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Isolation and characterisation of monoclonal antibodies against hydrophobic membrane subunit 9 of the yeast mitochondrial H^+-ATPase

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Five stable lines of myeloma-spleen cell hybrids, producing antibodies against the proteolipid subunit 9 of the yeast mitochondrial H^+-ATPase F_0-sector, have been isolated by immunizing mice with a proteolipid preparation in the presence of sodium dodecyl sulphate. One of these monoclonal antibodies also reacted with subunit 8 of the enzyme complex indicating a shared epitope. The antibodies did not react with the holo-H^+-ATPase, suggesting that their epitopes are shielded by other subunits of the enzyme complex.

Monoclonal antibodies to various subunits of the H^+-ATPase of yeast [1,2], pig [3] and bovine heart [4] mitochondria, and of *Escherichia coli* [5] have been instrumental in defining the subunit composition of the enzyme complex, in the study of the structure and function of its subunits, and in the elucidation of its assembly process [6–10]. All of the anti-H^+-ATPase monoclonal antibodies which have been isolated to date, however, have been found to be directed against the hydrophilic subunits of the enzyme complex, such as the α- and β-subunits of the F_1-sector, the oligomycin-sensitivity-conferring protein (OSCP), and F_B [1–5]. While monoclonal antibodies against the hydrophobic subunits of the membrane F_0-sector would provide a powerful tool in the analysis of the structure and function of this sector, which forms the proton channel of the enzyme complex, one inherent difficulty in obtaining such antibodies has been the high degree of hydrophobicity of the F_0-subunits. In yeast, these subunits are mitochondrially synthesised, and designated subunits 6, 8 and 9 (the DCCD-binding proteolipid) (see Refs. 1 and 2).

In this communication, we report a procedure which has been successfully applied to raise monoclonal antibodies against the proteolipid subunits of the yeast F_0-sector. The procedure involves the use of the ionic detergent, sodium dodecyl sulphate (SDS) to increase the antigenicity of the proteolipid preparation, and might have a general application in raising monoclonal antibodies to hydrophobic proteins.

The proteolipid antigen used in the present study was extracted from mitochondria of a wild-type strain of yeast as described by Velours et al. [11,12]. The procedure involved the extraction of proteolipids in chloroform/methanol (2 : 1, v/v) for 2 h at 50 °C or 16 h at room temperature followed by an extraction with a mixture of chloroform/methanol/water (50 : 3 : 47, v/v) to remove contaminating proteins. The procedure yielded almost exclusively subunits 8 and 9 (Fig. 1), although minor contaminants with M_r ranging from 15 000 to 60 000 were detected in some preparations (not shown).

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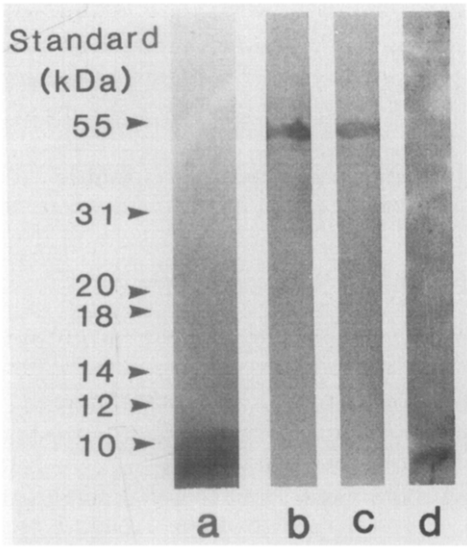

Fig. 1. The characterization of antisera from mice immunized with yeast mitochondrial proteolipid preparation in the presence and absence of SDS. Yeast mitochondrial proteolipid fraction containing the membrane F_0 subunits 8 and 9 of the H^+-ATPase was prepared as described by velours et al. [11,12]. Mice were immunized with this antigen preparation (100 μg per mouse and 10 μg per mouse for the primary and booster injections, respectively) in the absence (5 mice) or presence (10 mice) of SDS (0.5% w/v), following a published protocol [1]. Sera were obtained one week after the first booster injection, and serum antibodies were characterized by Western immunoblotting [1]. The dilution of antisera was 1:10, and the binding of antibodies to the antigens was detected by using an anti-rabbit immunoglobulin labelled with Horse Radish peroxidase, and 4-chloro-1-napthol as a substrate. Shown are the proteolipid preparation after separation by electrophoresis in 12.5% polyacrylamide gel in the presence of SDS [7], followed by Amido-black staining (a), or following detection by Western-immunoblotting with a monoclonal antibody (RH 48) against the β subunit of the yeast H^+-ATPase [1] (b), with a representative antiserum from mice immunized in the absence of SDS (c), and with a representative antiserum from mice immunized in the presence of SDS (d).

Fifteen mice were immunised with the proteolipid preparation, according to an immunisation protocol [1], which includes a primary injection of 100 μg antigen (in complete Freund's adjuvant) per mouse, followed by a booster injection with 10 μg antigen (in incomplete Freund's adjuvant) per mouse 21 days later. In ten of these mice, sodium dodecyl sulphate (SDS) was included in the antigen mixture at a final concentration of 0.5% (w/v), whereas in the other five mice, the antigen was injected in the presence of Freund's adjuvant only. Titres of antibodies ranging from 1:200 to more than 1:1000 were detected in all mice when tested with an ELISA against the proteolipid preparation.

No significant difference was observed in the titres of antibodies in mice injected with the proteolipids in the absence or the presence of SDS. However, when the antisera were further characterised by Western immunoblotting, the antisera from mice immunised in the absence of detergent was found to recognise primarily a high molecular weight protein of about 54 kDa (Fig. 1c). This protein is presumably the β-subunit of the H^+-ATPase complex, as the antisera reacted with this subunit when tested against a preparation of F_1-ATPase, isolated as described by Beechey et al. [13] (data not shown). Although the antigen preparations used for immunisation contained mainly the proteolipids subunit 8 and 9, minor amounts of contaminating α- and/or β-subunits of F_1, which have apparent M_r of 56000 and 54000, respectively, could still be observed in some preparations. This contaminating β-subunit can in fact be clearly demonstrated on a Western immunoblot, by using a previously isolated monoclonal antibody against the β-subunit of the yeast H^+-ATPase [1] (Fig. 1b). Thus, consistent with our previous observation [1], the yeast H^+-ATPase β-subunit appears to be strongly immunogenic in mice, as mice immunised with the proteolipid preparation in the absence of SDS produced antibodies recognising mainly the β-subunit, even though this subunit represents only a low-level contaminant in the preparation.

In contrast to the above situation, antisera from mice immunised with proteolipids in the presence of 0.5% SDS, contain antibodies which react with subunits 8 and 9 (apparent M_r 10000 and 7600, respectively in Fig. 1d). It appears, therefore, that the ionic detergent SDS has facilitated an immune response to subunits 8 and 9 in mice. The reason for this observation is not as yet clear, but is not limited to mice. Antibodies against yeast subunit 9 have been raised in rabbits in the presence of SDS [14].

The mouse that showed the highest titre of antibodies after immunisation in the presence of SDS was used for cell fusion. Spleen cells ob-

tained from this mouse were fused with P3X63Ag 8653 myeloma cells and plated into 288 Costar wells [1]. After 2–3 weeks, 216 wells showed sufficient growth of hybridoma cells to assay for antibody activity.

Antibodies produced by the hybridoma cells were first screened by an enzyme-linked immunosorbent assay (ELISA) using the proteolipid preparation as an antigen. For this assay, the antigen to be coated on the microtitre plate wells was dissolved in either phosphate buffered saline containing 0.5% SDS (PBS/SDS) or in chloroform/methanol ($CHCl_3$/MeOH) 2:1 (v/v). When the antigen was dissolved in PBS/SDS, a total of 15 strong positives was obtained ($A_{405} > 0.6$, Fig. 2a). However, only four of these antibodies reacted with the antigen when it was dissolved in $CHCl_3$/MeOH. In contrast, an additional 26 positives were recorded when the hybridomas were screened with $CHCl_3$/MeOH dissolved proteolipid ($A_{405} \geq 0.6$, Fig. 2b); these antibodies did not react with the antigen when the antigen was dissolved in PBS/SDS.

A possible explanation for the above interesting observation is that the antigenic molecules might have assumed different conformations in the two conditions, allowing different epitopes to be exposed or becoming inaccessible. All antibodies which gave a positive reaction with the proteolipid in either the organic solvents or in PBS/SDS were further analysed by Western immunoblotting. The supernatants from the 30 hybridomas which gave positive reaction when tested with $CHCl_3$/MeOH dissolved proteolipid were found to react in the Western immunoblot with either subunit 8 or subunit 9 or both (data not shown). However, no

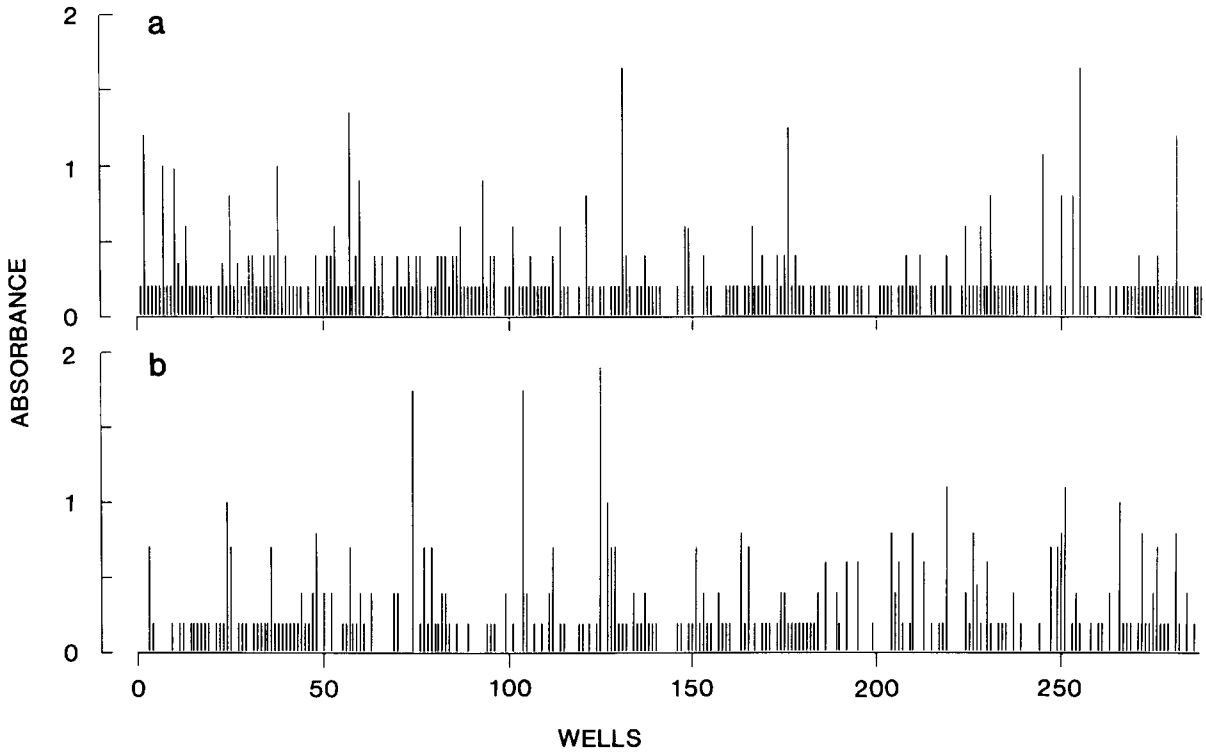

Fig. 2. Screening of anti-proteolipid monoclonal antibodies by enzyme-linked immunosorbent assay (ELISA). Proteolipids dissolved in either (a) phosphate-buffered saline containing 0.5% (w/v) SDS or (b) chloroform/methanol (2:1, v/v) were coated onto the wells of microtitre plates (1 μg) for 3 h at 37°C. After blocking the remaining binding sites with bovine serum albumin [1], 100 μl of hybridoma culture supernatants were added to the microtitre plate wells. Antiproteolipid antibodies were detected by the addition of anti-mouse immunoglobulins, conjugated to alkaline phosphatase, then *p*-nitrophenyl phosphate. Shown are the optical density readings recorded in a Titertek microtitre scanner at 405 nm.

reaction was observed in the Western immunoblot of supernatants from hybridomas which only gave positive reaction in ELISA when tested against proteolipids in PBS/SDS. All ELISA in the subsequent screening of hybridomas, therefore, was carried out using $CHCl_3$/MeOH-dissolved proteolipids as antigen.

After subcloning twice by limiting dilution, only seven cell lines were found to react strongly with the antigen. Five cell lines, designated 5B2, 4A2, 6A5, 10A4 and 27D8, grew as ascites tumors in mice, whereas the other two cell lines developed as solid tumors and did not produce ascites fluid. Antibodies from four cell lines 10A4, 6A5, 4A2 and 5B2 recognised the H^+-ATPase subunit 9 when tested by Western immunoblotting against the proteolipid preparation (Fig. 3a). Ascites fluid from the other cell line (27DB) was found to react with both subunits 8 and 9, suggesting the presence of a common epitope on these two subunits. Since the antibody can react with these subunits after separation in SDS-polyacrylamide gel, the common epitope is most likely to be a continuous one, consisting of a stretch of amino acids common to both subunits, rather than a conformational epitope. A comparison between the amino acid sequences of the H^+-ATPase subunit 8 [12] and subunit 9 [15] revealed only two common sequences of three amino acid residues: Glu-Leu-Val (amino acid residues 3–5 and 2–4 in subunits

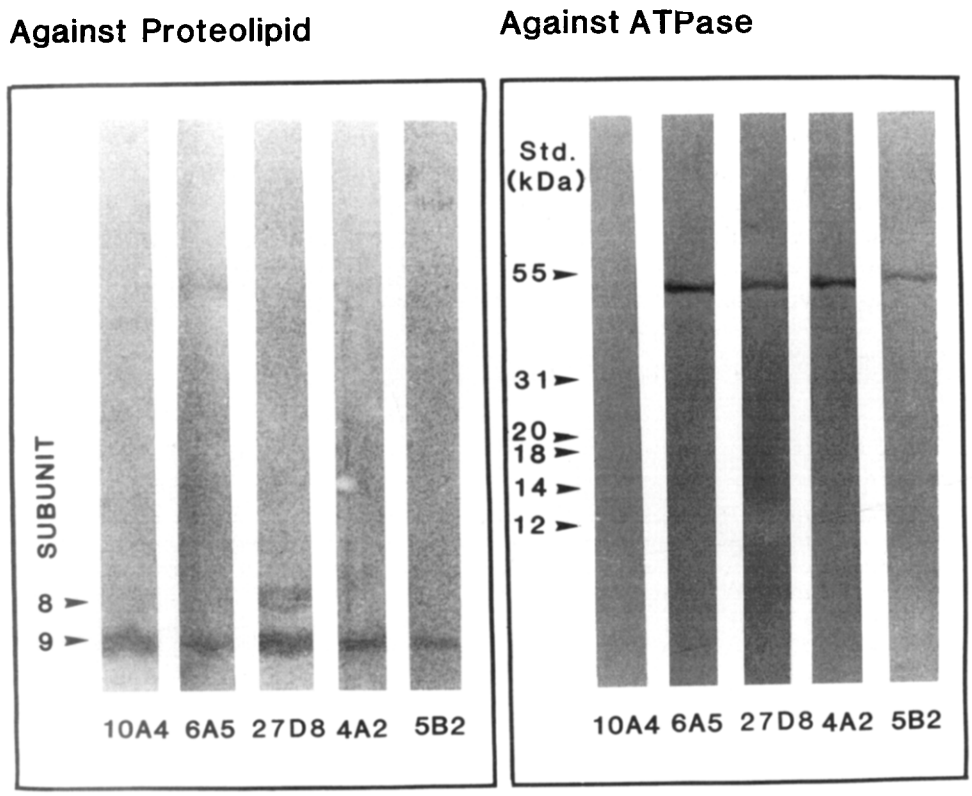

Fig. 3. The characterization of monoclonal antibodies to the proteolipid subunits of the yeast H^+-ATPase by Western-immunoblotting. Mitochondrial proteolipid preparation containing the H^+-ATPase subunits 8 and 9 (a), and glycerol gradient purified H^+-ATPase [16] (b), were electrophoresed on 12.5% SDS-polyacrylamide gels and electrophoretically transferred onto nitrocellulose strips [1]. The filter strips were incubated with ascites fluid from the five anti-proteolipid antibody secreting cell lines, diluted 1 : 50 with phosphate-buffered saline. Antigen-antibody reaction was detected by the addition of Horse Radish peroxidase conjugated anti-mouse immunoglobulins and 4-chloro-1-napthol as a substrate. The molecular masses (kDa) and position (▸) of standard proteins, electrophoretically separated in a parallel track, are indicated.

8 and 9, respectively) and Phe-Leu-Leu (amino acid residues 17–19 in subunit 8 and 70–72 in subunit 9). Overlapping with the second common sequence is a stretch of four residues (Leu-Leu-Phe-Glu) common to both proteins, but in a mirror image conformation to each other (residues 72–75 in subunit 9 and 19–16 in subunit 8).

The five monoclonal antibodies were also tested by Western immunoblotting against an F_0F_1-ATPase preparation (Fig. 3b). As expected, monoclonal antibodies 6A5, 4A2 and 5B2 reacted with a polypeptide with an apparent molecular mass of 45 kDa, which presumably represents the oligomeric form of subunit 9. This oligomer is commonly observed when yeast F_0F_1-ATPase is analysed by SDS-polyacrylamide gel electrophoresis [16,17]; the 45 kDa band was not observed when the monoclonal antibodies were tested against an F_1-ATPase preparation, isolated from yeast mitochondria by chloroform-methanol extraction [13] (data not shown). Antibody 27D8 also recognised the presumptive subunit 9 oligomer, but the relative amount of subunit 8 in the F_0F_1-ATPase was apparently too low for the detection of this subunit by antibody 27D8 in the Western immunoblotting analysis (Fig. 3b).

In contrast to the above antibodies, no reaction with the 45 kDa subunit 9 oligomer was detected when monoclonal antibody 10A4 was tested against the F_0F_1-ATPase by Western immunoblotting (Fig. 3b). Since this antibody recognises monomeric subunit 9 (isolated and purified by extraction in chloroform/methanol [14]), it appears that its epitope on subunit 9 is masked by the interaction of the proteolipid molecules to form the oligomer.

To determine whether the anti-proteolipid antibodies recognise subunits of the F_0-sector in intact ATPase, the effect of three of the antibodies (6A5, 27D8 and 10A4) on the ATPase activity was investigated, as well as their ability to immunoprecipitate the H^+-ATPase complex. Monoclonal antibodies 5B2 and 4A2 were not used because of their relatively low titres.

None of the antibodies appear to neutralise the ATPase activity (Table I) of either isolated mitochondria or glycerol gradient-purified H^+-ATPase; the ATPase activities in the presence of the anti-proteolipid antibodies were very similar to that observed in the absence of antibody. Furthermore, the antibodies did not affect the sensitivity of ATPase activity to oligomycin, an inhibitor of the yeast H^+-ATPase thought to bind to subunit 9 [19]. It appears, therefore, that the

TABLE I

THE EFFECT OF MONOCLONAL ANTIBODIES ON THE ATPase ACTIVITY OF ISOLATED MITOCHONDRIA AND PURIFIED H^+-ATPase

Mitochondria were isolated from a wild-type strain of yeast (strain J69-1b), and H^+-ATPase purified by glycerol gradient centrifugation as previously reported [16]. Antibodies were purified by affinity chromatography on a Protein A-Sepharose column [18]. Excess amount of purified immunoglobulins (200 μg in PBS) were incubated with 50 μg protein of mitochondria or 10 μg H^+-ATPase, in 0.9 ml ATPase reaction mixture (50 μmol Tris-HCl (pH 8.0), 3 μmol $MgCl_2$, 5 μg antimycin A, 1 μmol phospho*enol*pyruvate, 0.3 μmol NADH, 5 units lactate dehydrogenase and 2.5 units pyruvate kinase), for 2 h at 4°C. At the end of the incubation period, 20 μl of 50 mM ATP was added, and ATPase activity measured by following the decrease in the absorbance of NADH at 340 nm. 10 μg oligomycin per 10 μg H^+-ATPase or 100 μg oligomycin per mg mitochondrial protein was added as indicated.

Antibody added	Mitochondria		Purified H^+-ATPase	
	activity (μmol/min per mg protein)	oligomycin inhibition (%)	activity (μmol ATP/min per mg protein)	oligomycin inhibition (%)
None	1.8	83	1.7	71
27D8	2.0	85	2.0	70
10A4	1.9	84	1.8	72
6A5	1.6	80	1.5	68
Preimmune Ig	1.7	82	1.6	69

monoclonal antibodies are either 'null' antibodies which do not affect the activity of subunit 9 or its binding to oligomycin, or alternatively, they do not have access to their epitopes on the proteolipid molecule when the proteolipid is associated with the H^+-ATPase complex.

The ability of the antibodies to bind to subunit 9 and/or subunit 8 in intact H^+-ATPase, was examined by immunoprecipitating the enzyme complex from Triton-X100 solubilized mitochondria with purified monoclonal antibodies, conjugated to CNBr-activated Sepharose 4B, as described previously [1]. For this purpose, cells were labelled with [^{35}S]sulphate in the presence of cycloheximide, which allows incorporation of the isotope into mitochondrial translation products only. No H^+-ATPase subunit 9 (or subunit 8) could be detected when the immunoprecipitates were examined by electrophoresis in SDS-polyacrylamide gels [7] (data not shown), whereas immunoprecipitates obtained with a monoclonal antibody to the β subunit of the enzyme complex [1] showed the three ^{35}S-labelled mitochondrially synthesised subunits: subunit 6 (apparent M_r 20000), subunit 8 (apparent M_r 10000) and subunit 9 (apparent M_r 7600). It appears, therefore, that the three anti-proteolipid antibodies do not recognise subunit 9 in intact H^+-ATPase even though they are able to do so on a Western Immunoblot after the subunits of the enzyme complex have been dissociated by SDS.

The above observations suggest that the epitopes recognised by the antiproteolipid monoclonal antibodies are masked by other subunits of the F_0-sector, or by subunits of the F_1-sector. The characterisation of these epitopes, together with the investigation on whether the monoclonal antibodies would react with native subunit 9 after the removal of F_1- or other F_0-subunits might provide further insight into the folding and the geometry of the proteolipid molecule in the F_0-sector, and its interactions with other subunits of the enzyme complex.

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