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A test for screening monoclonal antibodies to membrane proteins based on their ability to inhibit protein reconstitution into vesicles

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The hypothesis that the binding of an antibody to a membrane protein is likely to prevent the reconstitution of the protein into liposomes was checked, by using the plant plasma membrane H^+-ATPase (EC 3.6.1.35) as a model system, and two reconstitution procedures: spontaneous insertion (SI) of purified H^+-ATPase into preformed liposomes, and a detergent-mediated reconstitution (DMR) procedure allowing the reconstitution of the whole membrane protein content. Nine monoclonal antibodies (MABs) raised against H^+-ATPase were tested. None affected the functioning of the enzyme reconstituted in liposomes, suggesting that the probability to obtain an inhibitory MAB is low. Five MABs inhibited its SI, and seven inhibited its reconstitution in the DMR procedure. These results indicate that it is possible to screen antibodies directed against membrane protein, by making use of their ability to inhibit the reconstitution of these proteins.

Introduction

Most transport proteins are devoided of enzymic activity, and thus can be characterized only by their vectorial operation. If a biochemical approach to their molecular identification is adopted, membrane proteins may be fractionated and functional reconstitution performed, e.g., in proteoliposomes. Monoclonal antibodies (MABs) raised against a membrane protein fraction can facilitate the identification of membrane transport systems. However, the probability to obtain an inhibitory MAB is low, because epitopes correspond to 6–8 amino acids.

One may assume that the binding of an antibody to a transport protein is likely to prevent its reconstitution into liposomes. In that case, the vectorial activity of this protein would no longer be detected or at least would be reduced in the proteoliposomes. Theoretically this may allow the extension of the screening to antibodies which recognise the protein without inhibiting its vectorial activity. We report here a study of the feasibility of such a screening test on a set of antibodies raised against plant plasma membrane H^+-ATPase (EC 3.6.1.35) [1]. This protein was chosen as a model system because it possesses both an enzymic and a transport activity. So it may be easily detected both in the solubilized state and after reconstitution.

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; CMC, critical micellar concentration; DMR, detergent-mediated reconstitution; DOC, deoxycholate; IRQ, initial rate of quenching; MAB, monoclonal antibody; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PNPP, *p*-nitrophenyl phosphate; SI, spontaneous insertion; V_{ATP}, ATP hydrolysis activity; V_{H^+}, ATPase H^+-pumping activity; V_{red}, plasma membrane $[Fe(CN)_6]^{3-}$ reduction activity.

Materials and Methods

Plant material and production of antibodies

Plasma membrane vesicles and H^+-ATPase were purified from 5-day-old corn roots according to Grouzis et al. [2]. 6-week-old female Balb/c mice were immunized with 10 μg plasma membrane proteins or 10 μg purified H^+-ATPase emulsified with Freund's complete adjuvant. Two 10 μg injections were made 3 weeks and 4 months later, and a final booster injection (15 μg protein) was made 3 days before the fusion. Hybridoma were screened by ELISA, and the antibodies-producing cells were cloned by limiting dilution.

MAB P4 was obtained by using plasma membrane proteins as antigen. The other MABs (A_n) were obtained by using purified H^+-ATPase as antigen. Isotype and subclasses were determined using an isotypage kit (Amersham, RPN 29).

Purification of antibodies

Ascitic fluids were diluted twice with 150 mM NaCl, 10 mM potassium phosphate buffer (pH 7.4), and lipids were removed by adding 14 mg/ml silica powder [3]. The mixture was agitated gently for 30 min at room temperature, and centrifuged for 15 min at $3000 \times g$. For IgM purification, the supernatant was loaded on a Sephacryl S-300 column. IgM-containing fractions were pooled, concentrated on an Amicon cell using 300000 MW cut-off membrane, and dialysed against 25 mM BTP-Mes (pH 7). For IgG purification (protein-A Spherodex, IBF), the supernatant was desalted on a Sephadex G-25 column (PD 10 column, Pharmacia) equilibrated with the binding buffer (1.5 M glycine, 3 M NaCl (pH 8.9)), and loaded on the protein A column. IgG were eluted with 100 mM Na^+-citrate buffer (pH 3). IgG containing fractions were pooled, concentrated (50000 MW cut-off membrane), and dialysed against 25 mM BTP-Mes (pH 7). Controls of purity were performed by electrophoresis (PhastSystem, Pharmacia). Antibodies were stored at −80°C in 10% glycerol.

Reconstitution procedures

Two reconstitution procedures were used: spontaneous insertion (SI) of purified H^+-ATPase into preformed liposomes, and a detergent-mediated reconstitution procedure (DMR) allowing the reconstitution of the whole membrane protein content.

The SI procedure has been described by Simon-Plas et al. [4]. Mixed soybean phospholipids (L-α-phosphatidylcholine, type II-S, Sigma) were dispersed (80 mg/ml) in 10 mM BTP-Cl (pH 6.5), 150 mM KCl by vigorous mixing on a vortex mixer in the presence of glass beads for 15 min. The liposomes suspension was clarified by sonication for 15 min under argon in a bath sonicator. Insertion of purified H^+-ATPase was achieved by mixing 2.5 mg of liposomes with 0.5 μg purified H^+-ATPase under vortex for a few seconds.

The DMR procedure was adapted from Perlin et al. [5]. Soybean lipids were dispersed in a buffer containing 10 mM Mes-Tris (pH 6.5), 50 mM K-acetate, 20% glycerol, and were clarified as described above for SI. Liposomes were added to plasma membrane vesicles (60 μg proteins) so that the lipid/plasma membrane proteins ratio was 15 w/w (final volume 200 μl). After vortexing for 10 s, liposomes and plasma membrane vesicles were solubilized by adding 20 μl of a deoxycholate (DOC) stock solution (10% w/v) so that the concentration of the detergent, higher than the CMC (5 mM), satisfied rho factor = 0.6 [6]. The proteins were reconstituted by detergent elimination using chromatography on a Sephadex G-50 gel. Spin column (Promega C1281) was filled with the gel equilibrated with 10 mM Mes-Tris (pH 6.5), 50 mM K-acetate, 20% glycerol, and centrifuged to remove the extra buffer. The protein/lipid/detergent mixture was loaded on the top of the gel, and the column was centrifuged for 5 min at $180 \times g$ to remove the detergent. The eluate (200–220 μl) containing proteoliposomes was stored in liquid nitrogen.

Plasma membrane protein activities

ATP hydrolysis activity (V_{ATP}) was determined by measuring the release of Pi according to the method of Ames [7]. The incubation medium (500 μl) contained 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl, 25 mM Tris-Mes (pH 6.5), and 50 μg/ml lysophosphatidylcholine [8]. The reaction was started by adding the enzyme, and proceeded for 30 min at 30°C.

ATPase H^+-pumping activity (V_{H^+}) was estimated using the pH-sensitive fluorescent probe 9-amino-6-chloro-2-methoxyacridine (ACMA). Plasma membrane vesicles or proteoliposomes were added in the assay medium (2 ml) containing 3 mM ATP, 1 μM ACMA, 150 mM KCl, 0.25 μM valinomycin, 10 mM BTP-Cl (pH 6.5). The reaction was started by adding Mg^{2+} (3 mM, as chloride salt). ACMA fluorescence was measured using excitation/emission wavelengths of 415/485 nm. The initial rate of quenching (IRQ) was linear with protein concentration, could be expressed in specific units (% quenching per min per mg protein), and was taken as an estimate of V_{H^+} [9].

Plasma membrane $[Fe(CN)_6]^{3-}$ reduction activity (V_{red}) was measured according to Brüggemann and Moog [10]. The assay medium contained 500 μM $K_3[Fe(CN)_6]$, 500 μM NADH, 250 mM sucrose, 0.02% Triton X-100, 20 mM BTP-Cl (pH 7). $[Fe(CN)_6]^{3-}$ reduction was measured by monitoring the decrease in absorbance at 420 nm.

Other methods

Lipid phosphorus was determined according to Gomori [11] with the following modification: samples were treated up to 170°C in nitric acid before adding perchloric acid. Proteins were assayed as described in Schaffner and Weissman [12] using BSA as standard.

Results and Discussion

Inhibition of H^+-ATPase spontaneous insertion by a monoclonal antibody

The anti-H^+-ATPase MAB A8 affected neither the rate of ATP hydrolysis by the solubilized enzyme (not shown), nor the H^+-pumping activity of the enzyme spontaneously inserted into liposomes (Fig. 1A, trace

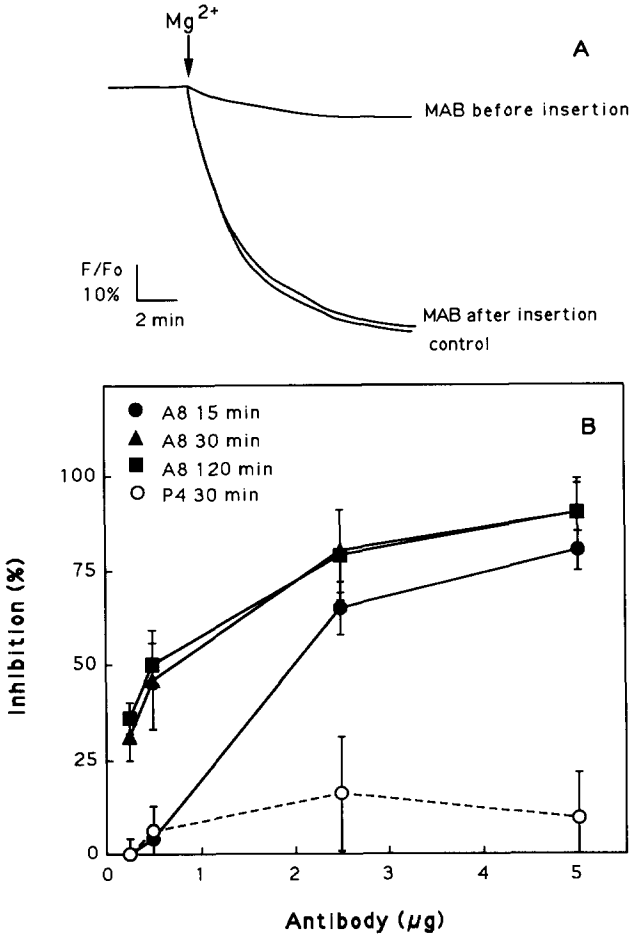

Fig. 1. Inhibition of H^+-ATPase spontaneous insertion into liposomes by a monoclonal antibody. (A) Effect of monoclonal antibody A8 on H^+-pumping activity. Monoclonal antibody (MAB) A8 was introduced in a H^+-ATPase containing medium, either before or after attempting spontaneous insertion (SI) of H^+-ATPase into liposomes. Trace 'MAB before insertion': H^+-ATPase (0.5 μg) and A8 (5 μg) were incubated together for 30 min at 30°C, then SI was attempted, and H^+-pumping was assayed. Trace 'MAB after insertion': SI was first attempted, then the proteoliposomes were incubated for 30 min at 30°C with 5 μg A8, and used for H^+-pumping assay. Trace 'control': H^+-ATPase was incubated 30 min at 30°C (no MAB), SI was attempted, and the proteoliposomes were transferred in the H^+-pumping assay medium. Traces show the quenching rate of ACMA fluorescence (F/F_0 as a percentage; F_0: steady-state initial fluorescence; F: time-dependent fluorescence). (B) Effect of antibody concentration and incubation time on H^+-pumping inhibition. H^+-ATPase (0.5 μg) was incubated with MAB A8 or MAB P4 as described above for the treatment 'MAB before insertion'. Various amounts of antibodies, and different incubation times were tested. H^+-pumping activity (V_{H^+}) was estimated from the initial rate of ACMA quenching. The difference between the values of V_{H^+} measured in the control and antibody treatments, expressed as percent of the control, was taken as an estimate of the inhibition of H^+-ATPase SI. Means of six replicates, and standard deviations values.

labelled 'MAB after insertion'). However, when this MAB was incubated with the H^+-ATPase before attempting SI of this enzyme into liposomes, a drastic decrease of ACMA fluorescence quenching was observed (Fig. 1A, trace labelled 'MAB before insertion'). Since the decrease in H^+-pumping activity could not be attributed to a direct effect of A8 on the enzyme functioning, an inhibition of the spontaneous insertion of the enzyme into the liposomes was hypothesized. Since a proportional relationship between the initial rate of ACMA quenching (IRQ) and the amount of H^+-ATPase inserted has been demonstrated [13], the decrease in the IRQ (expressed as a percentage of the IRQ in the control treatment) was taken as an estimate of the inhibition of the H^+-ATPase spontaneous insertion. The inhibition obtained with A8 increased with incubation time before insertion, and with the amount of antibody incubated with H^+-ATPase (Fig. 1B). Such an inhibition was not found with P4, a MAB obtained by using plasma membrane vesicles as antigen, and which did not recognize H^+-ATPase in ELISA (Table I): this MAB had a small inhibiting effect (around 15%) on H^+-pumping activity (Fig. 1B), similar to that observed when BSA or MABs (IgM or IgG) directed against erythrocyte plasma membrane were substituted for P4 in the incubation medium (data not shown).

H^+-ATPase was inserted into liposomes (SI), and proteoliposomes were layered on a sucrose gradient. Both ATP hydrolysis activity and H^+-pumping activity

TABLE I

Ability of antibodies to inhibit membrane protein reconstitution

The inhibition of H^+-ATPase reconstitution was inferred from the resulting inhibition of V_{H^+} (see Figs. 1 and 4). SI: spontaneous insertion. MAB alone: H^+-ATPase (0.5 μg) and the monoclonal antibody (2.5 μg) were incubated together for 30 min at 30°C; then SI was attempted. Ternary complex: H^+-ATPase and the MAB were incubated together with anti-antibodies (2.5 μg) for 30 min at 30°C before SI was attempted. DMR: detergent-mediated reconstitution (60 μg plasma membrane proteins, 300 μg antibodies; see legend to Fig. 3). ELISA: plates were coated with H^+-ATPase (0.25 μg per well). Antigen/antibody complex formation was revealed using the alkaline phosphatase/PNPP system (1 h incubation). Inhibition (%) of H^+-ATPase reconstitution: means of six replicates and standard deviation values.

MAB	Type	ELISA (absorbance)	Inhibition of insertion in the following procedures (%)		
			SI		DMR
			MAB alone	ternary complex	
P4	IgM	0.08	10±12	11±0	25± 3
A3	IgM	0.91	19± 0	n.d.	10± 0
A4	IgM	1.15	8± 4	55±7	100± 0
A8	IgM	1.73	80±11	76±4	66± 4
A12	IgM	1.88	15± 6	n.d.	85± 5
A17	IgM	0.87	79± 9	n.d.	100± 0
A29	IgM	0.20	64± 5	n.d.	71±14
A38	IgG	0.30	72± 2	n.d.	88± 3
A44	IgG	1.81	38± 9	n.d.	71± 8
A48	IgM	0.41	15±16	n.d.	9± 9

n.d., not determined.

co-sedimented with lipids (Fig. 2A). According to Scotto and Zakim [14], the co-sedimentation of lipids and the protein in such a gradient is proof that the protein had indeed been reconstituted. When H^+-ATPase was incubated with A8 before spontaneous insertion and centrifuged on the sucrose gradient, lipids and ATP hydrolysis activity were found in separate fractions. Furthermore, H^+-pumping activity was very low in all fractions (Fig. 2B). Such results indicate that the protein insertion failed [14]. Thus, the whole set of results supports the following hypothesis: the specific binding of the MAB on its target prevents the protein from spontaneously inserting into preformed liposomes. High MAB/antigen ratios (10:1, Figs. 1 and 2) were needed, probably because the incubation of A8 with the H^+-ATPase occurred in media containing about 20% glycerol. Such high glycerol concentrations are likely to affect the binding of antibodies on their target, by modifying hydrogen bonding interactions. Indeed, the intensity of ELISA response decreased when the glycerol content in the incubation medium was increased: about a 10-fold decrease in absorbance when the glycerol content was raised from 0 to 10% (Fig. 3). Similar ELISA experiments were performed with the other anti-H^+-ATPase MABs. A decrease in ELISA response due to increased glycerol concentration was observed systematically. The magnitude of the decrease was dependent on the antibody tested (not shown). Rapid and irreversible denaturation of H^+-ATPase occurred when glycerol concentration in the incubation medium was lowered below 20% ([15], and data not shown).

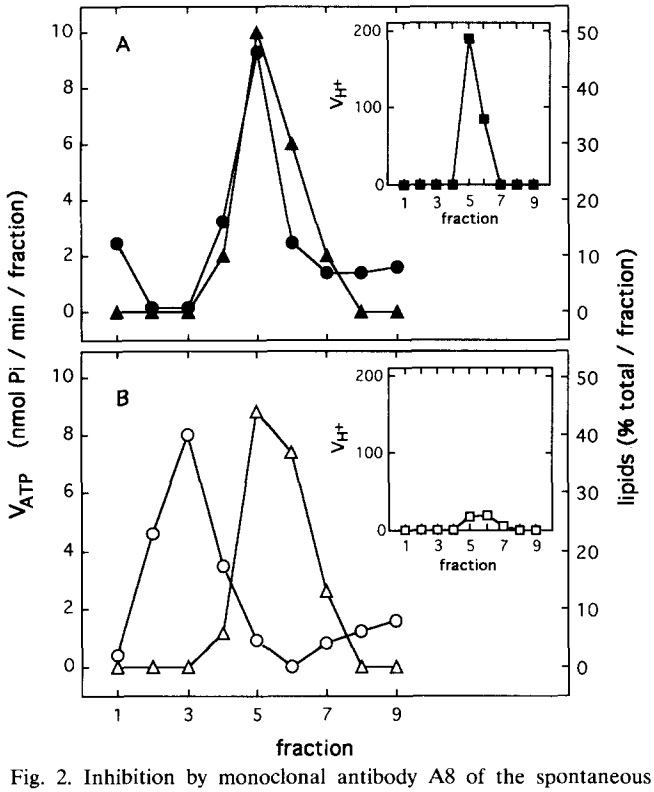

Fig. 2. Inhibition by monoclonal antibody A8 of the spontaneous insertion of H^+-ATPase into liposomes revealed by centrifugation on a density gradient. H^+-ATPase (6 μg) was incubated for 30 min at 30°C in 10 μl of a buffer containing 50 mM BTP-Mes, 20% v/v glycerol (pH 7), without (A) or with (B) MAB A8 (60 μg). The sample was then mixed (vortex) with 6 mg liposomes (SI procedure). The mixture was diluted with 250 μl of 150 mM KCl, 10 mM BTP-Cl (pH 6.5), and settled on the top of a discontinuous sucrose gradient (4, 15, 25 and 34% w/v sucrose, layers of 1 ml each). The gradient was centrifuged (250000 × g for 5 h, 4°C). Fractions (500 μl) were collected from the bottom. They were assayed for ATP hydrolysis activity (V_{ATP}, circles on the figure) and lipid content (% of the total content, triangles). H^+-pumping activity (V_{H^+}, inset) was estimated for each fraction from the initial rate of ACMA quenching (squares, % quenching/min); 150 μl of each fraction were introduced in the assay medium (Fig. 1).

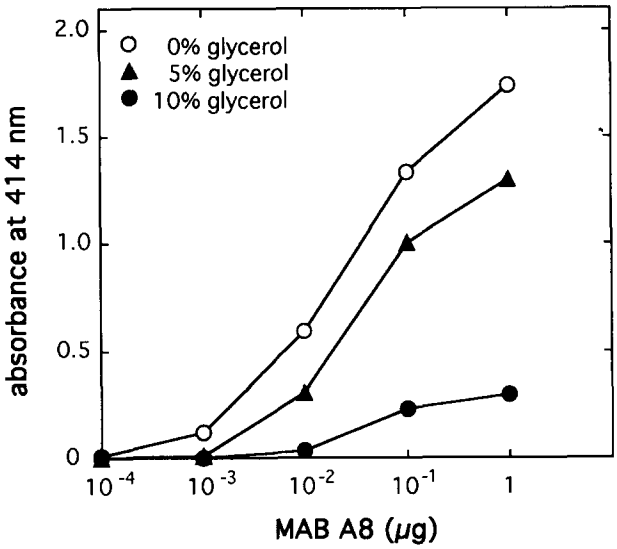

Fig. 3. Influence of glycerol concentration on ELISA response of MAB A8. ELISA plates were coated with 0.5 μg ATPase. MAB A8 was diluted in PBS containing 0.1% Tween and glycerol at various concentrations. The fixation of the MABs was revealed using anti-IgM or anti-IgG antibodies coupled with phosphatase alcaline (Sigma). The absorbance of the PNP produced was measured at 414 nm.

Inhibition of detergent mediated reconstitution of H^+-ATPase by a monoclonal antibody

A8 and P4 were incubated with plasma membrane vesicles for 30 min. Liposomes and DOC were added into the mixture, and the detergent was eliminated by passage through a column. The H^+-pumping and $[Fe(CN)_6]^{3-}$ reductase activities were measured in the eluate. The incubation of plasma membrane vesicles with A8 led to an important decrease of H^+-pumping activity (nearly 100% inhibition for a ratio MAB/plasma membrane protein of 10). P4 had a smaller effect: around 15% inhibition for a ratio of 10 (Fig. 4), a value similar to the one observed with controls in which BSA was substituted to MAB P4 (ratio BSA/plasma membrane protein of 10, data not shown).

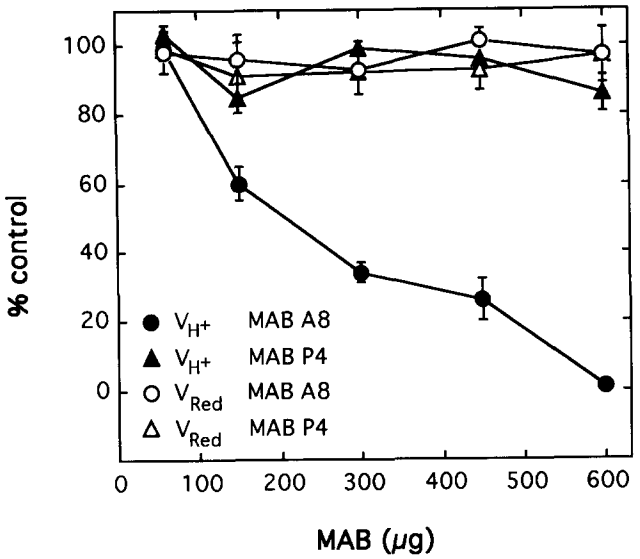

Fig. 4. Inhibition of H^+-ATPase insertion by a monoclonal antibody during a detergent mediated reconstitution procedure. Plasma membrane vesicles (60 μg membrane proteins) were incubated for 30 min at 30°C with various amounts of monoclonal antibodies A8 or P4, or with the antibody dilution buffer for the control treatment. Liposomes were added, the mixture was vortexed, solubilized by adding DOC, and loaded on a Sephadex G-50 column to remove the detergent. For each eluate, $[Fe(CN)_6]^{3-}$ reductase activity (V_{red}) and H^+-pumping activity (V_{H^+}, estimated from the initial rate of ACMA quenching) were measured, and expressed as % of the corresponding values for the control treatment. Means of six replicates and standard deviation values. A8: antibody raised against the H^+-ATPase; P4: no response in ELISA with the H^+-ATPase, cf. Table I.

Neither A8 nor P4 affected $[Fe(CN)_6]^{3-}$ reductase activity (Fig. 4). Thus, incubating plasma membrane vesicles with A8 led to a specific decrease in the H^+-pumping activity of the target enzyme, but seemed to have no effect on the activity of other enzymes. Plasma membrane vesicles (60 μg membrane proteins) were incubated with A8 (300 μg) for 30 min at 30°C, and the DMR procedure was attempted. The newly formed proteoliposomes were layered on a discontinuous sucrose gradient (as described in the legend to Fig. 2). The reductase acticity co-sedimented with lipids (data not shown), indicating that the reconstitution of the reductase activity had occurred [14]. Taken together, these results indicate that the decrease in H^+-pumping activity observed with A8 (Fig. 4) should be due to the fixation of this MAB on H^+-ATPase, preventing the H^+-ATPase from taking part in the formation of proteoliposomes.

MAB screening

Nine antibodies (ELISA data: see Table I) obtained by using purified H^+-ATPase as antigen (antibodies named A*n*), and one obtained by using plasma membrane vesicles as antigen (P4) were screened. None of the MABs investigated inhibited either ATP hydrolysis activity of solubilized H^+-ATPase or H^+-pumping activity after SI of H^+-ATPase into liposomes (data not shown). However, seven of the MABs inhibited the DMR of the enzyme (more than 50% inhibition), and four inhibited the SI (Table I). These results indicate that it is possible to screen antibodies directed against a transport system, by making use of their ability to inhibit the reconstitution of this system. The results obtained with MAB A4 indicate that it is possible to increase further the efficiency of the test, by adding anti-antibodies to the incubation media (Table I): MAB A4 alone was ineffective in SI experiments, but an inhibition of H^+-ATPase SI was observed when a ternary complex ATPase/anti-ATPase/anti-antibody was formed. In the same conditions, no variation of A8 or P4 inhibition ability was observed (Table I). The inhibition obtained when ternary complexes were formed could result from aggregation or precipitation of the complex formed.

For two MABs (A4 and A12), the response was quite different regarding the reconstitution procedure used: these antibodies were strongly inhibiting in DMR experiments, but were ineffective in SI experiments (Table I). The parts of the protein recognized by these antibodies may not have the same importance in the two reconstitution procedures. The use of such antibodies may allow a better understanding of the mechanisms of membrane protein reconstitution.

Conclusion

The reconstitution of a membrane protein into sealed phospholipid vesicles can be prevented by antibodies directed against the protein. One can make use of this to spot antibodies which bind to a transport protein without affecting its vectorial activity. The advantage of such a screening procedure is apparent from the following results: nine MABs were raised against H^+-ATPase, none of them affected the functioning of the enzyme reconstituted in liposomes, seven inhibited the reconstitution of the enzyme into vesicles (DMR procedure, Table I). Therefore, this sort of screening test could help the identification of ion transport systems, or any membrane protein for which a functional test is available.

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