane (compare Figs 2*a* and *b*). Clearly the cytosol counterpart represents only a small proportion of the total soluble protein, but it registers distinctively and reproducibly in the profiles of cytosol protein and its relative mobility and molecular weight (approx. 15000 daltons) coincide with those of the major plasma membrane protein. This protein is a well established component of *Acanthamoeba* plasma membrane and the prospect of it being a proteolytic artefact has been ruled out (Korn & Wright, 1973; Wilkins & Thompson, 1974). Moreover, the presence of a corresponding protein with the same relative mobility and molecular weight in polypeptide profiles of homogenates and cytosol fractions from the amoebae has been noted previously, and again its presence in the homogenate cannot be attributed to proteolytic artefact since the protein banding patterns of homogenates taken before and after a 6 h incubation at 30 °C are indistinguishable (Korn & Wright, 1973).

The nutritional dependence of *Acanthamoeba* on endocytosis decrees that large amounts of plasma membrane be routinely turned over. Yet, Goodall *et al.* (1972) observed that incorporation of labelled precursors into the plasmalemma occurred to the same extent in both phagocytosing and non-phagocytosing amoebae. This was thought to reflect molecular turnover occurring independently of phagocytosis which may be necessary for routine maintenance of the membrane. This study supports the feasibility of molecular turnover by showing that at least the major protein of the plasma membrane has a cytosol counterpart despite its apparent integral character.

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REFERENCES

- ARCHAKOV, A. I., BACHMANOVA, G. I., DEVICHENSKY, V. M., KARUZINA, I. I., ZHEREBKOVA, N. S., ALIMOV, G. A., KUZNETSOVA, G. P. & KARYAKIN, A. V. (1974). The reconstitution of microsomal redox chains. A comparative analysis of the effectiveness of membrane self-assembly and template binding of electron carriers. *Biochemical Journal* **144**, 1-9.
- BOWERS, B. & OLSZEWSKI, T. E. (1972). Pinocytosis in *Acanthamoeba castellanii*. *Journal of Cell Biology* **53**, 681-694.
- GOODALL, R. J., LAI, Y. F. & THOMPSON, J. E. (1972). Turnover of plasma membrane during phagocytosis. *Journal of Cell Science* **11**, 569-579.
- KOLB, E., FLEISCHER, G. A. & LARNER, J. (1970). Isolation and characterization of bovine lactate dehydrogenase. *Biochemistry* **9**, 4372-4380.
- KORN, E. D. & WRIGHT, P. L. (1973). Macromolecular composition of an amoeba plasma membrane. *Journal of Biological Chemistry* **248**, 439-447.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MARCHALONIS, J. J. (1969). An enzymatic method for the trace iodination of immunoglobulins and other proteins. *Biochemical Journal* **113**, 299-305.
- MARCHALONIS, J. J., CONE, R. E. & SANTER, V. (1971). Enzymatic iodination - a probe for accessible surface proteins of normal and neoplastic lymphocytes. *Biochemical Journal* **124**, 921-927.
- NICOLSON, G. L., MARCHESI, V. T. & SINGER, S. J. (1971). The localization of spectrin on the inner surface of human red blood cell membranes by ferritin-conjugated antibodies. *Journal of Cell Biology* **51**, 265-272.
- SCHULTZ, T. M. G. & THOMPSON, J. E. (1969). Enrichment of 5'-nucleotidase in membrane fragments isolated from *Acanthamoeba* sp. *Biochimica et biophysica acta* **193**, 203-211.
- ULSAMER, A. G., WRIGHT, P. L., WETZEL, M. G. & KORN, E. D. (1971). Plasma and phagosome membranes of *Acanthamoeba castellanii*. *Journal of Cell Biology* **51**, 193-215.
- WILKINS, J. A. & THOMPSON, J. E. (1974). The effects of cell population density on the plasma membrane of *Acanthamoeba castellanii*. *Experimental Cell Research* **89**, 143-153.