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# Immunochemical characterization of the modulator protein of the ATP,Mg-dependent protein phosphatase

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Polyclonal antibodies raised against the modulator protein of the ATP,Mg-dependent protein phosphatase completely neutralize all known properties of the purified modulator: inhibition or inactivation of the phosphatase catalytic subunit as well as the kinase $F_A$-mediated activation of the ATP,Mg-dependent phosphatase. They do not cross-react with phosphoinhibitor-1 or the phosphatase catalytic subunit. Direct analysis of boiled or unboiled skeletal muscle extracts by Western blotting reveals a 32 kDa polypeptide corresponding to the modulator protein as the most dominant protein staining band.

ATP,Mg-dependent protein phosphatase; Modulator protein; Polyclonal antibody

## 1. INTRODUCTION

The modulator (M) protein is the thermostable regulatory subunit of the cytosolic ATP,Mg-dependent protein phosphatase and as such, it modulates the transition of the catalytic subunit between its active ($F_C^*$) and inactive ($F_C$) conformation. In the free state (boiled), the modulator also causes an instantaneous inhibition of the phosphorylase phosphatase activity of the catalytic subunit $F_C^*$ [1].

Purification of 'free' modulator always includes a heat treatment [2] and produces a 32 kDa protein on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Its complete primary structure was determined recently [3]. The inclusion of the boiling step has raised some questions about the native structure of the modulator protein in tissue extracts [4–7]. We have shown that unboiled tissue extracts do not contain a 'free' modulator, but that the modulator activity is always associated in higher molecular mass complexes [4–6]. Such a complex is the inactive [$F_C$M] enzyme itself, but a second modulator containing the [MX] complex has been purified and characterized in rabbit skeletal muscle [6]. Both complexes were shown to contain a 32 kDa modulator subunit complexed to a 38 kDa heat-labile protein [4,6].

Earlier studies using polyclonal antibodies against the modulator protein either provided evidence for the existence of a 60 kDa modulator protein [7], or suggested that the modulator is present in tissues exclusively as a 32 kDa species [8]. In all cases, the antibodies were only shown to block the inhibitory properties of the modulator protein [7–9], while ATP,Mg-dependent phosphatase activity has been detected in some immunoprecipitates [9]. This would suggest that the antibodies raised against the free modulator recognize the [$F_C$M] enzyme, but do not prevent the kinase $F_A$-mediated activation of this inactive phosphatase.

In order to reinvestigate these contradictory and confusing results, we produced polyclonal antibodies against the modulator protein and examined their capability to block the different [1] modulator activities (activation, inactivation and inhibition); Western blotting experiments reveal a

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32 kDa protein in boiled or unboiled rabbit skeletal muscle extracts.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Nitrocellulose membranes were purchased from LKB, DEAE-Sephacel from Pharmacia, bovine serum albumin from Serva and complete Freund's adjuvant, horseradish peroxidase-conjugated rabbit anti-goat IgG, 4-chloro-1-naphthol, trypsin and trypsin inhibitor from Sigma. Modulator protein [2], protein kinase $F_A$ [10], inactive ATP,Mg-dependent phosphatase $F_CM$ [6], its active catalytic subunit $F_C^*$ [11] and inhibitor-1 [12] were purified from rabbit skeletal muscle.

The activity of the ATP,Mg-dependent phosphatase was commonly measured after a 10 min preincubation at 30°C with 0.2 mM ATP, 1 mM $Mg^{2+}$ and the minimal amount of kinase $F_A$ required for full activation. Before the phosphorylase phosphatase assay, a 2 min trypsin treatment was performed, followed by the addition of an excess of soybean trypsin inhibitor. The specific activity of the free inactive catalytic subunit $F_C$ [13] was 18000 U/mg in the presence and 1300 U/mg in the absence of an optimal amount of modulator, when measured as outlined above. The phosphatase assay time was 5 min. One unit of phosphatase releases 1 nmol [$^{32}$P]phosphate/min at 30°C from $^{32}$P-labeled phosphorylase *a* (1 mg/ml).

Inhibitor-1 was phosphorylated by the cyclic AMP-dependent protein kinase [14] and reisolated by an additional boiling step. The bovine heart cyclic AMP-dependent protein kinase (catalytic subunit) was a generous gift of D.A. Walsh (USA).

### 2.2. *Methods*

#### 2.2.1. Preparation and purification of antibodies against the modulator subunit

Antibodies against the modulator protein were raised in a

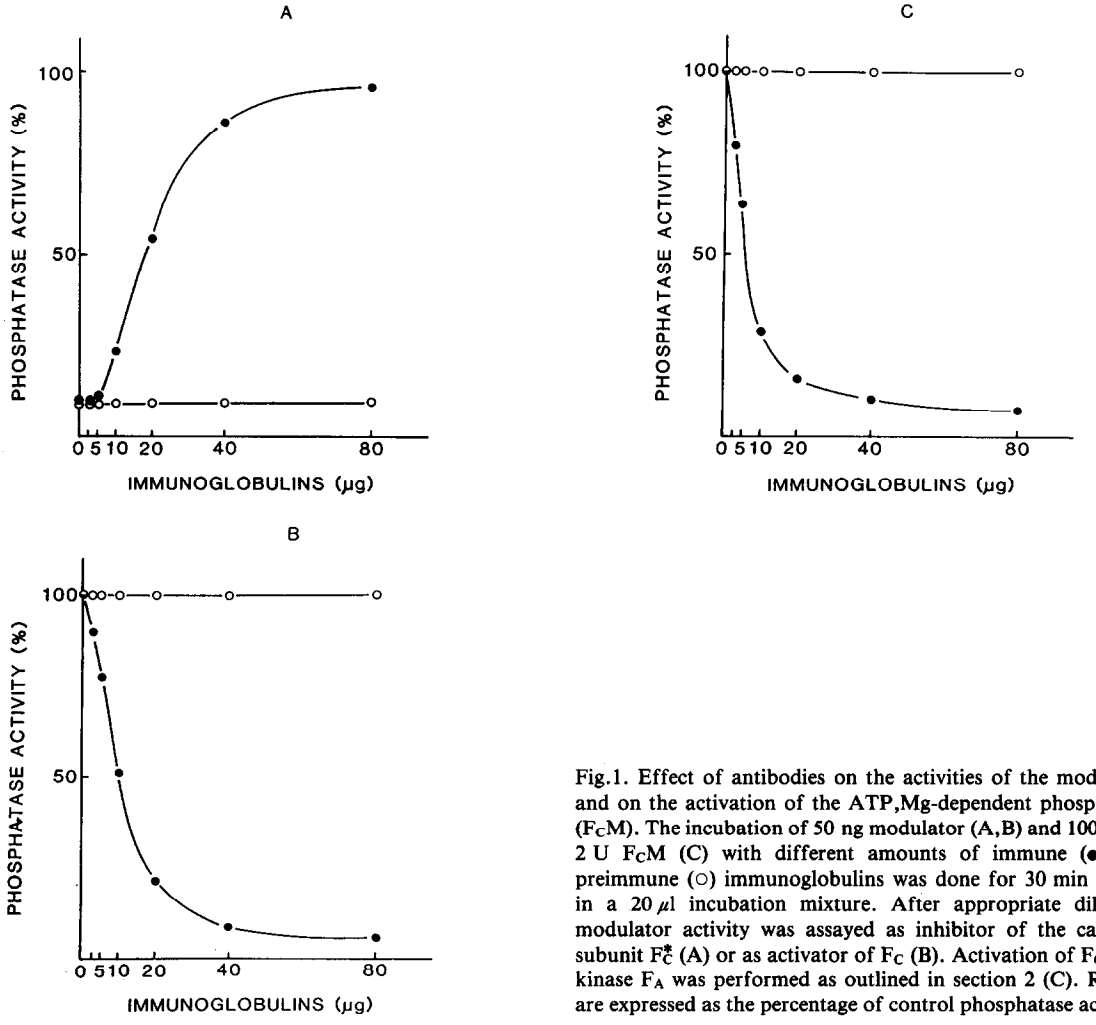



Fig.1. Effect of antibodies on the activities of the modulator and on the activation of the ATP,Mg-dependent phosphatase ($F_CM$). The incubation of 50 ng modulator (A,B) and 100 ng or 2 U $F_CM$ (C) with different amounts of immune (•) and preimmune (○) immunoglobulins was done for 30 min on ice in a 20 μl incubation mixture. After appropriate dilution, modulator activity was assayed as inhibitor of the catalytic subunit $F_C^*$ (A) or as activator of $F_C$ (B). Activation of $F_CM$ by kinase $F_A$ was performed as outlined in section 2 (C). Results are expressed as the percentage of control phosphatase activity.

female goat by injection of homogeneous modulator purified as in [2]. Antigen in complete Freund's adjuvant was injected subcutaneously at 3-week intervals using 1 mg for the primary and 0.5 mg for the booster injections. Blood was collected prior to immunization and after each injection. The production of antibodies was determined by Ouchterlony double immunodiffusion.

Immunoglobulins from antisera and preimmune serum were prepared as follows. Blood was allowed to clot and serum was collected. Immunoglobulins were precipitated with 40% saturated $(NH_4)_2SO_4$ and the pellet was washed twice in the original volume of 40% saturated $(NH_4)_2SO_4$. The centrifugation was repeated, the pellet resuspended in 10 mM Tris-HCl, 150 mM NaCl at pH 7.4 (buffer A) and extensively dialyzed against 10 mM Tris-HCl, pH 8 (buffer B). The sample was loaded on a column of DEAE-Sephacel (at least 10 ml bed volume for each 100 mg of protein) and washed with buffer B. When the absorbance of the eluate at 280 nm reached zero, the immunoglobulins were eluted with a linear gradient of 0–300 mM NaCl in buffer B (volume of the gradient = 10-fold the bed volume of the column) and concentrated by dialysis against 20% polyethylene glycol in buffer A. The IgG fractions were stored in aliquots at −20°C and used in all subsequent experiments. Analysis of the preparation by SDS-PAGE showed major bands corresponding to the heavy and light chains of immunoglobulin G (not shown).

2.2.2. Gel electrophoresis, Western transfer and immunoblotting

Polyacrylamide slab gel electrophoresis was performed in the presence of SDS, according to the method of Laemmli [15]. Western transfer of proteins onto nitrocellulose membranes was done with the Multiphor II Novablot System (LKB), using a discontinuous buffer system according to the manufacturers instructions. Following protein transfer, the membrane was incubated successively with 50 ml quenching solution (5% bovine serum albumin in buffer A) for 30 min to block nonspecific binding sites, with purified immunoglobulins in 50 ml quenching solution for 4 h, and with horseradish peroxidase-conjugated rabbit anti-goat IgG in 25 ml quench solution for 40 min. In between incubations the membrane was washed 4 times for 5 min with 50 ml of buffer A containing 0.1% (v/v) Triton X-100 and 2 times for 5 min with 50 ml of buffer A. Immunoreactive bands were then visualized by immersion in a mixture of 50 ml buffer A, 12 ml of 0.3% 4-chloro-1-naphthol in methanol and 25 μl of 30% $H_2O_2$. The positions of the marker proteins were localized by brief staining with 0.1% amido black in 45% methanol/10% acetic acid.

## 3. RESULTS AND DISCUSSION

The results presented in this study demonstrate that the polyclonal antibodies raised in goat against the modulator protein are specific in blocking all known modulator activities. Incubation of the immunoglobulins with a purified modulator preparation resulted in a dose-dependent reduction of the modulator activities: inhibition of the phosphatase catalytic subunit $F_C^*$ (fig.1A) and of the activation of $F_C$ (fig.1B). No effects were seen when the same sample was incubated with preimmune IgG. Boiling the immunocomplex released the heat-stable modulator protein, which exhibited both of the activities mentioned above. The antibodies did not cross-react with phosphoinhibitor-1 or with the phosphatase catalytic subunit, not even when very high concentrations were used during incubations with the immunoglobulins (not shown).

The inactive ATP,Mg-dependent phosphatase $F_CM$ is activated via a transient phosphorylation of the modulator subunit by protein kinase $F_A$, inducing the isomerization of the catalytic subunit into the active conformation [13]. Incubation of $F_CM$ with the immunoglobulins blocked this activation in a dose-dependent manner (fig.1C). These results are in contrast with those presented by other investigators who were able to detect ATP,Mg-dependent phosphatase activity associated with their immunoprecipitates [9]; the reason for this discrepancy is not clear.

The phosphatase catalytic subunit $F_C^*$ is inac-

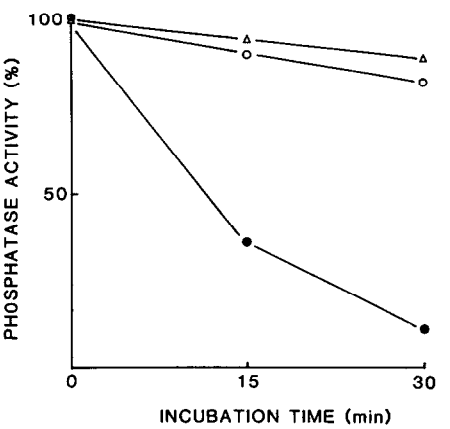
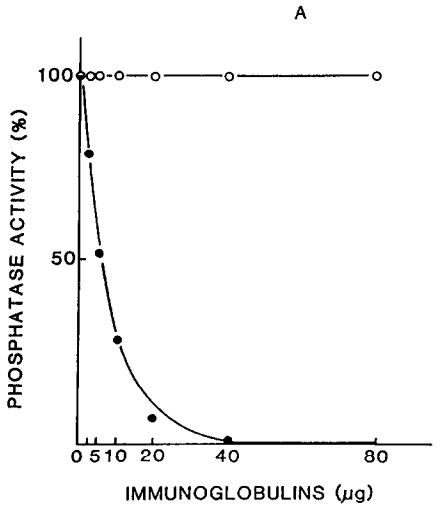



Fig.2. Effect of antibodies on the inactivation of the active catalytic subunit ($F_C^*$) by the modulator protein. Modulator (50 ng) was incubated with 80 μg of preimmune (○) and immune (●) immunoglobulins for 30 min on ice in a 20 μl incubation mixture. An aliquot of this mixture (5 μl) was incubated at 30°C with 250 mU of the catalytic subunit $F_C^*$ in a 100 μl incubation mixture. A control incubation without addition of modulator or immunoglobulins (△) was also performed. The phosphorylase phosphatase activity was determined at different time intervals after a 2 min trypsin treatment. Results are expressed as the percentage of control activity.



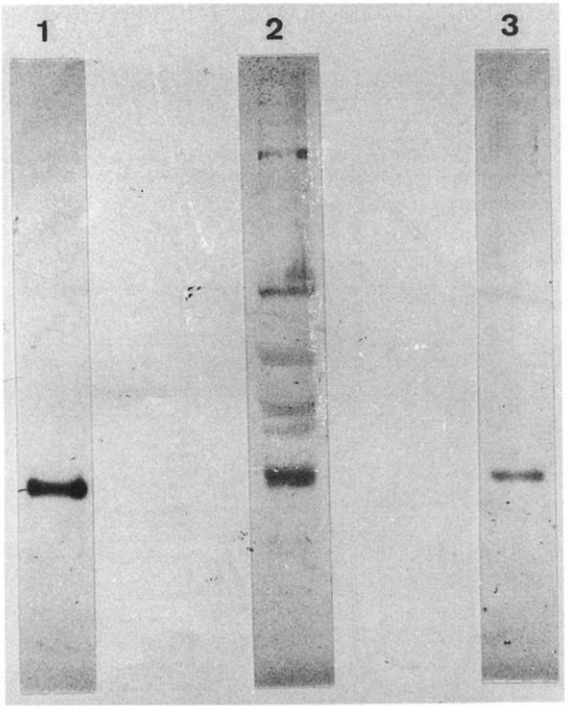




Fig.3. Effect of antibodies on the modulator activity of a boiled skeletal muscle extract and immunoblot analysis. (A) A boiled skeletal muscle extract (30 μl of 20 mg/ml) was incubated with different amounts of immune (●) and preimmune (○) immunoglobulins for 30 min on ice in a 40 μl incubation mixture. After appropriate dilution, modulator activity was assayed as activator of $F_C$. Results are expressed as percentage of control activity. (B) Immunoblot analysis of 40 ng purified modulator (lane 1) and of an equivalent amount of unboiled (lane 2) and boiled (lane 3) skeletal muscle extract.

tivated by exogenously added modulator, resulting in an enzyme that can be reactivated by kinase $F_A$. This inactivation is prevented when enough immunoglobulins are present to complex all the modulator (fig.2). These results suggest that the antibodies recognize not only both the inhibition and the activation site on the modulator protein, but also the site where modulator interacts with the active catalytic subunit thereby inducing the inactivation.

The antibodies inhibit not only the activity of purified modulator preparations but also that of boiled tissue extracts. Incubation of a boiled skeletal muscle extract (10000 × *g* supernatant fraction) with the immunoglobulins resulted in a dose-dependent reduction of the modulator activity, whereas preimmune IgG did not (fig.3A).

When a sample of the rabbit skeletal muscle extract was subjected to SDS-PAGE, transferred to nitrocellulose and probed with the immunoglobulins, several immunoreactive species were found. A 32 kDa polypeptide corresponding to the modulator protein (fig.3B) was always clearly present. Boiling the extract did not create new antigenic determinants and did not produce an increase in the amount of the 32 kDa modulator species. On the contrary, boiling the extract always resulted in some loss of immunoreactive 32 kDa protein. These results provide strong evidence that the modulator is indeed present as a 32 kDa protein both in unboiled and boiled rabbit skeletal muscle extracts.

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