Identification of a plasma membrane protein involved in P_i transport in the yeast *Candida tropicalis*

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A plasma membrane protein was found to contain antigenic determinants in common with a P_i-binding protein released by osmotic shock from *Candida tropicalis*. This plasma membrane protein (M.P. 30) has a molecular mass of 30 kDa as measured by SDS–PAGE and anti-M.P. 30 antibodies inhibit P_i uptake in protoplasts while only the corresponding Fab fragments inhibit P_i transport in whole cells. This plasma membrane protein may be the P_i plasma membrane carrier.

Yeast *Plasma membrane protein* *Antibody* *P_i carrier*

1. INTRODUCTION

High-affinity P_i transport activity in *Candida tropicalis* is drastically reduced by osmotic shock [1]. Characterization and partial purification of two P_i-binding proteins (P_i-binding protein 1 and P_i-binding protein 2) released by osmotic shock have been reported [2]. The inhibitory effect of antibodies raised against each of these proteins suggests that P_i-binding protein 2 (P_iBP2) is involved in P_i uptake at physiological pH values [3]. The release of this protein during protoplast formation is accompanied by a marked change in the K_m values of P_i uptake increasing from 5–6 μM in whole cells to 400–500 μM in protoplasts [4]. The high-affinity P_i transport system is thought to be composed of a P_i-binding protein (located near the cell surface within the cell wall) and a P_i-specific plasma membrane carrier. Unpublished data showed that antigenic determinants recognized by anti-P_iBP2 antibodies were present at a very low level in protoplasts. We report here the isolation by an immunoadsorbent column of two membrane-bound proteins bearing the same antigenic determinant(s) as P_iBP2. One has the same molecular mass as P_iBP2 and may be a membranous form of P_iBP2; the second one has a molecular mass of 30 kDa. Antibodies raised against the 30 kDa protein inhibit P_i uptake by protoplasts and the corresponding Fab fragments inhibit P_i uptake by whole cells. These results suggest that the 30 kDa plasma membrane protein is the P_i-specific plasma membrane carrier in *C.tropicalis*.

Abbreviation: SDS–PAGE, SDS–polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Strain and growth conditions*

C.tropicalis (CBS 6947) was grown and P_i-starved as in [1]. Cells were osmotically shocked as in [2,3]. Proteins present in the shock fluid were purified as in [2,3].

2.2. *Immunoadsorbent column*

Anti-P_iBP2 antibodies (in 0.25 M Na-carbonate buffer, pH 8.5) were coupled to Sepharose 4B CN (Pharmacia) as in [5,6].

2.3. *SDS–PAGE*

SDS–PAGE was performed as in [7].

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2.4. *Purification of plasma membrane proteins*

P_i-starved and osmotically shocked *C.tropicalis* cells were disrupted with a French press and the plasma membranes purified as in [8,9]. The plasma membrane proteins (10 mg·ml^{-1}) were solubilized in the presence of 2.5% Triton X-100, 20 mM Tris–HCl (pH 8.0), 0.05% NaN_3, and 10^{-4} M PMSF. The Triton concentration was then lowered to 0.5% in the same buffer and the plasma membranes were centrifuged (40000 × *g*, 1 h). The pellet was discarded. The supernatant fraction (protein mixture) was applied to a Sepharose 4B CN column coupled with control immunoglobulins, equilibrated with binding buffer (0.5% Triton X-100, 20 mM Tris–HCl (pH 8) and 0.05% NaN_3). The sample was recycled through the column continuously for 4 h at 4°C. The non-bound fraction was applied to a Sepharose 4B CN column coupled with anti-P_iBP2 antibodies and recycled through the column continuously at 4°C in binding buffer overnight. Proteins specifically retained were eluted with 50 mM diethylamine (pH 11.5), 0.5% Triton X-100, and immediately neutralized with 0.36 M Tris–acetate (pH 5.0). The proteins were dialyzed, concentrated on an Amicon membrane filter (Minicon concentrator B15) and suspended in 20 mM Tris–HCl (pH 8.0), 0.5% Triton X-100, 0.05% NaN_3, prior to SDS–PAGE. Protein concentration was determined as in [10].

2.5. *Preparation of antiserum*

The plasma membrane proteins specifically retained by the immunoadsorbent column (anti-P_iBP2 antibodies) were analyzed by SDS–PAGE. The band corresponding to the 30 kDa protein was cut out and homogenized in Na-phosphate buffer (0.1 M, pH 8.0) in a Potter homogenizer. The homogenates (30–50 μg protein) were mixed with Freund's adjuvant and injected subcutaneously into a male New Zealand rabbit. Two booster injections of the same mixture were made, 1 and 2 months later. Ten days after the last injection, the rabbit was bled. The antiserum was clarified by centrifugation and kept in the freezer (−20°C). Antibodies were precipitated with $(NH_4)_2SO_4$ (40% saturation), dialyzed overnight, concentrated in 10 mM Na-phosphate buffer (pH 8.0), 0.4% NaN_3, and chromatographed on a DEAE–Sephacel column (Pharmacia) in the same buffer. Antibodies were collected in the void volume and used as follows:

Before uptake experiments using whole cells, the antibodies were dialyzed, concentrated, and suspended in a 50 mM Mes–KOH buffer (pH 6.5). When experiments were run with protoplasts, the buffer contained 0.82 M sorbitol. Immunoglobulins from a control serum were treated similarly.

The Fab fragments from the antibodies, prepared as in [11], were separated from Fc fragments by chromatography on a DEAE–Sephacel column. For uptake experiments the Fab fragments were pooled, dialyzed, concentrated in 50 mM Mes–KOH buffer (pH 6.5) and stored at −20°C.

2.6. *Immunoblotting*

Electrophoretic transfer of proteins from SDS–PAGE to nitrocellulose sheets was carried out as in [12].

2.7. *P_i uptake*

Protoplasts were prepared from P_i-starved cells as in [13,14]. Prior to P_i uptake, protoplasts were incubated with antibodies (18 mg for 25 mg protoplast protein) or with control immunoglobulins for 10 min at 32°C in Mes–KOH buffer (pH 6.5) containing 0.82 M sorbitol. They were then diluted 6-fold in pH 5.4 uptake medium (for composition see [4]) and gently shaken at 32°C. Five min later, P_i uptake was started by adding $^{32}P_i$ at various concentrations. At 1, 3, 6, and 10 min, aliquots of 0.4 ml of the protoplast suspension were withdrawn and diluted in 10 ml of the cold uptake medium and centrifuged for 2 min at 6000 × *g*. The pellet was resuspended and sampled for protein determination and radioactivity.

Conditions for P_i uptake by cells were as follows: 5×10^6–10^7 P_i-starved cells were pelleted and suspended in 0.3 ml of 50 mM Mes–KOH buffer (pH 6.5) containing either immunoglobulins (1 mg), antibodies (1 mg) or Fab fragments (0.5 mg) and incubated at 32°C. After 20 min, the cell suspension was diluted 100-fold with uptake medium (50 mM Mes–KOH (pH 5.4), 0.5% glucose). After shaking for 5 min at 32°C, 2 ml of the cell suspension was injected into tubes containing $^{32}P_i$ at various concentrations. After a 30 s uptake period, the cells were filtered on a Sartorius membrane filter and rinsed with demineralized water. The radioactivity of the samples was measured.

3. RESULTS AND DISCUSSION

3.1. *Purification of two plasma membrane proteins by using an immunoadsorbent column*

Of the proteins released by osmotic treatment of *C.tropicalis* cells, only P_iBP2 was specifically retained on an immunoadsorbent column coupled with anti-P_iBP2 antibodies, as shown in fig.1, lane a. Its molecular mass was 52 kDa as determined by SDS–PAGE and 60 kDa by gel filtration on Sephadex G-100. In an earlier paper [2], we reported making a P_iBP2 preparation which was slightly contaminated and had a molecular mass of 70 kDa (determined by gel filtration). In contrast, when solubilized plasma membrane proteins were loaded on the same immunoadsorbent column, two major polypeptides were specifically retained (fig.1, lane b). One had the same molecular mass as P_iBP2 and may be a membranous form of P_iBP2. The second one had a molecular mass of 30 kDa; it is obvious that this polypeptide shares some antigenic determinants with the soluble and presumably membranous forms of P_iBP2. Antiserum was generated against this plasma membrane protein (30 kDa, called M.P. 30) prepared as described in section 2. It recognized this polypeptide, and to a lesser extent, another one of 52 kDa (the putative membranous form of P_iBP2) as shown by immuno-

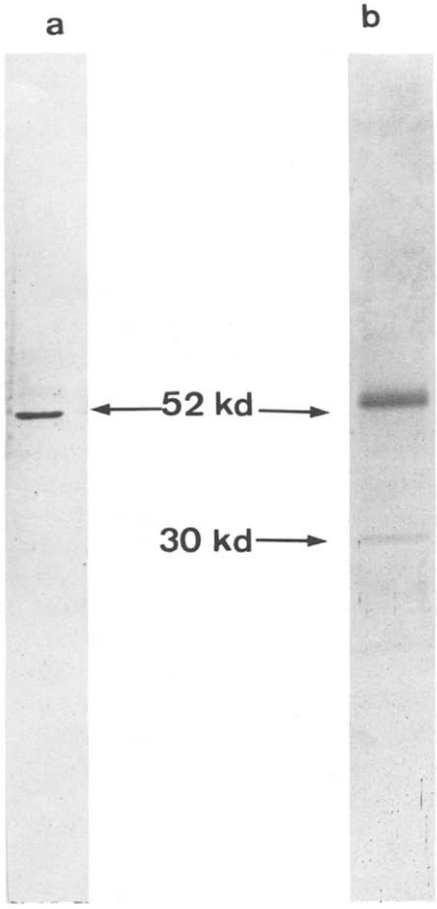

Fig.1. Electrophoretic separation by SDS–PAGE of: P_i-binding protein 2 from the shock fluid after its specific retention on an immunoadsorbent column coupled with anti-P_iBP2 antibodies (lane a); plasma membrane proteins specifically retained by the same immunoadsorbent column (lane b). kd, kDa.

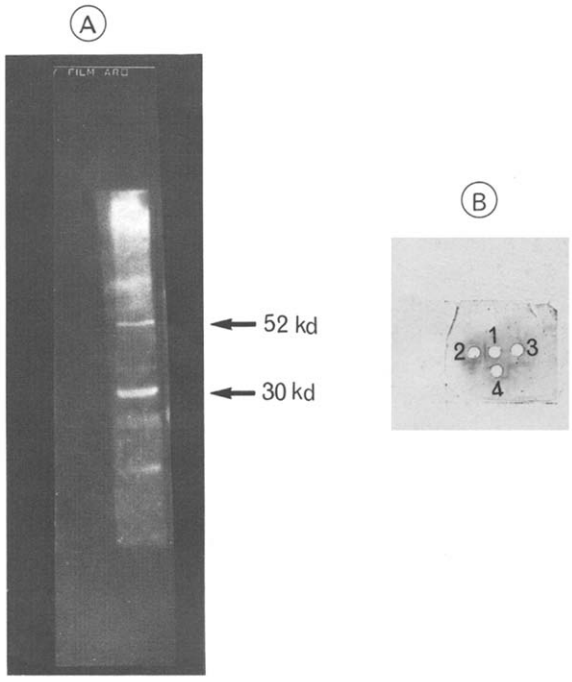

Fig.2. (A) Autoradiograms of immunoblotted plasma membrane proteins. The plasma membrane proteins were solubilized in 1% SDS, separated by electrophoresis and transferred to nitrocellulose sheets. The binding of antibodies to the plasma membrane proteins was detected by ^{125}I-protein A. Two major bands corresponding to 52 kDa and 30 kDa (the latter being more intensely labelled) were revealed by autoradiography. Exposure time: 16 h. (B) Ouchterlony tests: well 1, proteins released by osmotic shock; well 2, anti-P_iBP2 antiserum; wells 3 and 4 anti-M.P. 30 antibodies. A precipitation line can only be observed between well 1 and 2. There is no reaction of anti-M.P. 30 antibodies with the proteins present in the shock fluid.

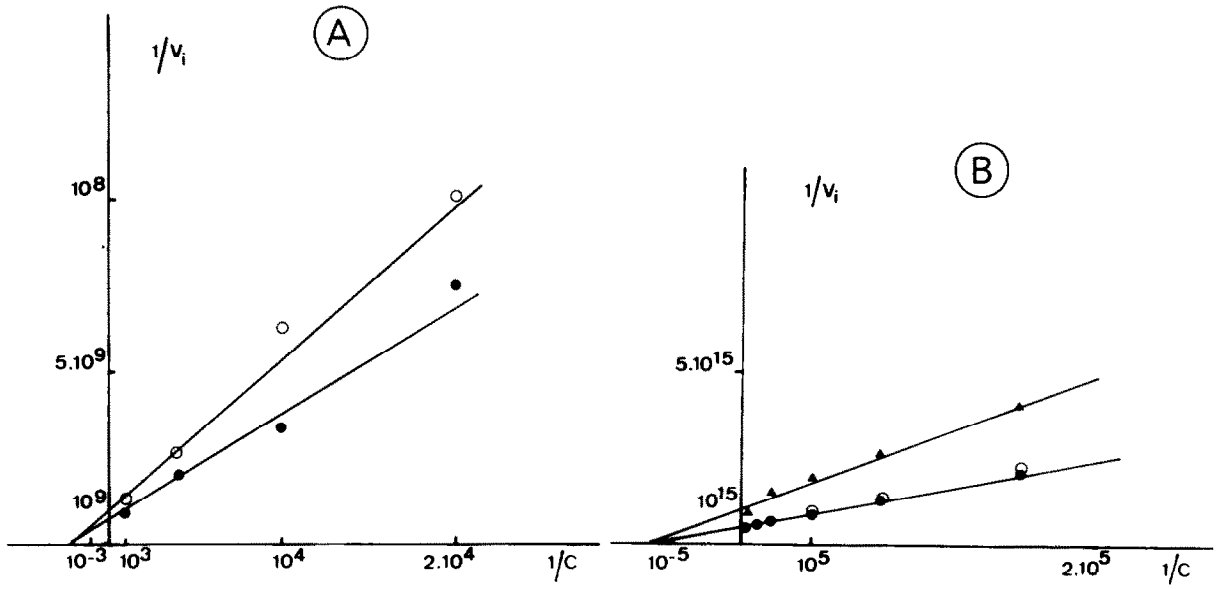

Fig.3. Inhibitory effect of whole anti-M.P. 30 antibodies and of the corresponding Fab fragments on the P_i uptake by protoplasts (A) and by whole cells (B). (●—●) Control (in the presence of immunoglobulin), (○—○) whole anti-M.P. 30 antibodies, (▲—▲) Fab fragments prepared from anti-M.P. 30 antibodies. V_i: initial velocities are expressed in mol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$ for protoplasts (A) and in mol $P_i \cdot cell^{-1}$ absorbed in 30 s for whole cells (B) C: molar P_i concentration.

blotting (fig.2A). Ouchterlony tests (fig.2B) showed that anti-M.P. 30 antiserum does not recognize the soluble form of P_iBP2 present in the shock fluid.

3.2. *Effects on P_i uptake of anti-M.P. 30 antibodies and of their derived Fab fragments*

The M.P. 30 antibodies inhibited P_i uptake by protoplasts (fig.3A) but not that by whole cells (fig.3B); however, Fab fragments prepared from the anti-M.P. 30 antibodies were effective in inhibiting P_i uptake by whole cells (fig.3B). Thus, it seems likely that while whole antibody molecules cannot enter and move through the cell wall to bind the plasma membrane protein, in the absence of cell wall (i.e. in protoplasts), the whole antibodies can directly reach the M.P. 30. Presumably, the lower molecular mass of the derived anti-M.P. 30 Fab fragments is responsible for the increased penetration of the cell wall and binding of the plasma membrane protein at the plasma membrane level, as evidenced by the inhibition of P_i uptake in whole cells. It is suggested that the M.P. 30 may be the membrane-specific P_i carrier in *C. tropicalis* based on the differential activity of M.P. 30 antibodies and their derived Fab fragments. The results reported here lead us to propose the following hypothesis: the high-affinity P_i transport system is composed of a P_i-binding protein, located near the cell surface within the cell wall and of a plasmalemma-specific P_i carrier of 30 kDa. This hypothesis is supported by the fact that P_i uptake in whole cells is equally inhibited by whole anti-P_iBP2 antibody molecules as by their corresponding Fab fragments (not shown). Moreover, it should be pointed out that the molecular mass of this membrane-specific P_i carrier is close to that found for P_i carriers in mitochondria [15–18] and in chloroplasts [19].

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