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Autophosphorylation-activated protein kinase inactivates the protein tyrosine phosphatase activity of protein phosphatase 2A

Zahi Damuni*, Haishan Xiong, Mei Li

Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, PO Box 850, Hershey, PA 17033, USA

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Abstract Phosphorylation of the catalytic subunit of protein phosphatase 2A (PP2A) on threonines with a distinct autophosphorylation-activated protein kinase [Guo and Damuni (1993) Proc. Natl. Acad. Sci. USA 90, 2500–2504] inactivated the phosphatase with ^{32}P-labelled myelin basic protein prepared by incubation with the kinase domain of the epidermal growth factor receptor, the src-family protein kinases p56^{lck} and p60$^{c\text{-}src}$, myelin basic protein kinase-1, or protamine kinase. Phosphoamino acid analysis demonstrated that the kinase domain of the epidermal growth factor receptor, p56^{lck} and p60$^{c\text{-}src}$ phosphorylated myelin basic protein on tyrosines, that the protamine kinase phosphorylated myelin basic protein on serines, and that myelin basic protein kinase-1 phosphorylated myelin basic protein on threonines. The results demonstrate that the autophosphorylation-activated protein kinase not only inactivates the protein serine/threonine phosphatase, but also the protein tyrosine phosphatase activity of PP2A. This autophosphorylation-activated protein kinase-mediated inactivation of PP2A may, in response to extracellular stimuli, not only contribute to the enhanced phosphorylation of cellular proteins on serines and threonines but also on tyrosines.

Key words: Phosphorylation/dephosphorylation; Protein kinase; Protein phosphatase

1. Introduction

Evidence has accumulated that the low but detectable protein tyrosine phosphatase activity of protein phosphatase 2A (PP2A)[1] [1–3], a multifunctional and ubiquitous protein serine/threonine phosphatase [4–6], may be physiologically relevant. For example, okadaic acid, a potent inhibitor of the protein serine/threonine and tyrosine phosphatase activities of PP2A [7,8], not only enhances the phosphorylation of cellular proteins on serines and threonines, but also on tyrosines [9]. Furthermore, immmunoprecipitates from polyoma-transformed cells with antibody to middle or small T antigens exhibit high okadaic acid sensitive protein tyrosine phosphatase activity [8]. These immunoprecipitates contain the PP2A catalytic C subunit of apparent M_r ~36,000 and the PP2A A subunit of apparent M_r ~60,000, both of which become associated with the middle or small T antigens following viral transformation [10,11]. Cayla et al. [12,13] also have identified a novel protein of apparent M_r ~37–39,000 designated PTPA (PhosphoTyrosyl Phosphatase Activator), which, in the presence of Mg^{2+} and ATP, activates markedly the protein tyrosine phosphatase activity of $PP2A_2$, a form of PP2A composed of the A and C subunits, but not $PP2A_1$, which contains a B subunit of apparent M_r ~55,000 in addition to the A and C subunits. The mechanism of action of PTPA is unknown, although phosphorylation has been ruled out [12,13]. Earlier studies demonstrated that the protein tyrosine phosphatase activity of $PP2A_2$ could also be enhanced markedly following incubation of the phosphatase with ATP [14,15], PP_i [14,15], or tubulin [16]. However, by contrast to PTPA and tubulin, incubation with ATP or PP_i, also inactivated the protein serine/threonine phosphatase activity of the phosphatase [14,15].

We have recently purified to apparent homogeneity a novel protein serine/threonine kinase of apparent M_r ~36,000 from extracts of bovine kidney [17]. Purified preparations of this enzyme underwent an unusually rapid ($t_{0.5}$~0.5–1 min) intramolecular autophosphorylation reaction which activated the kinase about 10-fold [17]. Hence, the enzyme was designated as the Autophosphorylation-activated protein Kinase (AK). Autophosphorylation and concomittant activation of AK were reversed by incubation with PP2A or PP1 [17]. Subsequent studies revealed that, in the presence of Mg^{2+} and ATP, AK inactivated PP2A [18], but not PP1 (unpublished observation), about 80% with several substrates including AK itself, a distinct insulin-stimulated cytosolic protein serine/threonine Protamine Kinase (cPK) [19,20], and ^{32}P-labelled myelin basic protein (MBP) prepared by incubation with cPK. Analysis by autoradiography after sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration chromatography to resolve AK from the PP2A preparations, showed that the C subunit of the phosphatase was phosphorylated following incubation with AK [18]. Phosphoamino acid analysis demonstrated that the C subunit was phosphorylated on threonines [18]. Analysis by thin layer chromatography established that PP2A underwent an autodephosphorylation reaction which was inhibited by microcystin-LR [18], another potent inhibitor of PP2A [21]. In the presence of this inhibitor, AK also incorporated about 1 mol of phosphoryl groups per mol of the A subunit of the PP2A preparations [18]. However, the functional significance of this phosphorylation was not determined.

The purpose of this study was to examine the effect of AK on the protein tyrosine phosphatase activity of PP2A. In this report, we show that, unlike PTPA [13], ATP [14,15], PP_i [14,15], and tubulin [16], AK inactivates the protein tyrosine phosphatase activity of PP2A. The results suggest that AK-mediated inactivation of PP2A may, in response to extracellular stimuli, not only contribute to the enhanced phosphorylation

*Corresponding author.

Abbreviations: PP2A, protein phosphatase 2A; AK, autophosphorylation-activated protein kinase; cPK, cytosolic protamine kinase; EGFRK, epidermal growth factor receptor kinase domain; MBP, myelin basic protein; MBPK-1, myelin basic protein kinase-1; PTPA, phosphotyrosyl phosphatase activator.

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of cellular proteins on serines and threonines, but also on tyrosines.

2. Materials and methods

2.1. Protein preparations

$p60^{c\text{-}src}$ and $p56^{lck}$ were from Oncogene Science and Upstate Biotechnology Inc., respectively. The kinase domain of epidermal growth factor receptor (EGFRK) was from Strategene. cPK [20], AK [19], $PP2A_1$ [22], $PP2A_2$ [22], MBP [23], and myelin basic protein kinase-1 (MBPK-1) [24] were purified to apparent homogeneity as described. All other materials are given in the references [20,24]. Protein was determined according to Bradford [25]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli [26].

2.2. Enzyme assays

Determination of the activities of $PP2A_1$ [22], $PP2A_2$ [22], cPK [20], MBPK-1 [23], and AK [19] was performed as described. One unit of PP2A activity was defined as the amount of enzyme that catalyzed the release of 1 nmol of phosphoryl groups from ^{32}P-labelled MBP/min. To ensure linearity, the extent of phosphoryl group release was limited to < 10%. One unit of cPK activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of phosphoryl groups into protamine sulfate/min. One unit of AK and MBPK-1 activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of phosphoryl groups into MBP/min.

The activities of $p56^{lck}$, $p60^{c\text{-}src}$ and EGFRK were determined using MBP as a substrate. The incubations were performed in a microcentrifuge tube and contained 50 mM Tris-chloride, pH 7.0, buffer containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 14 mM β-mercaptoethanol, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM [γ-^{32}P]ATP (2,000 cpm/pmol), 0.4 μM microcystin-LR, 0.2 mM sodium orthovanadate, and 50 μg MBP in a final volume of 0.05 ml. Reactions were initiated with MBP and terminated after 10 min at 30°C with 1 ml of 10% trichloroacetic acid. After centrifugation at 12,000 × g, the pellets were washed five times with 1 ml portions of trichloroacetic acid. The radioactivity in the pellets was then determined after addition of 1 ml of scintillant to the microcentrifuge tubes. Kinase was omitted from control incubations. One unit of $p56^{lck}$, $p60^{c\text{-}src}$, and EGFRK activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of phosphoryl groups into MBP/min.

2.3. Preparation of ^{32}P-labelled substrates

^{32}P-labelled MBP was prepared by incubation with cPK or MBPK-1 as described [18] or by incubation with $p56^{lck}$, $p60^{c\text{-}src}$ or EGFRK. The incubations with $p56^{lck}$, $p60^{c\text{-}src}$ or EGFRK were performed in a microcentrifuge tube and contained 50 mM Tris-chloride, pH 7.0, buffer containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 14 mM β-mercaptoethanol, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM [γ-^{32}P]ATP (2,000 cpm/pmol), 1.25 mg MBP, and 50 units of protein tyrosine kinase in a final volume of 0.25 ml. Reactions were initiated with MBP, and terminated with 1 ml of 10% (w/v) trichloroacetic acid after 60 min of incubation at 30°C. The mixtures were then centrifuged for 2 min in a microcentrifuge and the supernatant was discarded. The pellets were washed eight times with 1 ml portions of trichloroacetic acid followed by three times with 1 ml portions of 99% ethanol. The pellets were resuspended in 50 mM Tris-chloride, pH 7.0, buffer containing 10% glycerol and 1 mM benzamidine. The solutions were aliquoted and stored at −70°C. Phosphoamino acid analysis was performed as described [27]. Autoradiography was performed with Kodak X-Omat AR5 film.

3. Results

3.1. Phosphorylation of MBP on serines, threonines and tyrosines

Up to 1.5 mol, 0.9 mol, and 0.4 mol of phosphoryl groups were incoporated per mol of MBP following incubation with purified preparations of cPK, MBPK-1 and EGFRK, respectively. Phosphoamino acid analysis demonstrated that EGFRK phosphorylated MBP exclusively on tyrosines, that cPK phosphorylated MBP on serines, and that MBPK-1 phosphorylated MBP on threonines (Fig. 1). Similar analysis showed that up to 0.3 mol and 0.2 mol of phosphoryl groups were incorporated per mol of MBP exclusively on tyrosines following incubation with $p60^{c\text{-}src}$ and $p56^{lck}$, respectively (not shown).

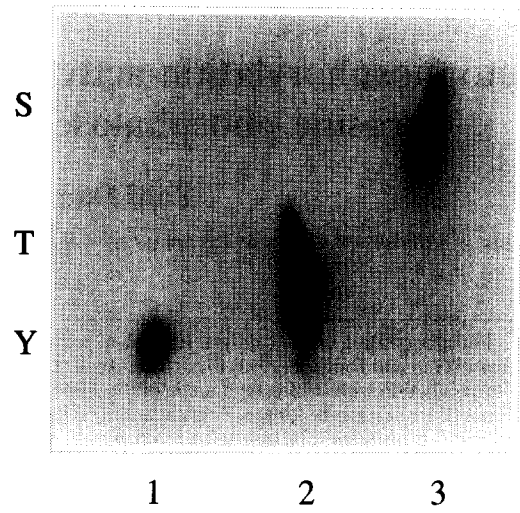


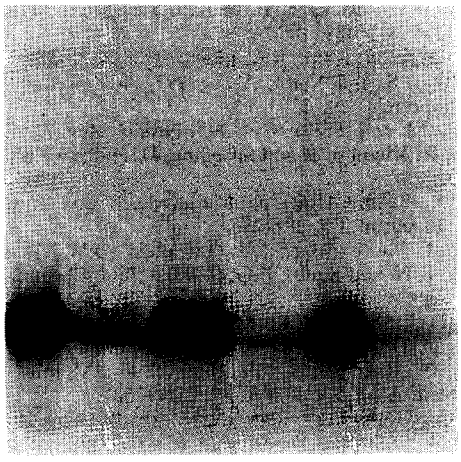
Fig. 1. Phosphorylation of MBP on serines, threonines and tyrosines. ^{32}P-Labeled MBP was prepared by incubation with EGFRK (lane 1), MBPK-1 (lane 2) or cPK (lane 3) as described in section 2. HCl to a final concentration of 6 N was then added and the solutions were heated at 110°C for 1.5 h. After thin layer electrophoresis at pH 3.5 [27], the cellulose plate was dried and then exposed to X-ray film. The figure shows an autoradiogram of the dried plate. The positions of the phosphoserine, phosphothreonine and phosphotyrosine standards, which were run alongside the samples, is shown and was identified by staining with ninhydrin.

3.2. Dephosphorylation of [^{32}P]serine/threonine- and [^{32}P]tyrosine labelled MBP by PP2A

The results presented in Fig. 2 show that $PP2A_1$ dephosphorylated the MBP preparations prepared by incubation with EGFRK, cPK or MBPK-1. Similar results were obtained with $PP2A_2$ (not shown). The activities of $PP2A_1$ and $PP2A_2$ were inhibited by okadaic acid or microcystin-LR with the [^{32}P]tyrosine-, the [^{32}P]serine- or the [^{32}P]threonine-labelled MBP preparations as substrate (not shown). In the standard assays, half-maximal inhibition occurred at about 1 nM okadaic acid or microcystin-LR. These results are consistent with earlier observations (e.g. [8]) that both the protein serine/threonine and protein tyrosine phosphatase activities of PP2A are inhibited by okadaic acid and microcystin-LR. With the [^{32}P]serine- and [^{32}P]threonine-labelled MBP preparations, $PP2A_1$ and $PP2A_2$ exhibited an apparent K_m of about 0.1 μM and 0.25 μM, respectively. In contrast, with the [^{32}P]tyrosine-labelled MBP prepared with EGFRK, $PP2A_1$ and $PP2A_2$ exhibited an apparent K_m of about 10 μM and 30 μM, respectively.

3.3. Inactivation of the protein tyrosine phosphatase activity of PP2A by AK

The effect of AK on the activity of $PP2A_1$ with the [^{32}P]tyrosine-labelled MBP prepared by incubation with EGFRK, the [^{32}P]serine/threonine-labelled MBP prepared by incubation with cPK, and the [^{32}P]threonine labelled MBP prepared by incubation with MBPK-1 was examined next. The results



1 2 3 4 5 6

Fig. 2. Dephosphorylation of [^{32}P]serine, [^{32}P]threonine- and [^{32}P]tyrosine-labelled MBP by PP2A. ^{32}P-Labelled MBP prepared by incubation with EGFRK (lanes 1 and 2), cPK (lanes 3 and 4), or MBPK-1 (lanes 4 and 5) as described in section 2 was incubated in a final volume of 0.025 ml in 50 mM Tris-chloride, pH 7.0, buffer containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, and 14 mM β-mercaptoethanol, in the absence (lanes 1, 3 and 5) and presence (lanes 2, 4 and 6) of 40 ng $PP2A_1$. After 30 min at 30°C, sample buffer [26] was added to the incubations, the solutions were heated to 100°C, and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed [26]. The gel was stained with Coomassie blue, washed extensively, dried and exposed to X-ray film. An autoradiogram of the dried gel is shown. The arrow denotes the position of MBP.

presented in Fig. 3 demonstrate that AK inactivated $PP2A_1$ with all of the ^{32}P-labelled MBP preparations employed as substrates. Similar results were obtained when $PP2A_2$ was incubated with AK, and when [^{32}P]tyrosine-labelled MBP prepared by incubation with $p60^{c\text{-}src}$ or $p56^{lck}$ was employed as substrate (not shown). Analysis of incubations containing AK, PP2A and [γ-^{32}P]ATP by autoradiography after gel filtration chromatography of the incubations on Superose 12 by Fast Protein Liquid Chromatography and by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described earlier [18] showed that the catalytic subunits of $PP2A_1$ and $PP2A_2$ were phosphorylated in the incubations with AK (not shown).

4. Discussion

The results presented in this communication establish that AK not only inactivates the protein serine/threonine, but also the protein tyrosine phosphatase activities of $PP2A_1$ and $PP2A_2$ (Figs. 1–3). These AK-mediated effects are clearly different from the effects of PTPA [12], ATP [14,15], PP_i [14,15], and tubulin [16], which, by contrast to AK, activate the protein tyrosine phosphatase activity of PP2A. Moreover, unlike PTPA [12], ATP [14,15], PP_i [14,15], and tubulin [16], which exhibit activity with $PP2A_2$ but apparently not with $PP2A_1$, AK inactivated the protein serine/threonine and protein tyrosine phosphatase activities of $PP2A_1$ (Figs. 1–3) and $PP2A_2$ (not shown). Also unlike PTPA [12] and tubulin [16], which have little or no effect on the protein serine/threonine phosphatase activity of PP2A, AK inactivated the protein serine/threonine phosphatase activity of the PP2A preparations (Figs. 1–3). The AK-catalyzed phosphorylation and concomitant inactivation of PP2A is nonetheless similar to the PTPA- and tubulin-mediated activations of the protein tyrosine phosphatase activity of PP2A [12,16] in that it also is transient [20] (this study, not shown). Whether the AK-catalyzed phosphorylation of PP2A has any effect on the activation of the phosphatase by PTPA or tubulin or vice versa remains to be determined.

Several hormones and other extracellular stimuli enhance the phosphorylation of cellular proteins on serines, threonines and tyrosines (reviewed in [28]). These phosphorylations control the activities of rate-limiting enzymes involved in diverse cellular processes and are therefore considered important in signal transduction [28]. Because the state of phosphorylation of any protein depends on the relative activities of the protein kinases and phosphatases, enhanced phosphorylation on serines, threonines, and tyrosines may result from the activation of protein kinases (reviewed in [28]) and/or the inactivation of protein phosphatases. Because PP2A is a 'dual specificity' protein phosphatase (e.g. Fig. 2), the results presented herein suggest that AK-mediated inactivation of PP2A (Fig. 3) may, in response to hormones and other extracellular stimuli, not only contribute to the enhanced phosphorylation of cellular proteins on serines and threonines, but also on tyrosines. Consistent with these possibilities is a recent report indicating that PP2A is inactivated, albeit by an unknown mechanism, following incubation of rat skeletal muscle cells with insulin [29]. In addition, okadaic acid, a potent inhibitor of the protein serine/threonine and protein tyrosine phosphatase activities of PP2A [7,8], not only enhanced the phosphorylation of cellular

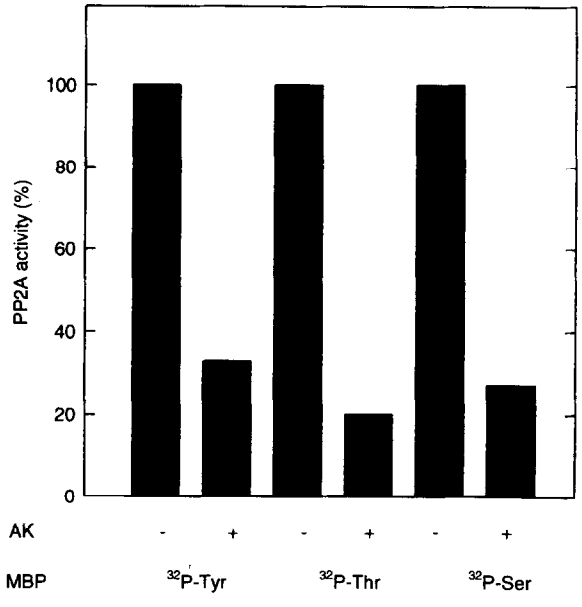


Fig. 3. Inactivation of the protein serine, threonine and tyrosine phosphatase activities of PP2A by AK. A 0.005 ml aliquot containing 12.5 ng of $PP2A_1$ was mixed with 0.035 ml of a solution made up of 50 mM Tris-chloride, pH 7.0, buffer containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 14 mM β-mercaptoethanol, 0.2 mM ATP, and 1 mM $MgCl_2$, and that had been incubated at 30°C for 10 min in the absence and presence of AK (12 ng). After a further 20 min of incubation at 30°C, 0.01 ml of ^{32}P-labelled MBP prepared by incubation with EGFRK, MBPK-1, or cPK was added as indicated. After an additional 3 min at 30°C, dephosphorylation was terminated with 0.1 ml of 10% trichloroacetic acid and phosphatase activity was determined as described [22].

proteins on serines and threonines, but also on tyrosines [9]. However, whether okadaic acid inhibition of PP2A was directly and/or indirectly responsible for the enhanced protein tyrosine phosphorylations in the cells was not established [9] perhaps, in part, because physiological substrates specific for the protein tyrosine phosphatase activity of PP2A have not yet been identified.

Recent studies have indicated that PP2A may itself be its own physiologically relevant protein tyrosine phosphorylated substrate [30]. Thus, it was reported that, in the presence of okadaic acid, the C subunit of PP2A could be phosphorylated stoichiometrically on tyrosine residues by immunoprecipitates of $p60^{v\text{-}src}$ from cells overexpressing the gene for this enzyme, the src-family protein kinase $p56^{lck}$, and purified preparations of the protein tyrosine-specific epidermal growth factor and insulin receptors [30]. Because phosphorylation of the C subunit was less than stoichiometric when okadaic acid was omitted from the incubations, the results were interpreted to indicate that okadaic acid inhibited PP2A from undergoing an auto-tyrosine-dephosphorylation reaction [30]. The observations reported herein suggested that this PP2A auto-tyrosine-dephosphorylation reaction may be inactivated by the AK catalyzed threonine phosphorylation of the C subunit of the phosphatase. However, at variance with the earlier observations [30], we have, in many attempts, observed little or no phosphorylation of the C subunit of PP2A with purified preparations of $p60^{c\text{-}src}$, $p56^{lck}$ and EGFRK in the absence or presence of okadaic acid. The reason for these discrepancies is uncertain. In this connection, it is noteworthy that the PP2A preparations employed in this study can be methylated at the carboxyl terminal Leu^{309} of their C subunit by purified preparations of a methyltransferase from bovine brain [31]. Therefore, the C subunit of the PP2A preparations employed in this study is not partially cleaved and contains the putative Tyr^{307} phosphorylation site [30]. Whether methylation affects the phosphorylation of the C subunit on threonines and/or tyrosines is of considerable interest and is currently under investigation in this laboratory.

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