Monoclonal antibodies that immunoreact with a cation-stimulated plant membrane ATPase

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Hybridoma technology has been used successfully to generate monoclonal-antibody probes against protoplast membrane antigens. Hybridomas secreting monoclonal antibodies that either inhibit or stimulate a putative plasma-membrane marker enzyme, ($K^+ + Mg^{2+}$)-stimulated pH 6.5 ATPase, have been identified and cloned. The specificity of monoclonal-antibody probes on the activity of other phosphate-hydrolysing enzymes has also been examined. The production and identification of monospecific antibodies capable of immunoreacting with particular component proteins in a complex plant membrane mixture highlight the usefulness of hybridoma methodology for the enzymologist, especially since such monoclonal antibodies can be used in the purification of proteins by immunoaffinity techniques.

The identification of suitable markers to characterize the plasma membrane of plant cells has been a matter of considerable controversy (Hendriks, 1976). Several enzymes, including glucan synthase (Van Der Woude *et al.*, 1974) and ATPase (Hodges & Leonard, 1974), have been considered prime candidates as plasma-membrane markers. However, unequivocal data in support of this are not available, and it may be necessary to develop highly specific antibody probes before confirmatory evidence can be obtained by alternative methods such as immunoelectron microscopy.

In view of the difficulties associated with purifying plant membrane proteins to homogeneity (Benson & Tipton, 1978) for use as antigen in antibody production by conventional methods, and also because of the lack of specificity of such polyclonal antibodies, we decided to generate membrane-specific high-avidity monoclonal antibodies by using the hybridoma techniques developed by Kohler & Milstein (1975). The present paper describes the production and identification of monoclonal antibodies that immunoreact with a putative plasma-membrane marker, ($K^+ + Mg^{2+}$)-stimulated pH 6.5 ATPase.

Materials and methods

Source of antigen

Protoplasts were isolated from barley (*Hordeum vulgare*) HRI-1 cell suspension cultures (Chin,

1978; Chin & Scott, 1979) lysed in hypo-osmotic buffer and used as a source of microsomes. The procedure of Hodges & Leonard (1974) was used to prepare plant plasma membranes enriched in ($K^+ + Mg^{2+}$)-ATPase.

Immunization of mice

Female Balb/c mice (10–12 weeks old) were primed by intraperitoneal injection of 2×10^7 barley protoplasts emulsified in Freund's complete adjuvant. Subsequent injections without adjuvant were given 14, 28 and 50 days later. Spleens were removed from immunized mice 3 days after the last injection.

Hybridization and cell culture

Spleen cells from each mouse were pelleted in serum-free Dulbecco's modified Eagle's medium (DME medium) and resuspended in 5 ml of cold 0.17M-NH₄Cl to lyse erythrocytes. Lymphocytes were then recovered by underlaying the cell suspension with 20% horse serum in DME medium and centrifuging at 100g for 5 min. Pelleted lymphocytes were re-adjusted to 5×10^7 cells and fused with 3×10^7 NS-1 myelomas essentially as described by Kennett *et al.* (1978), except that potassium dextran sulphate (1%, w/v) was included in the poly(ethylene glycol) 4000 fusion medium. The hybrids were resuspended in 8.5 ml of HAT (hypoxanthine/aminopterin/thymidine) medium described by Litfield (1964) and distributed in 50 μ l portions to 7 $\times$ 24-well Nunc culture plates. Each well had already been inoculated 1 day previously with 10^5 mouse peritoneal macrophages.

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Hybrid clones usually appeared about 10–14 days later and culture supernatants were assayed for anti-(plasma membrane) activity by a filter-paper binding assay (Rathjen *et al.*, 1981). Culture supernatants selected from positive filter-paper-binding-assay responders were further assayed for anti-ATPase activity. Responders were then re-cloned by limit dilution (Goding, 1980). Supernatants from re-cloned cultures (reclones) were routinely screened for inhibiting (I) or stimulating (S) activity against pH 6.5 ATPase.

Filter-paper binding assay

Plasma membrane proteins were coupled to CNBr-activated Whatman (no. 542) filter-paper discs. A single disc was inserted into each of 96 wells of a Linbro microtitre plate (Catalogue no. 76-003-05), punched previously with a 1 mm-diameter hole. Discs were washed with Buffer A [10 mM-Tris/HCl (pH 8.0)/0.15 M-NaCl/0.5% (w/v) bovine serum albumin/5% (w/v) calf serum/0.17% (w/v) gelatin/0.02% (v/v) NaN_3]. Culture supernatants (10 μl) were spotted on to each disc and incubated at 37°C for 30 min. Unbound antibody was eluted with Buffer A by using a vacuum manifold connected to the microtitre plate. Antibody was detected by adding 50 μl of ^{125}I -labelled rabbit anti-mouse immunoglobulin (100 000 c.p.m.). Unbound radioactivity was flushed through with Buffer B [1% (v/v) Tween 20 in phosphate-buffered saline (Oxoid, code BR 14a)]. Discs were air-dried and then counted for radioactivity in a Packard gamma counter.

Enzyme assays

Hybridoma supernatant (20 μl) was incubated for 1 h at 4°C with $(\text{K}^+ + \text{Mg}^{2+})$ -ATPase-enriched membrane fraction (adjusted to 40 μg of protein and 500 units of activity, where 1 unit = 1 mmol of P_i released/h). The mixture was then further incubated at 37°C for 5 min. Enzyme activity was assayed by the addition of 1 ml of reaction mixture, which contained 5 mM-ATP, 3 mM- MgSO_4 , 50 mM-KCl and 35 mM-Tris/Mes (4-morpholine-ethanesulphonic acid), pH 6.5. The reaction was terminated after 1 h at 37°C with trichloroacetic acid at a final concentration of 5% (w/v). P_i was determined as described by Dulley (1975). Assays for phosphate-hydrolysing enzyme activity utilizing IDP, ITP, AMP, *p*-nitrophenyl phosphate or glucose 6-phosphate as substrate were carried out as described by Hodges & Leonard (1974).

Protein was determined by the Lowry procedure as described by Dulley & Grieve (1975).

Results

Anti-(plasma membrane) activity was determined by filter-paper binding assay, and the results of a typical fusion (Fusion 6) are shown in Fig. 1. Three categories of response designated 'high' (>10 000 c.p.m.), 'intermediate' (5000–10 000 c.p.m.) and 'low' (<5000 c.p.m.), were identified, and only the culture supernatants from those wells with a high or intermediate response were

Fig. 1. Detection of anti-(plasma membrane) activity in hybridoma culture supernatants by the filter-paper binding assay

Spleen lymphocytes were fused with NS-1 myelomas and after washing were distributed in 50 μl portions to 7 $\times$ 24-well Nunc culture plates containing 10^5 mouse peritoneal macrophages in each well. Supernatants were assayed for anti-(plasma membrane) activity by the filter-paper binding assay 13 days after culture.

used in assays for immunoreactivity against ($K^+ + Mg^{2+}$)-ATPase. Of 168 wells examined, only 29 (or 17.3%) displayed a significant filter-paper-binding-assay response. This frequency tended to vary quite dramatically from one fusion to another. The average low response was usually about 3000 c.p.m./filter after correcting for background radioactivity.

The scatter of immune reactivity against pH 6.5 ($K^+ + Mg^{2+}$)-ATPase of the high and intermediate responders is depicted in Fig. 2. Of 29 responders assayed in Fusion 6, only wells 6/22 and 6/97 inhibited ($K^+ + Mg^{2+}$)-ATPase activity, by 64 and 40% respectively. ($K^+ + Mg^{2+}$)-ATPase activity was stimulated by 60% when made to react with antibodies present in culture well 6/13. Other culture supernatants exerted various amounts of inhibition (I) and stimulation (S), but these were within 2 s.d. of the input ATPase or control activity. The result of similar assays carried out with filter-paper-binding-assay responders in Fusion 2 and Fusion 5 are included in Fig. 2 for comparison.

Hybridomas present in each of the seven culture wells from three separate fusions (Fusions 2, 5, and 6) were recloned and the frequency of reclones

retaining either I or S activity is shown in Table 1. It can be seen that I- or S-hybridomas do not revert, and retain their inhibitory or stimulatory activity.

Fig. 2. Distribution of anti[pH 6.5 ($K^+ + Mg^{2+}$)-ATPase] activity in hybridoma supernatants selected as high and intermediate responders by the filter-paper binding assay

Table 1. Frequency of recloned hybridomas retaining either inhibitory (I) or stimulating (S) activity against membrane-associated pH 6.5 ($K^+ + Mg^{2+}$)-ATPase

Designation of hybridoma (positive filter-paper binding assay)	Frequency of reclones retaining either I- or S-activity against pH 6.5 ($K^+ + Mg^{2+}$)-ATPase (%)		Designation of reclones selected with maximum I- or S-activity
	I	S	
2/17	3	0	I 217/8; I 217/125
2/62	4	0	No survival
5/3	0	2	No survival
5/43	8	0	I 543/23
6/13	0	2	S 613/80
6/22	11	0	I 622/4
6/97	0	0	No survival

Table 2. Effect of culture supernatants from selected reclones on the phosphate-hydrolysing enzyme activity of barley root microsomes using different substrates

Abbreviations and symbols: Glu 6-P, glucose 6-phosphate; —, Inhibition; +, stimulation; 0, no effect on input activity

Substrate ...	Activity								Glu 6- <i>P</i>
	(K ⁺ + Mg ²⁺)ATP	Mg ²⁺ ATP	ATP	ITP	IDP	AMP	pNPP		
Assay pH ...	6.5	6.5	9	6	7.5	8.5	5.55	9.5	6.5
Designation of selected reclone									
I 217/8	—	—	—	0	0	—	—	—	0
I 217/125	—	0	0	0	0	0	0	0	0
I 543/23	—	0	0	0	0	0	0	0	0
S 613/80	+	+	+	0	0	0	0	0	0
I 622/4	—	0	0	0	0	0	0	0	0

Altogether four reclones with maximum I-activity and one with maximum S-activity were selected and propagated. The specificity of I- or S-activity of the selected reclones on different phosphate-hydrolysing enzyme activities in root microsomes utilizing a range of substrates was examined (Table 2). I217/8 was relatively non-specific and inhibited phosphate hydrolysing activity with all substrates tested except ITP, IDP and glucose-6-phosphate. I 217/125, I 543/23 and I 622/4 only inhibited the activity of pH 6.5 ($K^+ + Mg^{2+}$)-ATPase. S 613/80 stimulated ATPase activity when the substrate was presented as ($K^+ + Mg^{2+}$)ATP, Mg^{2+} ATP or just ATP.

All selected I and S hybridomas continued to produce antibodies of defined specificity when grown in flask cultures. Selected I and S hybridomas have also been established in mice as ascites.

Discussion

One of the most daunting aspects of hybridoma technology is the screening procedure used to assay for antibody reactivity in the supernatants of all the culture wells generated from a single fusion. This problem becomes more pronounced when it is also necessary to assay for anti-enzyme activity. With complex antigens like protoplast plasma-membrane vesicles, it was necessary to use initially a general screening assay, such as the filter-paper binding assay, that permitted the rapid detection of culture wells with high anti-membrane activity. This then decreased the number of enzyme assays to be carried out in the search for monoclonal antibodies that specifically reacted with membrane ATPase. By restricting assays to high and intermediate filter-paper-binding-assay responders, it must be stressed that some low responders such as 6/138 (Fig. 1) without either S- or I-activity and yet possessing a high binding affinity for purified ($K^+ + Mg^{2+}$)-ATPase (J. C. Chin, unpublished work) may be missed. This could represent a third class of monoclonal antibody with 'null' anti-enzyme activity.

The present investigation has confirmed a very important role for hybridoma methods in the enzymologist's repertoire. With a small amount of impure antigen, it is now feasible to generate high-avidity and monospecific antibodies that will always recognize the same determinant every time.

Provided suitable assay procedures are available, it is no longer necessary to use pure antigens to produce stimulatory (Choo *et al.*, 1980) or inhibitory (Cotton *et al.*, 1980) monoclonal antibodies.

Monoclonal anti-(protoplast membrane ATPase) antibody can now be used to probe the architecture of the plant plasma membrane, to evaluate the role of ATPase in ion transport, to provide a means of purifying any plasma-membrane-associated enzyme of interest to homogeneity (Chin & Mermelstein, 1981) and, perhaps most important of all, to provide a tool for the assembly or disassembly of enzyme subunits.

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