# Phosphorylation of the modulator protein of the ATP,Mg-dependent protein phosphatase by casein kinase TS

## Reversal by PCS phosphatases and control by distinct phosphorylation site(s)

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The phosphorylation by casein kinase TS (II) of the modulator protein of the ATP,Mg-dependent phosphatase increases after preincubation with the $PCS_{H1}$ phosphatase or with the catalytic subunit of the ATP,Mg-dependent phosphatase. Dephosphorylation by the two phosphatases combined leads to the incorporation of 2 mol phosphate per mol modulator (at Ser residues). Occupancy of the ATP,Mg-dependent phosphatase phosphorylation site(s) is a negative determinant in the phosphorylation of the modulator by kinase TS. Among the PCS phosphatases $PCS_{H1}$ shows the highest activity toward the $^{32}$P-Ser residues labeled by kinase TS in untreated or previously dephosphorylated modulator, while the ATP,Mg-dependent phosphatase is totally ineffective. Protamine stimulates all phosphatase activities, so that the catalytic subunit of the ATP,Mg-dependent phosphatase becomes almost as effective as the $PCS_C$ phosphatase in dephosphorylating the kinase TS sites.

*Polycation-stimulated protein phosphatase* *ATP,Mg-dependent protein phosphatase* *Modulator protein* *Protein kinase $F_A$* *Casein kinase TS (II)*

## 1. INTRODUCTION

The modulator protein (inhibitor-2) is the thermostable regulatory subunit of the ATP,Mg-dependent protein phosphatase which modulates the transition of the catalytic subunit between its active and inactive conformation [1]. Its complete primary structure was determined recently [2]. The phosphorylation of modulator protein by kinase $F_A$ on Thr residue 72 [2] induces the conversion of the inactive catalytic subunit into its activated conformation [3,4]. Phosphorylation of the modulator protein by casein kinase TS (II) on Ser residues 86, 120 and 121 [5] does not affect the enzyme activity directly, but was reported to potentiate the phosphorylation and activation by kinase $F_A$ [6].

Besides the intramolecular autodephosphorylation involved in the activation of the ATP,Mg-dependent protein phosphatase [4], the isolated modulator phosphorylated by kinase $F_A$ can be dephosphorylated also by calcineurin and by the catalytic subunit of the polycation-stimulated (PCS) protein phosphatases [7]. However, in vivo phosphoseryl residues largely predominate over phosphothreonine [8]. Recently we have purified a number of PCS phosphatases: $PCS_{H1}$, $PCS_{H2}$, $PCS_M$, $PCS_L$ phosphatase [9] and the catalytic subunit $PCS_C$ phosphatase [10]. To learn more about the activation-inactivation of the ATP,Mg-

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dependent protein phosphatase it was of interest to investigate the specificity of the different protein phosphatases toward the modulator in function of the phosphorylation by casein kinase TS.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

The PCS protein phosphatases [9,10], modulator protein [11], protein kinase $F_A$ [12] and the active catalytic subunit of the ATP,Mg-dependent protein phosphatase [10] were purified from rabbit skeletal muscle. One unit of phosphatase activity is the amount of enzyme that catalyzes the release of 1 nmol [$^{32}$P]phosphate per min at 30°C in a 30 $\mu$l assay containing 1 mg per ml of $^{32}$P-phosphorylase. Casein kinase TS was obtained from rat liver as in [13], 1 unit being the amount of enzyme incorporating 1 nmol of phosphate in 2 mg casein per ml and per min at 37°C. Phosphorylase *b* kinase [14] was a gift of Dr D.A. Walsh (Davis, USA). Whole casein was prepared as in [15]. Protamine hydrochloride was obtained from Sigma, Sephadex G-50 from Pharmacia [$\gamma$-$^{32}$P]ATP from the Radiochemical Centre, Amersham and the standard molecular mass markers used in SDS-PAGE from Bethesda Research Laboratories.

### 2.2. *Preparation of $^{32}$P-labeled protein substrates*

$^{32}$P-labeled phosphorylase *a* was prepared as described [16]. The modulator was phosphorylated by casein kinase TS during 15 min at 30°C, the incubation mixture (100 $\mu$l) containing 0.15 mg per ml modulator, 1 U per ml casein kinase TS, 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 25 mM $\beta$-mercaptoethanol, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM [$\gamma$-$^{32}$P]ATP (spec. act. 2000–3000 cpm per pmol). The reaction was stopped by boiling during 5 min. After being left on ice for 10 min, the sample was centrifuged through 1 ml Sephadex G-50 [17] equilibrated in 0.5 mM dithiothreitol, 20 mM Tris-HCl, pH 7.4, buffer, in order to reisolate the $^{32}$P-labeled modulator. When the modulator protein was dephosphorylated before the $^{32}$P-labeling with casein kinase TS, the regulatory protein was first incubated with the $PCS_{H1}$ and ATP,Mg-dependent phosphatase during 15 min at 30°C in 30 $\mu$l of a medium containing 20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol and 1 mg per ml bovine serum albumin; after boiling, cooling and centrifugation at 3200 × *g* during 2 min the phosphorylation reaction was carried out on the supernatant fraction prepared as described above.

### 2.3. *Dephosphorylation of the $^{32}$P-labeled modulator protein*

The reaction was carried out in a 10 $\mu$l incubation mixture containing 0.25 $\mu$M $^{32}$P-modulator, 20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol and 1 mg per ml bovine serum albumin and stopped after 10 min at 30°C by the addition of electrophoresis buffer (2% SDS, in the presence of 1% $\beta$-mercaptoethanol, 10% glycerol and 0.5 M Tris-HCl, pH 8.3) followed by gel-electrophoretic analysis according to the Laemmli method [18]. The gels were dried and autoradiograms were prepared using Curix RP1 X-ray film (Agfa-Gevaert) and intensifying screens. The phosphate incorporated was estimated by cutting out the radiolabeled modulator and counting the radioactivity in the scintillant liquid but also by densitometrical analysis of the autoradiograms with an LKB density laser ultroscan, representing the control values as 100%.

### 2.4. *Phosphoamino acid analysis*

To identify and quantify the phosphorylated amino acids, 10 $\mu$l samples containing 3 pmol of $^{32}$P-modulator were hydrolyzed during 4 h at 110°C in 6 N HCl. The HCl was removed by evaporation and the samples analyzed by thin layer electrophoresis at pH 1.9 with a buffer containing 2.5% formic acid and 7.8% acetic acid. After 4 h at 500 V the plate was dried and the autoradiogram prepared. The $^{32}$P-labeled amino acid was identified by comparison with the migration of the standard reference stained with ninhydrin.

## 3. RESULTS

### 3.1. *Phosphorylation of the modulator protein*

As illustrated in fig.1 the phosphorylation of the untreated modulator by casein kinase TS reaches a plateau after 15 min and no more than 0.5 mol phosphate per mol modulator can be incorporated. Prior dephosphorylation with $PCS_{H1}$ phosphatase or with the active catalytic subunit of the ATP,Mg-

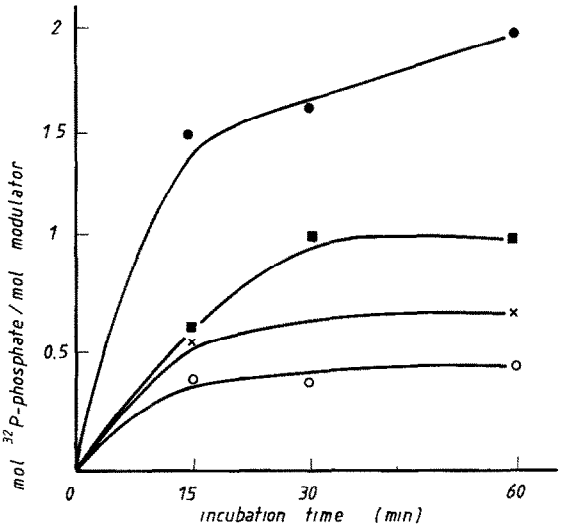


Fig.1. Time-dependent phosphorylation of untreated and previously dephosphorylated modulator protein by casein kinase TS. 0.1 mg modulator per ml was incubated in the presence of 0.4 U per ml kinase TS. At the times indicated aliquots of the incubation mixture were taken and proteins separated by SDS-PAGE. After slicing the gel, $^{32}$P-label in the modulator without pretreatment (○), after previous dephosphorylation by 2 U per ml $PCS_{H1}$ phosphatase (×), by 10 U per ml ATP,Mg-dependent phosphatase, catalytic subunit (■), or by both phosphatases (●), was determined on the basis of cpm. Procedures as described in section 2.

dependent phosphatase resulted in an increased incorporation of phosphate by casein kinase TS. After treatment of the modulator with the two types of phosphatases combined, up to 2 mol phosphate per mol modulator could be incorporated. No significant phosphorylation of the modulator protein by kinase $F_A$ was observed under conditions optimized for casein kinase TS activity (see section 2), after dephosphorylation of the modulator by $PCS_{H1}$ phosphatase and/or the catalytic subunit of the ATP,Mg-dependent phosphatase (table 1). A very low phosphorylation level by kinase $F_A$ could be found after a 16-fold longer exposure time of the autoradiograms, resulting in overexposure of the modulator labeled by kinase TS, an observation also made previously [6]. The increased phosphorylation of the dephosphorylated modulator by kinase TS is accompanied by a synergistic increase in phosphorylation by kinase $F_A$.

Table 1

Synergistic phosphorylation of the modulator protein by casein kinase TS and protein kinase $F_A$

| Modulator | mol [$^{32}$P]phosphate per mol of modulator | | |
|---|---|---|---|
| Pretreatment: | kinase TS | kinase $F_A$ | kinase TS + $F_A$ |
| None | 0.45 | n.d. | 0.8 |
| $PCS_{H1}$ phosphatase | 0.7 | n.d. | 1.1 |
| ATP,Mg-dependent phosphatase catalytic subunit | 0.8 | n.d. | 1.3 |
| Both phosphatases | 1.2 | n.d. | 2.0 |

The modulator was dephosphorylated by 7 U per ml of the protein phosphatases as described in section 2. 50 μg modulator per ml was then incubated with 0.4 U kinase TS and/or 5 μg kinase $F_A$ (100 ng per ml of which activates completely 0.6 U $F_CM$ per ml within 10 min in an assay described in [12]) per ml, during 15 min at 30°C. After slicing the gel, $^{32}$P-label in the modulator was determined on the basis of cpm as described in section 2

### 3.2. *Dephosphorylation of the $^{32}$P-labeled modulator protein*

Five different PCS protein phosphatases, termed $PCS_{H1}$, $PCS_{H2}$, $PCS_M$, $PCS_L$, $PCS_C$, and the active catalytic subunit of the ATP,Mg-dependent protein phosphatase, were tested for their ability to dephosphorylate modulator $^{32}$P-labeled by casein kinase TS. As shown in table 2 the $PCS_{H1}$ phosphatase displays the highest activity, while the $PCS_C$ phosphatase and the catalytic subunit of the ATP,Mg-dependent phosphatase are ineffective. These observations were not significantly different when the modulator protein was phosphorylated after prior dephosphorylation.

As shown in table 3 protamine stimulates the dephosphorylation of modulator ($^{32}$P-labeled by casein kinase TS) by the PCS phosphatases, but also allows for the expression of activity by the $PCS_C$ phosphatase and by the catalytic subunit of the ATP,Mg-dependent phosphatase. The optimal concentrations of protamine for stimulation of the different PCS phosphatases are in the same range or even slightly lower than with phosphorylase as the substrate [9]. Stimulation of the different phosphatases was also observed with 1 mM $Mn^{2+}$ (not shown).

Table 2

Dephosphorylation of the casein kinase TS $^{32}$P-labeled modulator

| Phosphatases (U/ml) | Pretreatment of the $^{32}$P-modulator | | | |
|---|---|---|---|---|
| | (none) native | $PCS_{H1}$ phosphatase | ATP,Mg-dependent phosphatase | Combined phosphatases |
| None | 100 | 100 | 100 | 100 |
| $PCS_{H1}$ phosphatase | | | | |
| 0.5 | 58 | 60 | 50 | 55 |
| 2 | 28 | 40 | 40 | 30 |
| 4 | 8 | 5 | 10 | 10 |
| $PCS_{H2}$ phosphatase | | | | |
| 0.5 | 100 | 98 | 72 | 100 |
| 2 | 89 | 81 | 65 | 88 |
| 8 | 73 | 71 | 62 | 61 |
| $PCS_{M}$ phosphatase | | | | |
| 0.5 | 100 | 100 | 83 | 73 |
| 2 | 75 | 87 | 65 | 54 |
| 4 | – | 61 | 47 | 38 |
| $PCS_{I}$ phosphatase | | | | |
| 1.6 | 100 | 88 | 80 | 100 |
| 5 | 83 | 70 | 60 | 80 |
| 10 | 58 | 55 | 40 | 70 |
| $PCS_{C}$ phosphatase | | | | |
| 8 | 100 | 100 | 100 | 100 |
| ATP,Mg-dependent phosphatase (catalytic subunit) | | | | |
| 18 | 100 | 100 | 100 | 100 |

The procedures were as described in section 2. Results expressed as % $^{32}$P-labeled modulator. $^{32}$P content of the modulator was estimated by densitometric analysis of the autoradiograms, the 100% control values representing the casein kinase TS $^{32}$P-labeled modulator without or after pretreatment with 7 U/ml of the $PCS_{H1}$ phosphatase, the catalytic subunit of the ATP,Mg-dependent phosphatase, or both phosphatases

## 4. DISCUSSION

As described in the introduction the modulator-subunit of the ATP,Mg-dependent phosphatase is known to undergo phosphorylation by different kinases. The phosphorylation state of the modulator protein appears to be of critical importance for the in vitro phosphorylation by protein kinases. The isolated modulator is apparently partially phosphorylated; it could only be phosphorylated up to ± 0.5 mol per mol by casein kinase TS, which is known to phosphorylate 3 Ser residues [5]. After pretreatment with the ATP,Mg-dependent or the $PCS_{H1}$ phosphatase, an additional incorporation of respectively 0.23 or 0.55 mol phosphate could be obtained with casein kinase TS, while the pretreatment with both phosphatases combined resulted in the incorporation of 1.42 mol over the control. These phosphorylations by casein kinase TS involved Ser

Table 3

Effect of protamine on the dephosphorylation of casein kinase TS $^{32}$P-labeled modulator

| Protamine (μg/ml) | Protein phosphatase | | | | | |
|---|---|---|---|---|---|---|
| | $PCS_{H1}$ 0.1 U/ml | $PCS_{H2}$ 0.1 U/ml | $PCS_M$ 0.5 U/ml | $PCS_L$ 0.5 U/ml | $PCS_C$ 1 U/ml | ATP,Mg-dependent 1 U/ml |
| – | 90 | 82 | 100 | 100 | 100 | 100 |
| 0.4 | 73 | 67 | 16 | 72 | – | – |
| 0.8 | 36 | 91 | – | 32 | – | – |
| 1.2 | 73 | – | 29 | – | – | – |
| 2 | 16 | 91 | 65 | 32 | 100 | 100 |
| 4 | 16 | 71 | 51 | 28 | 100 | 100 |
| 8 | – | – | 91 | – | 60 | 80 |
| 16 | 11 | 23 | – | 69 | 30 | 60 |
| 50 | 29 | 22 | – | – | 54 | 64 |
| 166 | 45 | – | – | – | 82 | 82 |

The procedures were as described in section 2. Results are expressed as % $^{32}$P-labeled modulator. $^{32}$P content of the modulator was estimated by densitometric analysis of the autoradiograms, the control value being referred to as 100%

residues, as demonstrated by thin layer electrophoresis after acid hydrolysis (not shown), which – together with the failure to activate the ATP,Mg-dependent phosphatase – proves that the kinase is not contaminated with protein kinase $F_A$. The increased phosphorylation efficiency by casein kinase TS after pretreatment with the two protein phosphatases is coupled to a synergistic effect on the phosphorylation by protein kinase $F_A$. An enhanced level of phosphorylation by kinase $F_A$ after phosphorylation by kinase TS was also observed with glycogen synthase [19] and the type II regulatory subunit of cyclic AMP-dependent protein kinase [20]. Neither the 'native' nor the dephosphorylated modulator were phosphorylated significantly by protein kinase $F_A$ alone at the concentration and conditions used in these experiments. The observed synergistic effect of casein kinase TS on the phosphorylation by protein kinase $F_A$ would corroborate the potentiating effect of kinase TS observed [6] on the activation of the ATP,Mg-dependent phosphatase by kinase $F_A$. Neither the dephosphorylation of modulator by any one phosphatase, separately or combined, nor the subsequent phosphorylation by casein kinase TS had any effect (not shown) on the efficiency of the heat-stable modulatory protein to stimulate the kinase $F_A$-mediated activation of 'free $F_C$' in an activation procedure described previously [1].

The PCS and ATP,Mg-dependent phosphatases show a clear difference in site specificity toward modulator protein $^{32}$P-labeled by casein kinase TS (table 2). It was observed that (i) among the PCS phosphatases the $PCS_{H1}$ type shows the highest activity toward the Ser residues phosphorylated by casein kinase TS, all the sites phosphorylated seem to be dephosphorylated equally well by the $PCS_{H1}$ phosphatase; (ii) the ATP,Mg-dependent protein phosphatase failed to dephosphorylate all the forms of modulator protein labeled in vitro by casein kinase TS; (iii) there is also a clear difference in site specificity toward the native or in vivo phosphorylated modulator: the site dephosphorylated by the ATP,Mg-dependent phosphatase is not a good substrate for the $PCS_{H1}$ phosphatase, since additional effects of both phosphatases are observed.

The unexpected finding that a previous dephosphorylation of native modulator by the

ATP,Mg-dependent phosphatase leads to an increased incorporation of phosphate by kinase TS in sites which cannot be dephosphorylated by the same phosphatase, is suggestive of a complex site-site interaction between the phosphorylated residues on the modulator protein. It would be logical to assume that the native modulator is phosphorylated at a site(s) different from the kinase $F_A$ site which can also be dephosphorylated by the ATP,Mg-dependent protein phosphatase, since in vitro phosphorylation of the modulator by kinase $F_A$ does not impair the subsequent incorporation of phosphate by kinase TS [6]. Moreover significant phosphorylation of Thr-72 in isolated modulator is not likely [2], and in vivo studies in mouse diaphragm have shown 90% $^{32}P$-incorporation in Ser residues and only 10% in Thr residues [8].

In conclusion there seems to exist a complex interaction between the phosphorylation sites in the modulator protein. Phosphorylation by casein kinase TS potentiates the phosphorylation by kinase $F_A$ and occupancy of in vivo phosphorylation site(s) appears to be a negative determinant in the phosphorylation by casein kinase TS. The increased phosphorylation by casein kinase TS after dephosphorylation by the $PCS_{H1}$ phosphatase can be explained by the involvement of identical sites. Dephosphorylation by all the PCS protein phosphatases shows an optimum concentration for protamine similar to that observed in the dephosphorylation of phosphorylase [9]. The stimulation observed with the catalytic subunit of the ATP,Mg-dependent phosphatase can also be substrate-directed, since dephosphorylation of phosphorylase by the ATP,Mg-dependent protein phosphatase is inhibited by polycations [21]. Similar effects of polycations were observed with autophosphorylated C kinase [22] and glycogen synthase phosphorylated on site 3 [23].

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