Phosphoprotein phosphatase inhibitor-2 is phosphorylated at both serine and threonine residues in mouse diaphragm

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Phosphoprotein phosphatase inhibitor-2 (i-2) was rapidly isolated from mouse diaphragm extracts by the use of specific antibodies. The i-2 so obtained was associated with ATP-Mg and F_A/GSK-3 dependent phosphatase activity, supporting the idea that i-2 is in fact a component of this form of phosphatase. Inhibitor-2 isolated from diaphragms incubated with [^{32}P]phosphate contained both phosphoserine ($\sim$ 90%) and phosphothreonine ($\sim$ 10%). Therefore, i-2 is multiply phosphorylated in mouse diaphragm and the potential exists for control of the ATP-Mg-dependent phosphatase via multiple phosphorylation sites in vivo.

ATP-Mg-dependent phosphatase *Antibody* *Type 1 phosphatase* *Protein phosphorylation* *Mouse diaphragm* *Inhibitor-2*

1. INTRODUCTION

Inhibitor-2 is a heat stable protein [1,2] able to inhibit phosphoprotein phosphatase of type 1 [3,4]. Recently it has been found to be a component [5] of the ATP-Mg-dependent phosphatase first described by Riley and Haynes [6], and later by Merlevede and Riley [7]. This enzyme requires a protein factor, F_A, and ATP-Mg [8,9] to elicit activity. The proposed mechanism is that F_A, also identified as a glycogen synthase kinase GSK-3 [10], phosphorylates the inhibitor-2 [11], in the ATP-Mg-dependent phosphatase, at a threonine residue. This phosphorylation causes a conformational change of the catalytic component [12,13], leading to activation of the phosphatase. Phosphorylation of inhibitor-2 at serine residues in vivo has also been reported. Casein kinase II is able to phosphorylate the inhibitor whether in an isolated form or as part of the ATP-Mg-dependent phosphatase complex [14]. This phosphorylation occurs at serine residues and by itself does not affect enzyme activity. However, the action of casein kinase II in combination with submaximal concentrations of F_A/GSK-3 greatly enhances phosphorylation by the latter protein kinase and the activation of the phosphatase. Cyclic AMP-dependent protein kinase also phosphorylates inhibitor-2 at serine residues but without detectable effect on the properties of the phosphatase [14]. The possibility exists, therefore, that inhibitor-2 might undergo multiple phosphorylation and this work was aimed at analyzing the phosphorylation state of inhibitor-2 in mouse diaphragms by the use of specific antibodies. The study also provides direct evidence for an association of inhibitor-2 and phosphatase activity in rapidly processed extracts.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{32}P]Orthophosphate was purchased from New England Nuclear. *Staphylococcus aureus* of strain Cowan 1 (Pansorbin) was obtained from Calbiochem-Behring. CNBr-activated Sepharose-4B and other chemicals were from Sigma. Mice of strain ICR were from Harlan (Indianapolis). Phosphatase inhibitor-2 was purified from rabbit skeletal muscle as in [14]. [^{32}P]Phosphorylase *a* was prepared as in [14]. Catalytic subunit of type 1 phosphatase was purified to homogeneity by a modification of the procedures in [4,15]. Analysis by polyacryl-

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amide gel electrophoresis (PAGE) in the presence of SDS indicated that the preparation contained a single polypeptide of apparent M_r 38 000. No other species of lower M_r were observed. The specific activity of the phosphatase was 27 000 units/mg (unit as defined in [14]). Phosphatase and inhibitor-2 assays and the phosphorylation of inhibitor-2 by casein kinase II [16] were performed as in [14].

2.2. *Preparation and affinity purification of antibodies against phosphatase inhibitor-2*

Antibodies against inhibitor-2 were raised in guinea pigs as described in [17]. For each injection the animals received 80 μg purified protein. Both immune serum and affinity purified antibodies were used here. Inhibitor-2 was coupled to CNBr-activated Sepharose and this matrix was used for affinity purification of the antibodies from the serum. The specificity of the antibodies was determined by their ability to immunoprecipitate ^{32}P-labeled inhibitor-2 phosphorylated up to 3 mol phosphate per mol inhibitor by casein kinase II. ^{32}P-labeled inhibitor removal after treatment with S. aureus was measured as well as the amount associated with the immunoprecipitate. All of the inhibitor-2 could be precipitated by the antibodies and recovered in the immunoprecipitates, as a species of M_r 32 000 as judged by SDS-PAGE (not shown). Also, addition of unlabeled inhibitor-2 before exposure to the antibodies reduced the amount of ^{32}P-labeled inhibitor associated with the immunoprecipitate. This competition experiment established that the antibodies recognized both phosphorylated and unphosphorylated inhibitor-2.

2.3 *Incubation of mouse diaphragm and immunoprecipitation of phosphatase inhibitor-2*

Diaphragm muscles from male mice (22–26 g) were carefully excised and incubated in 1 ml of medium containing 119 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 2.4 mM $CaCl_2$, 0.1 mM KH_2PO_4, 24.8 mM $NaHCO_3$ and 5 mM glucose. Each incubation contained two diaphragms and was carried out under an atmosphere of 95% O_2:5% CO_2. After 10 min preincubation the muscles were transferred to flasks containing 1 ml fresh medium and 0.7 mCi [^{32}P]phosphate was added. Incubations were continued for 1 h after which the tissues were frozen in liquid nitrogen. Homogenization was performed in 5 vols of 50 mM Tris-HCl, 100 mm NaF, 10 mM EDTA, 2 mM EGTA, 2 mM KH_2PO_4, 10 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM tosylphenylchloromethyl ketone, 0.1 mM tosyllysylchloromethyl ketone, 10 μg/ml leupeptin and 0.1% Triton X-100, pH 7.2. After centrifugation at 13 000 $\times g$ for 20 min the supernatant was retained for immunoprecipitations. Portions of the supernatants were heated at 100°C for 10 min followed by centrifugation as before. Untreated tissue extracts (125 μl) or boiled supernatants (125 μl) were incubated with antibodies (9.4 μl immune serum or 9 μg affinity-purified IgG). These amounts were sufficient to achieve maximal removal of inhibitor-2 from the tissue extracts. Controls contained the same amount of non-immune serum or purified non-immune IgG. After 30 min at room temperature, the samples were placed on ice and excess Pansorbin (5%, w/v, 140 μl for serum or 31 μl for purified IgG) was added, respectively. Following a 30 min incubation at 0°C the bacterial pellet was collected by centrifugation at 3000 $\times g$ for 5 min and washed twice by consecutive resuspension and centrifugation in buffer consisting of 10 mM sodium phosphate, 1 mM EDTA, 0.4 M NaCl, 0.1% Triton X-100, pH 7.5. A third wash with NaCl reduced to 0.1 M was also included. To elute ^{32}P-labeled inhibitor-2, the pellet was either extracted with 200 μl of 70% formic acid or resuspended in 50 mM Tris-Cl, 0.1 mM EDTA, 125 mM β-mercaptoethanol and treated at 100% C for 10 min. The supernatant obtained after centrifugation at 8000 $\times g$ for 10 min was dried using a centrifugal evaporator (Speedvac, Savant Instruments). The samples were analyzed on 6–2% acrylamide gradient gels using a modification [18] of the procedure of Laemmli [19]. Phosphoamino acid analysis was also performed as described in [14]. For measurements of inhibitor-2, spontaneously active phosphatase and ATP-Mg-dependent phosphatase, diaphragm muscles were incubated in the absence of [^{32}P]phosphate. ATP-Mg-dependent phosphatase was determined as in [14] using a suspension of antigen-antibody-*S. aureus* complex. Inhibitor-2 was released from the antibody-protein A complex by treatment for 10 min at 100°C in 50 mM Tris-HCl, 0.1 mM EDTA and 50 mM β-mercaptoethanol, pH 7.0. The

bacteria were removed by centrifugation and the supernatant was used to measure inhibitor-2 by its ability to inhibit the low- M_r type 1 phosphatase.

3. RESULTS

3.1. *^{32}P labeling of phosphatase inhibitor-2 in mouse diaphragm*

Incubation of mouse diaphragms with [^{32}P]-phosphate led to the incorporation of ^{32}P into numerous polypeptides separated by SDS-PAGE (fig.1A, track 1). As could be expected, many of these labeled species were not detected in extracts that had been heat treated (fig.1A, track 2). Antibodies against purified rabbit skeletal muscle phosphatase inhibitor-2 were able to immunoprecipitate inhibitor-2 from extracts of mouse diaphragm incubated with [^{32}P]phosphate (fig. 1B and C). Analysis of material released at 100°C from immunoprecipitates indicated a single, domi-

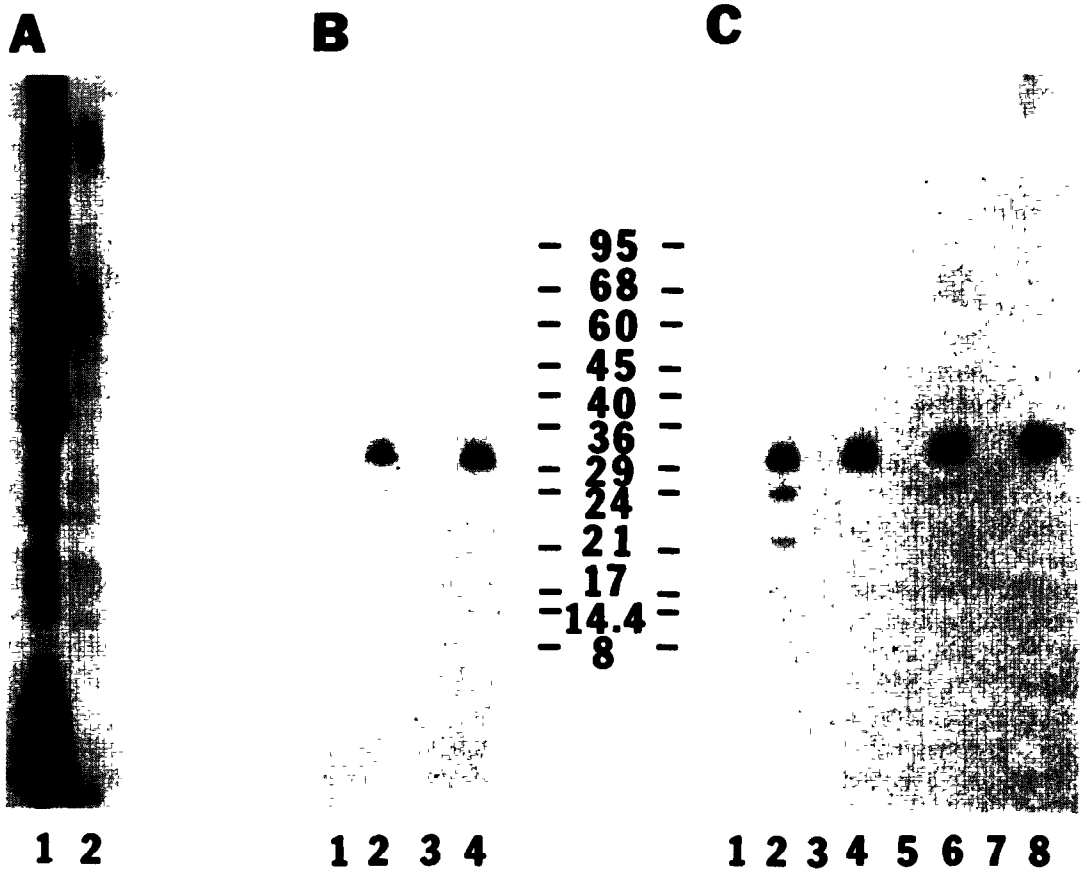


Fig.1. Analysis of immunoprecipitates from mouse diaphragms incubated with [^{32}P]phosphate. Tissue extracts or immunoprecipitates prepared as described in section 2, were analyzed by polyacrylamide gel electrophoresis in the presence of SDS. Portions of a corresponding autoradiogram are shown. Panel A: unboiled (track 1) or boiled (track 2) extracts, equivalent to 1.25 mg tissue. Panel B shows material immunoprecipitated with affinity-purified antibodies and released from the bacterial pellet by heat treatment. Immunoprecipitates prepared from boiled (tracks 3 and 4) or unboiled (tracks 1 and 2) extracts, equivalent to 14 mg tissue were analyzed. Either antibodies (tracks 2 and 4) or a corresponding control (non-immune IgG) were used (tracks 1 and 3). Panel C, tracks 1–4, shows the results of a similar analysis except that the immunoprecipitates were extracted with 70% formid acid. Panel C, tracks 5–8, shows results using serum for the immunoprecipitation and heat treatment for extraction of the bacterial pellet. Tracks 5 and 7, non-immune serum; tracks 6 and 8, immune serum; tracks 5 and 6, unboiled extract; tracks 7 and 8, boiled extract. The numbers denote M_r values ($\times 10^{-3}$).

nant radioactive species of M_r 32 000, similar to that of purified inhibitor-2 (fig. 1B and C, tracks 2, 4, 6 and 8). A much lesser proportion of a higher mobility species, apparent M_r 34 000, was also visible, as well as a species of apparent M_r 27 500. These polypeptides were absent from control precipitations using non-immune IgG or serum (fig. 1B and C, tracks 1, 3, 5 and 7). Similar results were obtained whether the immunoprecipitate was derived from unboiled or boiled tissue extracts. In particular, the intensity of the radioactive band in both cases was very similar, suggesting that inhibitor-2 was unaffected by the treatment of the extract at 100°C. Both immune serum and affinity purified antibodies immunoprecipitated the same dominant 32-kDa labeled polypeptide. Since it could be argued that proteins present in the immunoprecipitates from unboiled extracts might become insoluble after boiling, the immunoprecipitates were also extracted with 70% formic acid, in which most proteins would be expected to be soluble. As seen in fig. 1C (tracks 1–4), no significant difference in the SDS–PAGE analysis resulted when affinity purified antibodies had been used. We did observe an additional ^{32}P-labeled species of M_r 43 000, released by formic acid, when serum was used to prepare immunoprecipitates from unboiled (but not boiled) extracts (not shown). As explained below, the significance of this species is unclear.

Competition experiments were also carried out to help confirm the identity of the 32-kDa species. Thus, different amounts of unlabeled rabbit muscle inhibitor-2 were added to boiled or unboiled extracts before exposure to antibodies. As shown in fig. 2 for affinity-purified antibodies, the unlabeled inhibitor effectively competed with the 32-kDa and 34-kDa ^{32}P-labeled species in a concentration-dependent manner. The 27 500-kDa species did not compete (fig. 2) and in other experiments the 43-kDa species was not associated with the use of unfractionated serum (not shown).

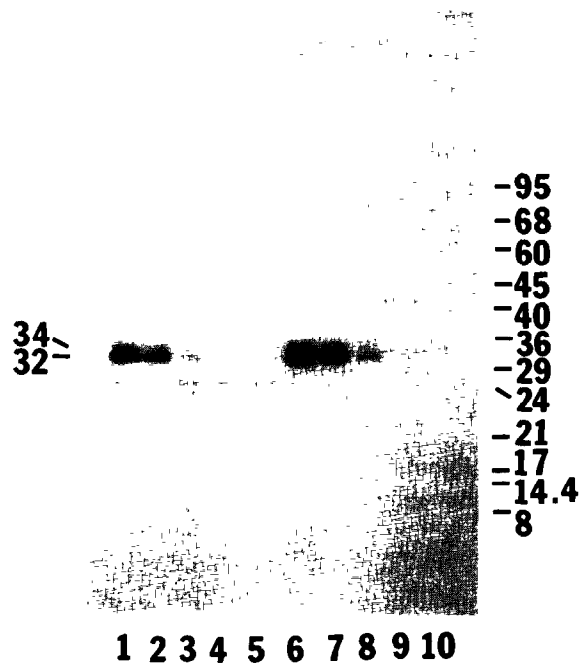


Fig. 2. Competition of the immunoprecipitated mouse inhibitor-2 with rabbit inhibitor-2. Immunoprecipitates were prepared as described for fig.1 from unboiled (tracks 1–5) or boiled (tracks 6–10) extracts of ^{32}P-labeled mouse diaphragms. Before exposure to affinity-purified antibodies, purified rabbit skeletal muscle inhibitor-2, 0 (tracks 1 and 6), 50 ng (tracks 2 and 7), 150 ng (tracks 3 and 8), 450 ng (tracks 4 and 9) or 900 ng (tracks 5 and 10) were added to the extracts. Shown is an autoradiogram prepared after SDS-polyacrylamide gel electrophoresis of the material released from the bacterial pellet by heat treatment. The numbers alongside the gel represent M_r values ($\times 10^{-3}$).

3.2. *Phosphoamino acid analysis of ^{32}P-labeled inhibitor-2*

Inhibitor-2 immunoprecipitated from boiled extracts by affinity-purified antibodies (thus lacking the 27-kDa species) was subjected to acid hydrolysis and the resulting ^{32}P-labeled amino acids were analyzed by thin-layer electrophoresis. Autoradiography of the plate showed that most of the radioactivity was associated with phosphoserine and only a small amount, approx. 10%, was present as phosphothreonine (fig. 3B). Only a trace of phosphoserine was associated with the corresponding non-immune IgG. In fig. 3A is shown the analysis, by SDS–PAGE and autoradiography, of the material used for the phosphoamino acid determinations shown in fig. 3B.

3.3. *ATP-Mg-dependent phosphatase and inhibitor-2 activity associated with the immunoprecipitates*

Immunoprecipitates obtained with unboiled mouse diaphragm extracts were analyzed for both

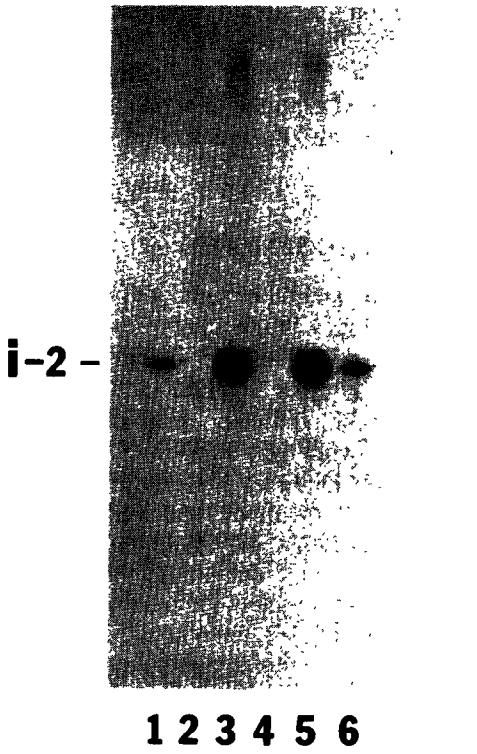
ATP-Mg-dependent phosphatase activity and phosphatase inhibitory material (table 1). Little or no spontaneously active phosphatase was detected whereas in the presence of F_A/GSK-3 and ATP-Mg significant activity was measured. Heat treatment of the bacterial pellets released material that was able to inhibit purified rabbit muscle type 1 phosphatase. The parallel analyses for non-immune IgG indicated neither phosphatase activity nor phosphatase inhibitor associated with the *S. aureus*.

Table 1

Analysis of phosphatase and inhibitor-2 activity in immunoprecipitates of mouse diaphragm extracts

	Phosphatase activity (units/ml) F_A/GSK-3		Inhibitor-2 activity (% inhibition)
	−	+	
Immune IgG	0.05	3.10	66
Non-immune IgG	0.0	0.0	5

Immunoprecipitates from unboiled diaphragm extracts (equivalent to 2 mg tissue) were analyzed for phosphatase and inhibitor-2 activity as described in section 2. The entries under 'inhibitor-2 activity' indicate the percentage inhibition of an assay containing 7.5 munits of purified rabbit muscle type 1 phosphatase, 1 unit corresponding to 1 nmol of $^{32}P_i$ released per min



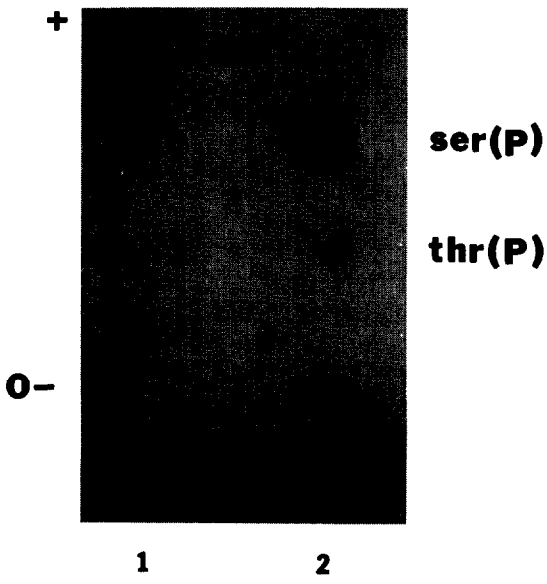


Fig. 3. Phosphoamino acid analysis of immunoprecipitated mouse diaphragm inhibitor-2. Immunoprecipitates from boiled extracts of ^{32}P-labeled diaphragms were prepared as described in section 2 using affinity-purified antibodies. Panel A shows an autoradiogram resulting from gel electrophoretic analysis of the material released from the bacterial pellet by heat treatment (tracks 3 and 5). The corresponding analyses with non-immune IgG are shown in tracks 2 and 4. Tracks 1 and 6 contained purified rabbit muscle inhibitor-2 which had been phosphorylated using [^{32}P]ATP and casein kinase II. Another portion of the material analyzed in tracks 2–5 was hydrolyzed, and subjected to thin-layer electrophoresis, as described in section 2. Panel B, autoradiogram of the thin-layer plate: track 1, control IgG; track 2, affinity-purified antibodies. The origin and the anode are indicated by 0 and +, respectively. The migration of phosphoserine (ser(P)) and phosphothreonine (thr(P)) standards is also noted.

4. DISCUSSION

Here, we set out to examine the phosphorylation strate of the inhibitor in intact muscle cells by using mouse diaphragm incubated with [^{32}P]phosphate. With the use of specific antibodies we were able to isolate rapidly a phosphorylated species of 32 kDa from crude muscle extracts. This species was recognized by immune but not by non-immune IgG, could compete with purified inhibitor-2, and was able to inhibit purified type 1 phosphatase. These results argue strongly that the 32-kDa polypeptide was indeed inhibitor-2. The results also demonstrate immunological cross-reactivity between rabbit skeletal muscle and mouse diaphragm inhibitor-2.

One of the most striking findings was that immunoprecipitates prepared with inhibitor-2 antibodies contained detectable ATP-Mg-dependent phosphatase activity. This result, for which both mild and rapid isolation methods were employed, provides strong evidence for an association of inhibitor-2 with the type 1 phosphatase catalytic subunit in the cell. Such an association had been inferred previously only from studies using conventionally purified components, often subjected to relatively harsh purification steps. However, the possibility has also been raised by Tonks and Cohen [20] that ATP-Mg-dependent phosphatase 'may not exist in freshly prepared extracts'. More work is needed for unequivocal resolution of this important question but the present study does demonstrate that ATP-Mg-dependent phosphatase activity is detectable shortly after homogenization of mouse muscle.

^{32}P labeling clearly indicated that phosphorylation of the inhibitor occurs in mouse diaphragm and that phosphate turns over on the time scale of the experiments. In addition, ^{32}P was introduced into both phosphoserine and phosphothreonine. Therefore, a protein kinase(s) besides F_A/GSK-3 must be operating in the cell. Currently, only two possible candidates are known, cyclic AMP-dependent protein kinase and casein kinase II, since these two protein kinases have been shown to phosphorylate inhibitor-2 at serine residues [14]. Since only casein kinase II action affected the properties of the phosphatase, it is tempting to speculate that the phosphoserine observed in association with inhibitor-2 in muscle cells might result from casein kinase II action and that this protein kinase might have a functional role in regulating phosphatase activity. In any event and whatever protein kinases are responsible, demonstration of phosphoserine in inhibitor-2 implies that control of this protein is likely to involve multiple phosphorylation sites and an important question now raised is whether physiological controls of phosphatase activity, such as by insulin and epinephrine, are correlated with alterations in serine and/or threonine phosphorylation. Future studies will address this question.

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