

Immunological cross-reactivity of fungal and yeast plasma membrane H^+ -ATPase

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The plasma membrane H^+ -ATPases from fungi and yeasts have similar catalytic and molecular properties. A structural comparison has been performed using immunoblot analysis with polyclonal antibodies directed toward the 102 kDa polypeptide of the plasma membrane H^+ -ATPase from *Neurospora crassa*. A strong cross-reactivity is observed between the fungal H^+ -ATPase and the enzyme from the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Structural homologies are indicated also by the analysis of the cross-reactive peptides originated by proteolytic digestion of *Neurospora* and *S. cerevisiae* purified enzymes. Neither enzyme from these two sources appears to be glycosylated by a highly sensitive concanavalin A affinity assay on blotted proteins. A glycoprotein of M_r 115000 and pI 4.8–5, which comigrates with a cell cycle-modulated protein on 2D gel, is present in partially purified preparations of plasma membrane H^+ -ATPase of *S. cerevisiae* and it is shown to be structurally unrelated to H^+ -ATPase.

H⁺-ATPase 2D gel electrophoresis Immunoblotting Peptide mapping

1. INTRODUCTION

The plasma membrane of fungi and yeasts contains a proton-translocation ATPase (H^+ -ATPase) which constitutes the primary transport system of these cells. The H^+ -ATPase plays vital physiological functions for these microorganisms since a series of H^+ -dependent co-transport system relies on it [1,2] and it also plays a central role in the regulation of intracellular pH [1].

Since the purification of the H^+ -ATPase from the fission yeast *Schizosaccharomyces pombe* [3], followed by that from the mycelial fungus *Neurospora crassa* [4] and by that from the budding yeast *Saccharomyces cerevisiae* [5,6], much information about the molecular properties of the H^+ -ATPases has been gathered.

The plasma membrane H^+ -ATPase appears to be composed of a single catalytic subunit of about

100 kDa, as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of purified enzyme preparations from *N. crassa* [4] and *Sc. pombe* [3] and of partially purified preparations from *S. cerevisiae* [5,6]. The subunits of about 100 kDa from *Sc. pombe* [7,8], *N. crassa* [9] and *S. cerevisiae* [5,10] were also shown to form a phosphorylated intermediate (a β -aspartyl phosphate) during the reaction cycle.

On the basis of these properties, the plasma membrane H^+ -ATPase belongs to the wider group of cation-transport ATPases, which includes the $(Na^+ + K^+)$ -ATPase of mammalian cells, the Ca^{2+} -ATPase of sarcoplasmic reticulum and the $(H^+ + K^+)$ -ATPase of gastric mucosa, characterized by the formation of a covalent phosphorylated intermediate and by the sensitivity to inhibition by vanadate [1].

Here, we have used polyclonal antibodies directed against purified *N. crassa* H^+ -ATPase and the technique of peptide mapping to probe the

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relatedness of the structure of *S. cerevisiae* and *N. crassa* H⁺-ATPases.

2. MATERIALS AND METHODS

2.1. Purification of plasma membrane H⁺-ATPase of *S. cerevisiae* and *N. crassa*

Partially purified preparations of H⁺-ATPase from *S. cerevisiae*, obtained from the wild-type strain Σ 1278b (α) according to [11], were kindly provided by Dr A. Goffeau (University of Louvain-la-Neuve, Belgium). The preparations examined were the pool of the active fractions of sucrose gradients on which plasma membrane enriched fractions solubilized by lysolecithin were separated by density. Purified plasma membrane H⁺-ATPase from *N. crassa* (a gift from Dr C.W. Slayman, Yale University, New Haven, USA) was prepared according to [4].

2.2. Sample preparation

For the analysis by SDS-PAGE, the H⁺-ATPase preparations, provided as lyophilized powder, were reconstituted in water and to appropriate aliquots an equal volume of double-strength SDS sample buffer [12] was added. The samples were then denatured at 37°C for 20 min.

Total protein extract of *S. cerevisiae*, mutant strain 321 [13], and *Sc. pombe*, wild-type strain 972h⁻ [14], were obtained as follows: 10⁸ cells from exponentially growing cultures were rapidly filtered, washed with cold water and resuspended in a test-tube by addition of 400 μ l SDS sample buffer containing 1 mM PMSF and 10 μ g/ml of pepstatin. After removal of the filter an equal volume of glass beads was added and cells broken by vortex-mixing for 4 min. The samples were denatured and after removal of glass beads by centrifugation, aliquots of the supernatants were analyzed by SDS-PAGE.

For the analysis by two-dimensional (2D) gel electrophoresis the lyophilized samples were directly resuspended in urea lysis buffer [15]. ³⁵S-labeled total protein extract from *S. cerevisiae* were prepared as described in [13].

2.3. Electrophoretic procedures

Proteins were resolved by SDS-PAGE according to Laemmli [12] or 2D gel electrophoresis according to O'Farrell [15]. In some cases after elec-

trophoresis gels were stained with Coomassie blue R-250 and destained with 45% methanol, 9% acetic acid in water.

Electrophoretic transfer of proteins from gel to nitrocellulose sheet was carried out as described by Towbin et al. [16], at a constant current of 200 mA for 16–22 h.

2.4. Immunodecoration of blots

After transfer blots were extensively washed in TBS (0.01 M Tris, pH 7.4, 0.9% NaCl) and processed as reported by Zippel et al. [17]. Blots were incubated with 40 μ g/ml of purified antibodies' anti-plasma membrane H⁺-ATPase of *N. crassa*

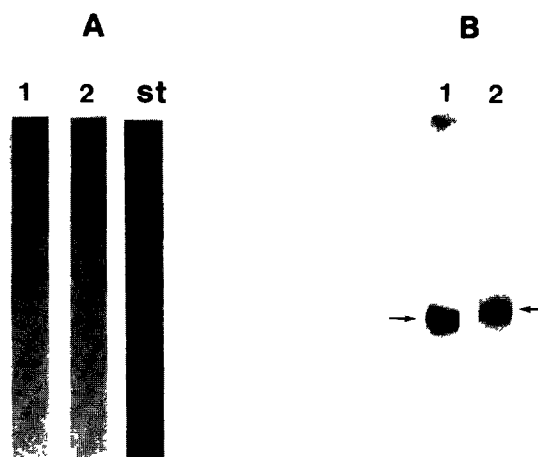


Fig.1. Electrophoretic and immunoblot analysis of H⁺-ATPase preparations from *S. cerevisiae* and *N. crassa*. About 3 μ g of a purified H⁺-ATPase from *Neurospora* (lane 1) and about 5 μ g of a partially purified preparation of purified H⁺-ATPase from *S. cerevisiae* (lane 2) were separated on an 8% polyacrylamide gel. After the run proteins were stained with Coomassie blue (A) or transferred to nitrocellulose sheets and immunodecorated with *Neurospora* antibodies and ¹²⁵I-protein A. Autoradiograms are shown (B). Arrows indicate the 102 kDa and 104 kDa polypeptides. The molecular mass standards (st) are: myosin (200 kDa), β -galactosidase (116.25 kDa), phosphorylase b (92.5 kDa), BSA (66.2 kDa) and ovalbumin (45 kDa).

(kindly provided by Dr C.W. Slayman) in TBS containing 5% bovine serum albumin (BSA). Bound antibodies were revealed by ^{125}I -labeled protein A (NEN). Dried blots were exposed to Kodak X-Omat R films with intensifying screen.

The antibody used was prepared by immunization of rabbits with the 102 kDa band of plasma membrane H^+ -ATPase of *Neurospora* and purified by chromatography on protein A-Sepharose.

2.5. Identification of glycoproteins on nitrocellulose blots

Glycoproteins immobilized on nitrocellulose sheets were detected by their binding to concanavalin A. After transfer, blots were treated using the procedure indicated by Hawkes [18], with the following modifications: 2.5% BSA fraction V in TBS (50 mM Tris-HCl, pH 7.4, 200 mM NaCl) instead of 10% fetal calf serum was used as blocking solution; the first incubation of the filters in blocking solution was prolonged to 1 h.

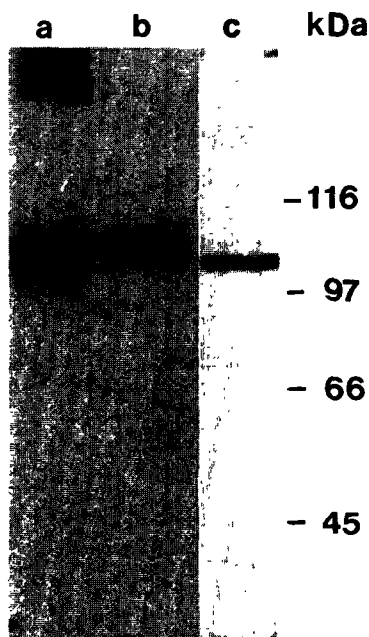


Fig.2. Cross-reactivity of antibody against plasma membrane H^+ -ATPase from *N. crassa* with H^+ -ATPase from *S. cerevisiae* and *Sc. pombe*. Immunoblot of purified plasma membrane H^+ -ATPase from *N. crassa* (about 1 μg) (lane a), of total protein extracts from *S. cerevisiae* (lane b) and *Sc. pombe* (lane c) fractionated by SDS-PAGE.

2.6. Immunoprecipitation and peptide mapping

Exponentially growing cells of *S. cerevisiae* (strain 321) were labeled for 3 h with [^{35}S]methionine (20 $\mu\text{Ci}/\text{ml}$) and broken as previously described except for the presence of 1% SDS in buffer A containing 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ pepstatin [13]. The extract was heated at 37°C for 15 min and about 3×10^7 trichloroacetic acid-precipitable dpm of labeled extract were used for the immunoprecipitation. The extract was added with RIPA buffer [19] containing 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ pepstatin and 10 $\mu\text{g}/\text{ml}$ leupeptin, so that the final concentration of SDS was 0.1%. The clarified supernatant was immunoprecipitated first with pre-immune antibodies and then with *Neurospora* antibody. The immunocomplexes

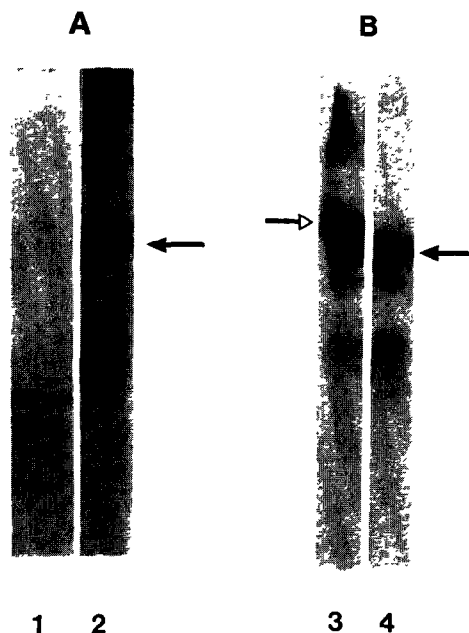


Fig.3. Assay of the affinity to concanavalin A of preparations of plasma membrane H^+ -ATPase. About 1 μg purified H^+ -ATPase from *N. crassa* (A) and about 3 μg of a partially purified preparation of *S. cerevisiae* H^+ -ATPase (B) were separated on an 8% SDS-polyacrylamide gel and after blotting to nitrocellulose each lane was cut into two strips; one was treated with concanavalin A and peroxidase (lanes 1,3), the other was immunodecorated with *Neurospora* antibodies and ^{125}I -protein A (lanes 2,4). Closed arrows indicate the 102 kDa and 104 kDa polypeptides while the open arrow denotes the 115 kDa glycoprotein.

were absorbed with protein A-Sepharose. The final pellet was resuspended in SDS sample buffer, denatured for 30 min at 56°C and analyzed by SDS-PAGE. The stained and dried gel was exposed to a Kodak film. The bands of H⁺-ATPase stained in Coomassie blue or the [³⁵S]methionine-labeled spots were excised from 1D or 2D gels and rerun on a 15% SDS-polyacrylamide gel in the presence of *Staphylococcus aureus* V8 protease (0.5, 0.05 or 0.005 µg) according to Cleveland et al. [20].

3. RESULTS AND DISCUSSION

Preparations of purified plasma membrane H⁺-ATPase from *N. crassa* and from *S. cerevisiae* were fractionated by SDS-PAGE. After staining

with Coomassie blue a single band is detected in both preparations (fig.1A), its molecular mass being about 102 kDa for the *N. crassa* enzyme (lane 1) and 104 kDa for the *S. cerevisiae* enzyme (lane 2).

The fractionated proteins were transferred to nitrocellulose sheets and immunodecorated with a polyclonal antibody raised against purified *N. crassa* H⁺-ATPase. As shown in fig.1B, the antibody detects only the 102 kDa band in *N. crassa* (lane 1) and the 104 kDa band in *S. cerevisiae* (lane 2). The reaction is specific, since no other cross-reacting material is detected in total proteins from exponentially growing cultures of *S. cerevisiae* (fig.2, lane b). In total proteins from exponentially growing *Sc. pombe* cells the antibody detects only a 102 kDa band (fig.2, lane c).

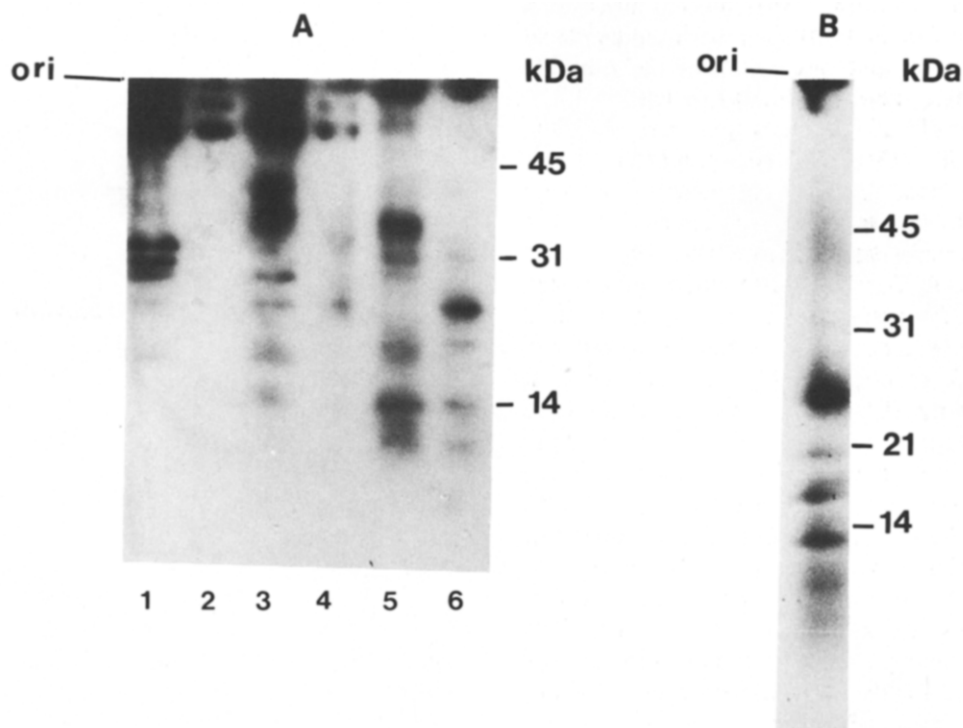


Fig.4. Peptide mapping analysis of *S. cerevisiae* and *N. crassa* H⁺-ATPase. (A) Preparations of H⁺-ATPase were separated by SDS-PAGE as described in fig.1A. The 102 kDa and 104 kDa protein bands were excised from several tracks and rerun on a 15% SDS-polyacrylamide gel with increasing concentrations of *St. aureus* V8 protease (lanes 1,2, 0.005 µg; lanes 3,4, 0.05 µg; lanes 5,6, 0.5 µg). Lanes: 1,3,5, H⁺-ATPase of *N. crassa*; 2,4,6, H⁺-ATPase from *S. cerevisiae*. Immunodetection of cross-reactive peptides as described in text. (B) The ³⁵S-labelled protein band immunoprecipitated by antibodies against H⁺-ATPase of *Neurospora* was excised from an 8% gel and run on a 15% polyacrylamide gel in the presence of 0.5 µg *St. aureus* V8 protease. The gel was fluorographed and the autoradiogram is shown.

To ascertain whether the plasma membrane H^+ -ATPase is a glycosylated protein the concanavalin A (Con A) binding assay was performed according to Hawkes [18]. The preparation of *N. crassa* H^+ -ATPase was fractionated by SDS-PAGE and blotted on a nitrocellulose filter. One strip was stained with amido black (not shown) and the other cut into two strips, one of which was treated with Con A while the other was immunodecorated as usual with the antibody against *N. crassa* H^+ -ATPase. As shown in fig.3A, lane 1, no reaction with Con A occurred at the level of the 102 kDa band of the *N. crassa* H^+ -ATPase (lane 2), thus indicating that the enzyme is not glycosylated.

The preparation of *S. cerevisiae* H^+ -ATPase was then processed in the same way (fig.3B) and again no positive reaction occurred at the level of the 104 kDa H^+ -ATPase polypeptide (lane 4), although the Con A assay detects the presence of a contaminating glycoprotein of slightly higher molecular mass (about 115 kDa) (lane 3).

The analysis by 2D gel electrophoresis according to O'Farrell [15] of the preparations of H^+ -ATPase from *N. crassa* and *S. cerevisiae* were unsuccessful: the enzyme (as detected by the specific antibody) did not move in the first dimension (isoelectric focusing) also under denaturing conditions known to solubilize membrane proteins [21]. The preparations were then subjected to reverse 2D electrophoresis [22]. Proteins were first fractionated by SDS-PAGE, then gel slices were cut out in the region around 100–110 kDa and the gel slices subjected to isoelectric focusing in the presence of various detergents (NP40, CHAPS, sulfobetain SB12, and a modified sulfobetain). The proteins were then transferred to nitrocellulose and immunodecorated with the antibody against *Neurospora* H^+ -ATPase and ^{125}I -protein A. The apparent pI determined in this way was about 8.0 for the *N. crassa* enzyme and about 8.0–8.5 for the *S. cerevisiae* enzyme (not shown). The cloned gene sequence of the *S. cerevisiae* H^+ -ATPase has been reported [23]. From the deduced amino acid composition, the pI of the protein has been calculated [24] and is quite different from that found experimentally. We believe therefore that the apparent pI of 8.0–8.5 is an artefact due to the known poor solubility of fungal and yeast plasma membrane H^+ -ATPases [5].

To investigate further the structural relatedness between these plasma membrane H^+ -ATPases, the 104 kDa polypeptide from *S. cerevisiae* and the 102 kDa polypeptide from *Neurospora* were fractionated by SDS-PAGE as described in fig.1A. The bands containing the proteins were cut from wet stained gels and rerun on 15% polyacrylamide gel in the presence of increasing concentrations of V8 protease, transferred to a nitrocellulose sheet and then immunodecorated with antibody against H^+ -ATPase of *N. crassa* and ^{125}I -protein A.

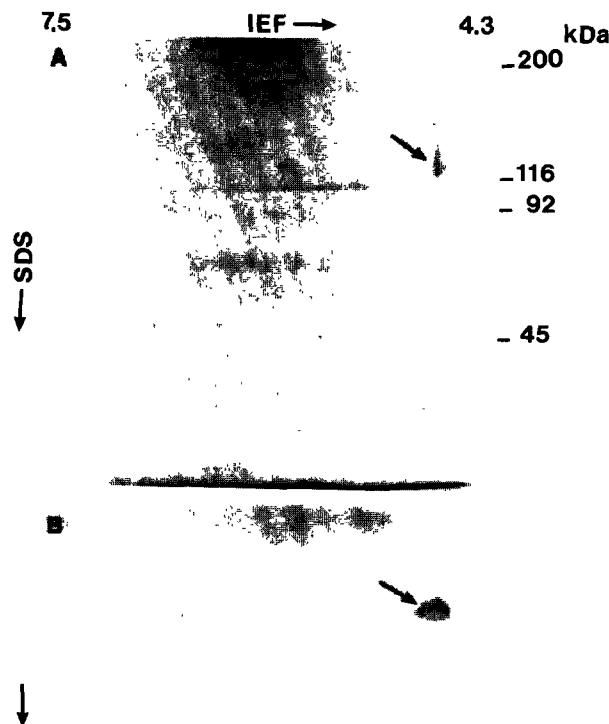


Fig.5. 2D gel of partially purified plasma membrane H^+ -ATPase of *S. cerevisiae*. About 45 μ g protein were dissolved in urea lysis buffer [15] and subjected to 2D gel electrophoresis. (A) Coomassie-stained gel. (B) Blot treated with concanavalin A and peroxidase. Arrows indicate the 115 kDa glycoprotein.

As shown in fig.4A the immunoblot of the peptide mapping is fairly different for the two enzymes at the lower protease concentrations used, while it appears more similar at the highest one. In fact, at 500 ng protease (lanes 5,6), the *N. crassa* H⁺-ATPase gives rise to 5 major bands of about 35, 30, 20, 15 and 12 kDa, while the 5 bands yielded for *S. cerevisiae* H⁺-ATPase have molecular masses of about 30, 24, 20, 15 and 12 kDa.

A similar pattern of peptide mapping was found for *S. cerevisiae* H⁺-ATPase when the antibody was used to immunoprecipitate the cross-reacting protein labelled with [³⁵S]methionine, which was then subjected to proteolytic digestion by V8 protease. Fig.4B shows the ensuing radioactive peptides of molecular mass around 24, 20, 15 and 12 kDa.

In conclusion, the plasma membrane H⁺-ATPases of *Neurospora* and *S. cerevisiae*, which are not glycosylated proteins, appear to

have a fair degree of molecular similarity since they yield several proteolytic peptides of the same molecular mass which react strongly with the antibody against *Neurospora* H⁺-ATPase. One *S. cerevisiae* peptide (24 kDa, fig.4A) has an especially high level of cross-reactivity suggesting a very conserved protein sequence. Elucidation of the sequence of *Neurospora* H⁺-ATPase and comparison with that of *S. cerevisiae* [23] will allow these results to be placed in the right perspective.

From our data the single potential N-linked glycosylation site present in the protein sequence of *S. cerevisiae* H⁺-ATPase [23] does not appear to be glycosylated. The contaminant 115 kDa glycoprotein, gp115, present in the preparation of H⁺-ATPase of *S. cerevisiae* (fig.3B) was further characterized. It migrates on 2D gel electrophoresis [15] with a pI of 4.8–5.0 (fig.5A) and reacts strongly with Con A (fig.5B).

In experiments of coelectrophoresis of the

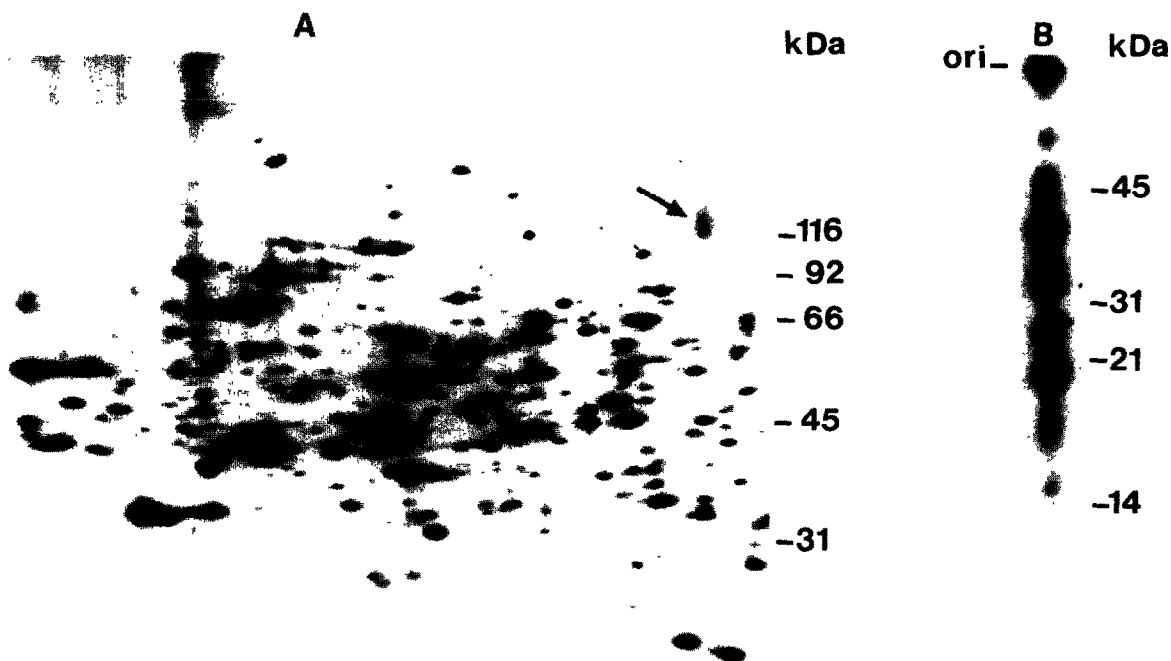


Fig.6. 2D gel of total protein of *S. cerevisiae* and peptide map of gp115. (A) Exponentially growing cells were labelled for 3 h with [³⁵S]methionine (20 μ Ci/ml). An extract for 2D gel electrophoresis was prepared. The arrow indicates gp115. (B) The radioactive spot corresponding to gp115 was excised and subjected to partial proteolytic digestion, with 0.5 μ g of protease as described previously.

purified preparation of H^+ -ATPase from *S. cerevisiae* with ^{35}S -labeled proteins from exponentially growing cells of *S. cerevisiae*, the 115 kDa polypeptide comigrates on 2D gel, in an area in which very few proteins are present, with the in vivo labeled gp115 (fig.6A), a glycoprotein whose synthesis and processing are known to be cell-cycle modulated [13,25]. Comparison of the peptide map of ^{35}S -labeled gp115 (fig.6B) and of H^+ -ATPase (fig.4B) shows many differences, thus indicating that the two proteins are structurally unrelated. The tight association of gp115 with H^+ -ATPase may be due to similar performance during the purification procedure or to a physical association.

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