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Phosphorylation and inactivation of protein phosphatase 1 by pp60$^{v\text{-}src}$

(tyrosine protein kinase/cell transformation/oncogenes/retrovirus)

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ABSTRACT **Protein phosphatase 1, one of four major protein phosphatases involved in cellular regulation, was phosphorylated *in vitro* by pp60$^{v\text{-}src}$, the transforming gene product of Rous sarcoma virus. Phosphorylation was accompanied by a loss of protein phosphatase activity. The inactivation of protein phosphatase 1 was time-dependent and the extent of inactivation correlated closely with the stoichiometry of phosphorylation. Under optimal conditions, 0.34 ± 0.01 mol of phosphate were incorporated per mol of protein phosphatase and the activity of the enzyme was decreased by 39 ± 2%. The inactivation required the presence of both MgATP and pp60$^{v\text{-}src}$.There was no loss of activity when adenosine 5′-[βγ-imido]triphosphate was used in place of ATP. Phosphorylation of protein phosphatase 1 occurred exclusively on tyrosine residues and was blocked by specific antibodies to pp60$^{v\text{-}src}$. During preincubation of pp60$^{v\text{-}src}$ at 41°C, its protein kinase activity towards casein was lost rapidly. The ability of pp60$^{v\text{-}src}$ to phosphorylate and inactivate protein phosphatase 1 declined in parallel with the loss of casein kinase activity. Limited chymotryptic digestion of ^{32}P-labeled protein phosphatase 1 (M_r 37,000) resulted in its quantitative conversion to a M_r 33,000 species. Conversion to this species was accompanied by the loss of ^{32}P-labeling and by reactivation of the protein phosphatase. When various concentrations of chymotrypsin were used in the digestion, there was a close correlation between conversion to the M_r 33,000 species and the restoration of protein phosphatase activity. pp60$^{v\text{-}src}$ was unable to phosphorylate or inactivate a partially proteolyzed species of protein phosphatase 1 (M_r 33,000/34,000).**

The *src* oncogene is solely responsible for transformation of cells by Rous sarcoma virus (RSV) (1). The protein product of this gene, pp60$^{v\text{-}src}$, is a member of a unique class of protein kinases that specifically phosphorylate substrate proteins on tyrosine residues and that have been linked to the control of cell growth in normal and transformed cells (2–4). Other members of this family include the cellular receptors of four growth factors and the products of five additional retroviral oncogenes (5, 6). Several lines of evidence suggest that the protein kinase activity of pp60$^{v\text{-}src}$ is relevant to cell transformation by RSV. First, pp60$^{v\text{-}src}$ is the sole protein encoded by the *src* oncogene (2); therefore, some activity of this protein is clearly responsible for transformation. Second, the only known activity of pp60$^{v\text{-}src}$ is that of a protein kinase. Third, phosphorylation of cellular proteins on tyrosine residues is increased 10-fold after transformation by RSV (7). Fourth, in cells infected with a mutant RSV that is temperature-sensitive for transformation, the increase in phosphorylation of proteins on tyrosine residues is observed only at the permissive temperature (7). pp60$^{v\text{-}src}$ isolated from cells infected with the temperature-sensitive mutant was found to be 6–7 times more labile following incubation at 41°C (nonpermissive temperature) than pp60$^{v\text{-}src}$ isolated from cells infected with wild-type RSV (8). A definitive understanding of the physiological significance of the tyrosine protein kinase activity of pp60$^{v\text{-}src}$ requires identification of physiologically relevant cellular substrates. At least seven proteins have been identified that exhibit increased phosphorylation on tyrosine residues following transformation by RSV (5, 6). However, in all cases where it is known, the stoichiometry of phosphorylation is low (≤0.1 mol/mol) and no alteration in activity of any of these proteins has been demonstrated.

Relevant cellular substrates may be identified by investigating the ability of pp60$^{v\text{-}src}$ to phosphorylate and alter the activity of purified cellular proteins whose function might explain some aspect of the transformed phenotype. Transformation of cells by RSV has been found to increase the phosphorylation state of several proteins on serine and threonine residues (9, 10). In view of the absolute specificity of pp60$^{v\text{-}src}$ for tyrosine residues, it is clear that the increase must result from activation of protein kinases or inhibition of protein phosphatases with specificity for serine or threonine residues. In this communication we demonstrate that one such protein phosphatase, termed protein phosphatase 1, is an *in vitro* substrate for pp60$^{v\text{-}src}$ and, further, that this phosphorylation results in a significant decrease in its activity.

MATERIALS AND METHODS

Protein Preparations. Phosphorylase *b* (11), inhibitor 1 (12), phosphorylase kinase (13), and protein phosphatase 1 (14, 15) were purified to homogeneity from rabbit skeletal muscle. The specific activity of protein phosphatase 1 was 28,000 units/mg when assayed with 3 μM phosphorylase *a*. Protein phosphatase 1 was separated from two active proteolytic fragments (M_r 34,000 and 33,000) by gel-permeation HPLC using a TSK-G3000 SW column (60 × 0.5 cm). The undegraded form of the enzyme was used in all experiments unless otherwise stated. pp60$^{v\text{-}src}$ was highly purified by immunoaffinity chromatography (8) and stored in 20 mM Hepes, pH 7.1/1 mM EDTA/5 mM dithiothreitol/50% (vol/vol) glycerol. IgG from normal and tumor-bearing rabbits was provided by R. L. Erikson (Harvard University).

Casein Kinase Activity of pp60$^{v\text{-}src}$. The reaction mixture (25 μl) contained pp60$^{v\text{-}src}$, 1 mg of α-casein/ml, 25 mM Tris·HCl (pH 7.1 at 25°C), 1 mM EDTA, 5 mM magnesium acetate, 0.005% Brij 35, 30 mM 2-mercaptoethanol, and 250 μM [γ-^{32}P]ATP (3000 cpm/pmol). Incubations were at 30°C and were terminated by the addition of 1 ml of 10% (wt/vol) trichloroacetic acid, and the reaction mixtures were pro-

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Abbreviations: RSV, Rous sarcoma virus; TBR-IgG, IgG from rabbits bearing RSV-induced tumors; Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate.

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cessed as described (16). [γ-^{32}P]ATP was prepared from ^{32}P-labeled inorganic phosphate (17). One unit of protein kinase activity is that amount which incorporates 1 nmol of phosphate per minute into casein.

Phosphorylation of Protein Phosphatase 1. The standard reaction mixture (25 μl) contained 40 pmol of protein phosphatase 1/ml, 40 milliunits of pp60$^{v\text{-}src}$/ml, 25 mM Tris·HCl (pH 7.1 at 25°C), 1 mM EDTA, 5 mM magnesium acetate, 0.005% Brij 35, 30 mM 2-mercaptoethanol, 10% glycerol, and 250 μM unlabeled ATP or [γ-^{32}P]ATP (40,000–60,000 cpm/pmol). After incubation for 90 min at 30°C, reactions were terminated by one of two methods. Reactions with unlabeled ATP were terminated by 180-fold dilution and assayed for protein phosphatase 1 activity (see below). Reactions with ^{32}P-labeled ATP were terminated by addition of 8 μl of sample buffer (70 mM Tris·HCl, pH 6.8 at 25°C/11% glycerol/5% 2-mercaptoethanol/3% NaDodSO$_4$/0.01% bromophenol blue) followed by incubation for 3 min at 95°C. Phosphoproteins were resolved by NaDodSO$_4$/10% PAGE (18) in slab gels. Gels were silver-stained (19) and dried onto either Whatman 3MM paper or cellophane sheets. Radiolabeled proteins were visualized by autoradiography on Kodak XRP-5 film with the aid of Dupont Cronex Lightning Plus intensifying screens. The extent of ^{32}P incorporation was quantitated by liquid scintillation spectroscopy of excised stained protein bands or by densitometric analysis of autoradiograms using a Gilford Response spectrophotometer. Molecular weights were estimated using phosphorylase *b* (M_r 97,500), bovine serum albumin (M_r 68,000), horseradish peroxidase (M_r 48,000), ovalbumin (M_r 45,000), and carbonic anhydrase (M_r 29,500) as standards.

Protein Phosphatase 1 Assay. Activity was determined by the release of [^{32}P]P$_i$ from ^{32}P-labeled phosphorylase *a* (20). The assay mixture (60 μl) contained protein phosphatase 1, 1.0 μM ^{32}P-labeled phosphorylase *a*, 50 mM Tris·HCl (pH 7.0 at 25°C), 1 mM EGTA, 2 mM caffeine, 0.3 mg of bovine serum albumin/ml, and 50 mM 2-mercaptoethanol. One unit of protein phosphatase 1 activity is that amount which catalyzes the release of 1.0 nmol of inorganic phosphate/min.

Phospho Amino Acid Analysis. Protein phosphatase 1 was phosphorylated under standard conditions and subjected to NaDodSO$_4$/PAGE. The appropriate band in the wet gel was localized by autoradiography, excised, eluted, lyophilized, and subjected to partial acid hydrolysis in the presence of 6 N HCl for 3.5 hr at 100°C. Phospho amino acids were separated by electrophoresis at pH 3.5 (3). The positions of ^{32}P-labeled phospho amino acids were visualized by autoradiography and compared to those of standard phospho amino acids localized by ninhydrin staining.

Protein Determinations. The protein concentration of purified protein phosphatase 1 was determined by the methods of Bradford (21) and Hazra *et al.* (22) using bovine serum albumin ($A_{280}^{1\%} = 6.5$) as a standard. The protein concentration was also estimated from analysis of its amino acid composition. The values obtained from these three independent methods agreed within ±20% and the average was used for the purpose of quantitation. The $A_{280}^{1\%}$ for protein phosphatase 1 was 16.3.

RESULTS

Phosphorylation of Protein Phosphatase 1 by pp60$^{v\text{-}src}$. Incubation of protein phosphatase 1 with pp60$^{v\text{-}src}$ in the presence of Mg[γ-^{32}P]ATP resulted in the appearance of a labeled band (Fig. 1*B*) that coincided with the silver staining (Fig. 1*A*) of the protein phosphatase after NaDodSO$_4$/PAGE. The phosphorylated species was not evident when either pp60$^{v\text{-}src}$ or protein phosphatase 1 were incubated alone with Mg[γ-^{32}P]ATP. pp60$^{v\text{-}src}$ that had lost all casein kinase activity after incubation for 10 min at 95°C was unable to phosphorylate the protein phosphatase (Fig. 1*B*). Phosphorylation conditions were optimized with respect to substrate concentration, pp60$^{v\text{-}src}$ concentration, and time of incubation. Optimal phosphorylation conditions are given in *Materials and Methods*. The initial rate of phosphorylation of protein phosphatase 1 was 50% the rate at which pp60$^{v\text{-}src}$ phosphorylated casein (1.2 μg/ml) under identical incubation conditions. The time course for the phosphorylation of protein phosphatase 1 is depicted in Fig. 2. In nine separate experiments under these conditions, 0.34 ± 0.01 mol of phosphate was incorporated per mol of the protein phosphatase.

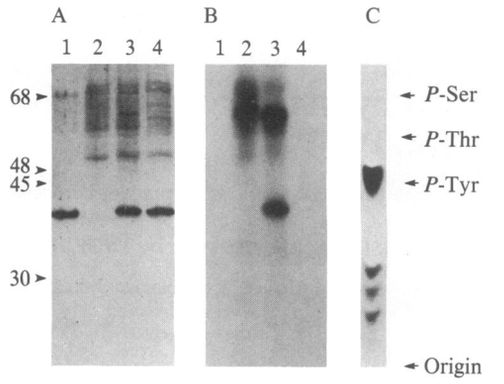

FIG. 1. Phosphorylation of protein phosphatase 1 by pp60$^{v\text{-}src}$. (*A* and *B*) Silver-stained NaDodSO$_4$/10% polyacrylamide slab gel and the corresponding autoradiogram. Lanes: 1, 2 pmol of protein phosphatase 1 (M_r 37,000); 2, 0.5 milliunit of immunoaffinity-purified pp60$^{v\text{-}src}$; 3, protein phosphatase 1 plus pp60$^{v\text{-}src}$; 4, protein phosphatase 1 plus inactivated (10 min at 95°C) pp60$^{v\text{-}src}$. All reaction mixtures contained [γ-^{32}P]ATP and were incubated for 90 min at 30°C under standard assay conditions. Molecular weight markers ($M_r \times 10^{-3}$) are indicated at left. (*C*) Phospho amino acid analysis of ^{32}P-labeled protein phosphatase 1. An autoradiogram of the cellulose sheet is presented (see *Materials and Methods*). The migration positions of standard phospho amino acids are indicated. Fifty percent of total radioactivity appeared as free phosphate in the hydrolyzate.

Three lines of evidence indicate that the phosphorylation of protein phosphatase 1 was specifically due to pp60$^{v\text{-}src}$ and not a trace contaminant in the preparation. Phospho amino acid analysis of ^{32}P-labeled protein phosphatase 1 indicated that the phosphorylation occurred exclusively on tyrosine residues (Fig. 1*C*). Specific antibodies to pp60$^{v\text{-}src}$ (TBR-IgG), recovered from rabbits bearing tumors induced by RSV, block the autophosphorylation of pp60$^{v\text{-}src}$ as well as its ability to phosphorylate exogenous substrates (8). Preincubation of 0.5 milliunit of pp60$^{v\text{-}src}$ with 6.5 μg of TBR-IgG for 30 min at 4°C totally inhibited the protein kinase activity of pp60$^{v\text{-}src}$ toward casein and decreased the phosphorylation of protein phosphatase 1 by 92%. The phosphorylation of casein and protein phosphatase 1 was unaffected after preincubation of pp60$^{v\text{-}src}$ with the same amount of IgG from normal rabbits. The casein kinase activity of pp60$^{v\text{-}src}$ is rapidly lost after preincubation of pp60$^{v\text{-}src}$ at 41°C (ref. 8 and Fig. 3). The ability of pp60$^{v\text{-}src}$ to phosphorylate protein phosphatase 1 decayed in parallel with the casein kinase activity (Fig. 3).

pp60$^{v\text{-}src}$ Inactivates Protein Phosphatase 1. Incubation of protein phosphatase 1 with pp60$^{v\text{-}src}$ and MgATP resulted in a time-dependent loss of activity that correlated closely with the extent of phosphorylation of the molecule (Fig. 2). The inactivation of protein phosphatase 1 required both MgATP and pp60$^{v\text{-}src}$. No loss of activity was observed when adenosine 5′-[β,γ-imido]triphosphate (Ado*PP*[NH]*P*), a nonhydrolyzable analog of ATP, was substituted for ATP or when inactive pp60$^{v\text{-}src}$ (pretreated at 95°C) was used in the reaction (Table 1). During preincubation of pp60$^{v\text{-}src}$ for various times

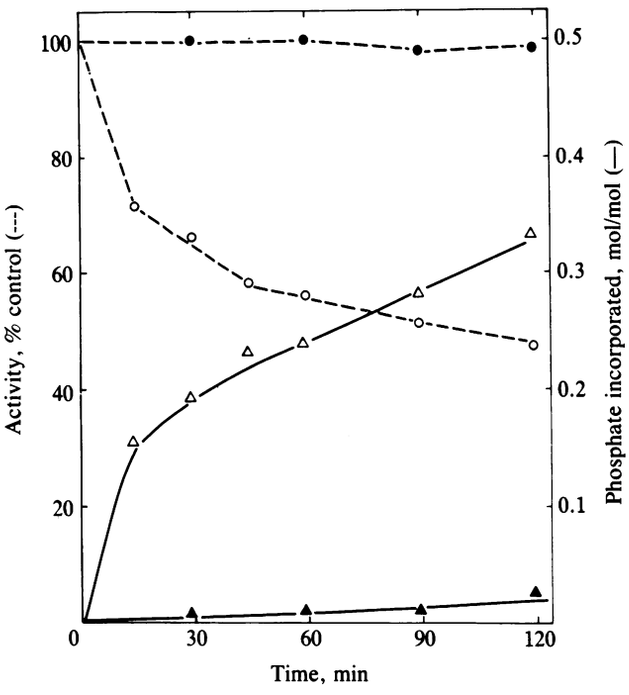

FIG. 2. Time course of the phosphorylation and inactivation of protein phosphatase 1 by pp60$^{v\text{-}src}$. Phosphate incorporated (mol/mol of protein phosphatase 1; △) at each time point was determined after analysis by NaDodSO$_4$/PAGE of reaction mixtures incubated under standard phosphorylation conditions. The stoichiometry of phosphorylation of partially proteolyzed protein phosphatase 1 (60:40 mixture of M_r 33,000 and 34,000 species) by pp60$^{v\text{-}src}$ (▲) was also determined. The activities of undegraded (○) and partially proteolyzed (●) protein phosphatase 1 were measured at each time point. No effect on the activity of either form of the protein phosphatase was observed after incubation with inactivated (10 min at 95°C) pp60$^{v\text{-}src}$.

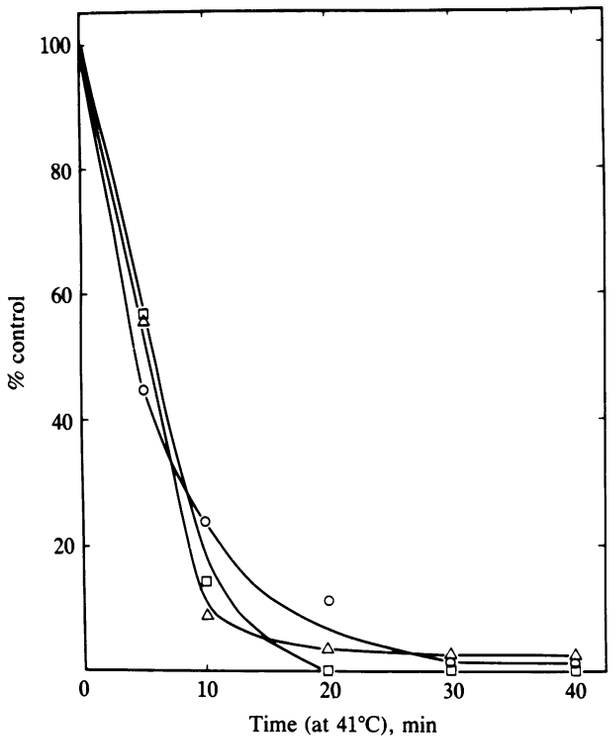

FIG. 3. Thermolability of the protein kinase activity of pp60$^{v\text{-}src}$ at 41°C and the effect on the phosphorylation and inactivation of protein phosphatase 1. pp60$^{v\text{-}src}$ was preincubated for various times at 41°C in 25 mM Tris·HCl, pH 7.1 (25°C)/1 mM EDTA/30 mM 2-mercaptoethanol/25% glycerol. Protein kinase activity then was determined at each time point using either casein (○) or protein phosphatase 1 (△) as substrates in their respective assays. The ability of pp60$^{v\text{-}src}$ to inactive protein phosphatase 1 after preincubation at 41°C (□) was determined at each time point in concurrent incubations. Control activity (100%) represents 0.4 milliunit of pp60$^{v\text{-}src}$ activity in the casein assay, 0.20 mole of [^{32}P]P$_i$ incorporated per mole of protein phosphatase 1, and 40% inactivation of protein phosphatase activity.

at 41°C, the ability of pp60$^{v\text{-}src}$ to inactivate protein phosphatase 1 decayed in parallel with the loss of protein kinase activity (Fig. 3). In 12 separate experiments under optimal conditions, the activity of protein phosphatase 1 was decreased by 39% ± 2%.

The results from the following two experiments showed that the loss of protein phosphatase activity was not due to the generation of an inhibitor during the incubation with pp60$^{v\text{-}src}$ and MgATP. First, the percent inactivation of the protein phosphatase did not vary when the reaction mixture was assayed over a 10-fold range of dilutions. Second, when pp60$^{v\text{-}src}$ and MgATP were incubated alone and then added to assays containing protein phosphatase 1, no inhibition of the protein phosphatase was observed.

Localization of the Phosphorylation Site on Protein Phosphatase 1. Following the purification of protein phosphatase 1, three species with apparent molecular weights of 37,000, 34,000, and 33,000 are obtained. The lower M_r species arise as a result of limited proteolysis during the purification (15). The two partially proteolyzed species of protein phosphatase 1 were not phosphorylated by pp60$^{v\text{-}src}$ under optimal conditions (Fig. 2). This result suggested that the pp60$^{v\text{-}src}$ phosphorylation site was on a fragment released during proteolytic conversion of the protein phosphatase.

To examine this possibility in more detail, protein phosphatase 1 was phosphorylated under standard conditions and subjected to limited chymotryptic digestion (Fig. 4). This treatment resulted in the quantitative conversion of the protein phosphatase to an active M_r 33,000 species (ref. 15 and Fig. 4). The ^{32}P-labeling associated with the protein phosphatase declined in parallel with the amount of protein staining in the M_r 37,000 band (Fig. 4*A*). This result supported the idea that the phosphorylated band (M_r 37,000) in the NaDodSO$_4$/polyacrylamide gel was in fact protein phosphatase 1. No ^{32}P radioactivity was detected in the M_r 33,000 band.

The results presented above suggest that the inactivation of protein phosphatase 1 was a consequence of its phosphorylation by pp60$^{v\text{-}src}$. The ability to selectively remove a small

Table 1. Dependence of protein phosphatase 1 (PrP-1) inactivation on pp60$^{v\text{-}src}$ and ATP

Additions	Activity, % control
PrP-1 + Mg	100
PrP-1 + Mg + pp60$^{v\text{-}src}$	104 ± 2
PrP-1 + MgAdo*PP*[NH]*P*	100 ± 6
PrP-1 + MgAdo*PP*[NH]*P* + pp60$^{v\text{-}src}$	101 ± 5
PrP-1 + MgATP	98 ± 2
PrP-1 + MgATP + pp60$^{v\text{-}src}$	61 ± 2
PrP-1 + MgATP + pp60$^{v\text{-}src}$ (95°C)	98 ± 5

Protein phosphatase 1 was incubated with the indicated additions under conditions described in *Materials and Methods*. Active (40 milliunits/ml) or the same volume of heat-inactivated (10 min at 95°C) pp60$^{v\text{-}src}$ were included as indicated. Unlabeled ATP or Ado*PP*[NH]*P* were added to give a final concentration of 250 μM. Reaction mixtures were assayed for phosphorylase phosphatase activity. Results are expressed as mean ± SEM of three separate experiments.

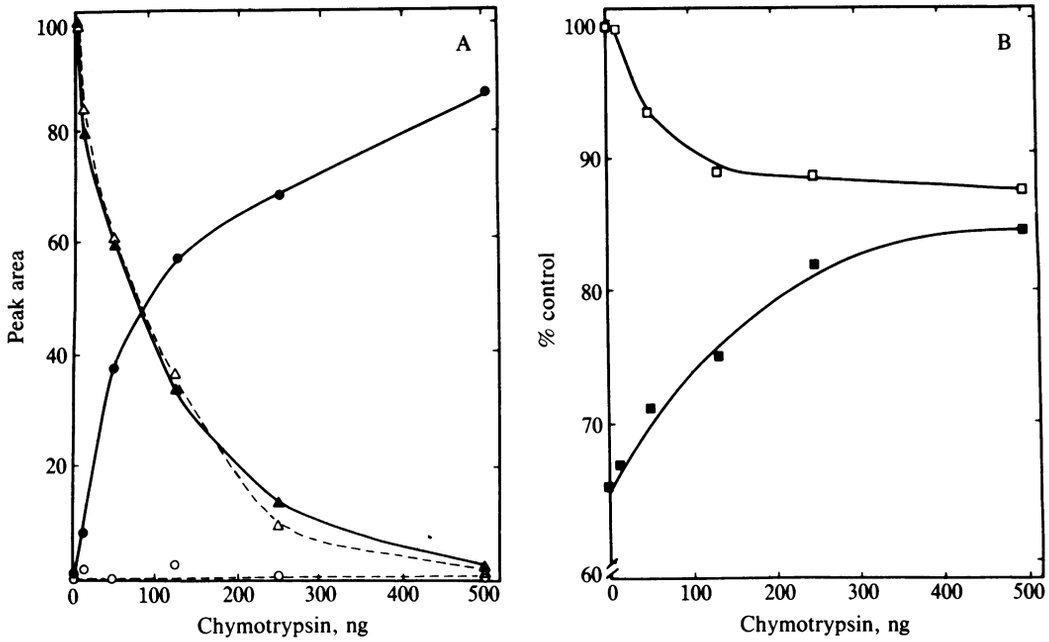

FIG. 4. Limited chymotryptic digestion of phosphorylated protein phosphatase 1. (*A*) Protein phosphatase 1 (24 pmol) was phosphorylated by pp60$^{v\text{-}src}$ under standard conditions. Aliquots (4 pmol) were further incubated with 0–500 ng of chymotrypsin in 30 μl for 10 min at 30°C. Reactions were terminated by the addition of 5 μl of 100 μM phenylmethylsulfonyl fluoride and analyzed by NaDodSO$_4$/PAGE. The slab gel was silver-stained and dried onto cellophane. Silver staining (▲) and the corresponding intensity of the autoradiogram (△) at the position of the protein phosphatase (M_r 37,000) was quantitated by densitometry at A_{500}. The peak areas of silver staining and intensity of ^{32}P-labeling of the M_r 37,000 band in the absence of chymotrypsin were arbitrarily set to 100 area units. Silver staining (●) and ^{32}P content (○) of the M_r 33,000 form of protein phosphatase 1 also were determined. (*B*) The experiment was repeated using unlabeled ATP and either active or inactive (10 min at 95°C) pp60$^{v\text{-}src}$ in the initial incubation. Aliquots (4 pmol) of unphosphorylated (□) or phosphorylated (■) protein phosphatase 1 were incubated with 0–500 ng of chymotrypsin under identical conditions. Reactions were terminated by the addition of phenylmethylsulfonyl fluoride, diluted, and assayed for protein phosphatase activity. The inactivation of control and activation of phosphorylated protein phosphatase 1 were not seen when phenylmethylsulfonyl fluoride was added prior to incubation with chymotrypsin. The activity of the partially proteolyzed protein phosphatase 1 (M_r 33,000/34,000) was not affected by incubation with chymotrypsin under identical conditions.

fragment of protein phosphatase 1 containing the phosphorylation site, without drastically altering the rest of the molecule, allowed us to further test this hypothesis. Incubation of partially proteolyzed protein phosphatase 1 (M_r 33,000/34,000), which lacked the phosphorylation site, with MgATP and pp60$^{v\text{-}src}$ did not result in any loss of activity (Fig. 2). When phosphorylated protein phosphatase 1 was incubated with chymotrypsin, the phosphatase was reactivated (Fig. 4*B*). The dose dependence of this reactivation closely paralleled the appearance of the M_r 33,000 silver-staining band (Fig. 4*A*). In contrast, limited proteolysis of unphosphorylated protein phosphatase 1 was accompanied by a slight (13%) decrease in activity (Fig. 4*B*), to the same level observed after digestion of the phosphorylated enzyme.

DISCUSSION

The results show that protein phosphatase 1 is a substrate for pp60$^{v\text{-}src}$ *in vitro*. Phosphorylation of protein phosphatase 1 occurred exclusively on tyrosine residues and was blocked by a specific antibody to pp60$^{v\text{-}src}$. The phosphorylation of protein phosphatase 1 was accompanied by a decrease in the activity of the enzyme. Several lines of evidence demonstrate that the inactivation was a consequence of the phosphorylation of the protein phosphatase. First, the inactivation was time-dependent and required both MgATP and pp60$^{v\text{-}src}$. No loss of activity was observed when a nonhydrolyzable analog, Ado*PP*[NH]*P*, was substituted for ATP. Second, the extent of inactivation closely paralleled the stoichiometry of phosphorylation during a time course and following thermal denaturation of pp60$^{v\text{-}src}$. Finally, there was a close correlation between the presence of the phosphorylation site and the ability of pp60$^{v\text{-}src}$ to inactivate the protein phosphatase. Proteolytic removal of the phosphorylation site by limited chymotryptic digestion led to reactivation of phosphorylated protein phosphatase 1. Conversely, a partially proteolyzed species of protein phosphatase 1, which lacked the phosphorylation site, was not inactivated by pp60$^{v\text{-}src}$.

The inactivation of protein phosphatase 1 through its direct phosphorylation by pp60$^{v\text{-}src}$ adds an additional element to the control of an enzyme that is already known to be an important target for cellular regulation. Previous studies have shown that protein phosphatase 1 is regulated through the phosphorylation and dephosphorylation of three heat-stable proteins, termed inhibitor 1, inhibitor 2, and DARPP-32, that specifically inhibit the protein phosphatase. Inhibitor 1 and DARPP-32 are structurally and functionally related proteins (23, 24). Each is phosphorylated on a specific threonine residue by cAMP-dependent protein kinase (25, 26) and these phosphorylation reactions are a prerequisite for the inhibitory activity of the proteins (23, 26, 27). The phosphorylation of inhibitor 1 in skeletal muscle and of DARPP-32 in D1-receptive neural cells is increased *in vivo* in response to epinephrine and dopamine, respectively, which elevate levels of cAMP in these cells (28, 29). An inactive protein phosphatase, termed the MgATP-dependent protein phosphatase, has been shown to consist of an equimolar complex of protein phosphatase 1 and inhibitor 2 (30, 31). The protein phosphatase activity of this complex is expressed after its incubation with MgATP and glycogen synthase kinase 3 as a result of the phosphorylation of inhibitor 2 on a threonine residue.

The physiological role of protein phosphatase 1 is best understood in skeletal muscle, where the enzyme represents the major activity towards glycogen phosphorylase *a*, the β-subunit of phosphorylase kinase, and sites 1a, 2, and 3a–c

of glycogen synthase (32). The dephosphorylation of these substrates by protein phosphatase 1 is thought to produce coordinate changes in glycogen metabolism that lead to increased rates of glycogen synthesis and the inhibition of glycogen breakdown (33). The broad substrate specificity of protein phosphatase 1 and the presence of the enzyme in a wide variety of tissues where glycogen metabolism is of little importance suggest that the enzyme may have other functions (32, 34, 35).

The discovery that $pp60^{v\text{-}src}$ was a tyrosine-specific protein kinase led to the suggestion that transformation of cells by RSV was a consequence of the phosphorylation of a set of proteins on tyrosine residues. Our results, together with the observation that transformation of cells by RSV results in increased turnover of inositol phospholipids (36, 37), suggest an extension of this model in which a primary function of $pp60^{v\text{-}src}$ is to regulate control elements in systems involving the phosphorylation and dephosphorylation of cellular proteins on serine and threonine residues. The increased turnover of inositol phospholipids may result from the phosphorylation of diacylglycerol and phosphatidylinositol by $pp60^{v\text{-}src}$ (36) or perhaps via the activation of a phosphatidylinositol phosphate (polyphosphoinositide) phosphodiesterase. The increased turnover of polyphosphoinositides is linked to the activation of protein kinase C and of calcium/calmodulin-dependent protein kinases through the elevation of levels of diacylglycerol and calcium ions, respectively (38, 39). As a consequence, the phosphorylation of a set of cellular proteins on serine and threonine residues is likely to be increased. The phosphorylation of protein phosphatase 1 by $pp60^{v\text{-}src}$ may enhance the phosphorylation of some of these proteins by inhibiting their dephosphorylation. Through a similar mechanism, the inactivation of protein phosphatase 1 may increase the phosphorylation of additional proteins that are substrates for other protein kinases. The phosphorylation of at least four proteins on serine and threonine residues is enhanced in RSV-transformed cells. These include the 40S ribosomal protein S6 (10) and three of the seven proteins that become phosphorylated on tyrosine residues in transformed cells (9). It is unlikely, however, that these represent the full spectrum of proteins whose phosphorylation on serine and threonine residues is revelant to transformation by RSV.

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