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ATP-citrate lyase as a substrate of protein histidine phosphatase in vertebrates☆

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Abstract

The first protein histidine phosphatase from vertebrates discovered recently was found in a variety of tissues, however, a physiological substrate protein was missing. Phosphorylation of liver extracts in the presence of EDTA, followed by SDS–PAGE and autoradiography showed labeling of three proteins. Acid- and alkaline-treatment revealed the existence of N-phosphates. Addition of histidine phosphatase exclusively resulted in dephosphorylation of a 110 kDa protein (denaturing conditions). Gelfiltration revealed its native molecular mass of ~450 kDa. That protein was purified and identified as ATP-citrate lyase. The results are in favor of histidine phosphatase playing an important yet unidentified role in metabolic processes.

Reversible phosphorylation of *histidine* residues has been extensively studied in prokaryotes (for reviews see [1,2]). Histidine kinases, histidine phosphatases, and the phosphate-transfer from histidine to aspartate via two-component systems are essential and well-established elements of signal transduction in bacterial cells. This is in sharp contrast to the knowledge of numerous kinases and phosphatases acting on O-phosphate esters of serine, threonine, and tyrosine residues in proteins from eukaryotes (for reviews see [3–5]). Indeed, information on phosphorylation and dephosphorylation of histidine residues (reversible N-phosphorylation) in vertebrates nowadays is still limited (for review see [6]), although the presence of phosphohistidine in vertebrate proteins was first described in the 1960s [7,8] and by now is estimated to account for 6% of total protein phosphorylation in eukaryotes [9]. Acid-labile phosphates in rabbit liver could be allocated to histones I and IV in the 1970s [10,11]. Today the number of substrates identified for covalent modification of histidine residues in vertebrates still is surprisingly modest: (i) Annexin I, a 37 kDa member of a family of Ca^{2+}-dependent phospholipid-binding proteins, was reported to undergo histidine phosphorylation in airway epithelia [12]; (ii) P-selectin, a leukocyte adhesion molecule, was shown to become phosphorylated on histidines following platelet activation with thrombin or collagen [13]; and (iii) guanine nucleotide-binding regulatory protein β-subunits (G_β) were shown to undergo phosphorylation at a histidine residue in human leukemia cells and in insulin secreting cells [14,15]. Thus, there is strong evidence for important and multiple roles of phosphohistidine in vertebrate signal transduction pathways.

Serine/threonine protein phosphatases type 1, 2A, and 2C were shown to be capable of dephosphorylating [^{32}P]his-histone H4 in vitro [16]. Vertebrate phosphatases specific for phosphohistidine had not been identified until 2002 when two groups independently discovered the same characteristics and sequence of a 14 kDa protein acting on phosphohistidine [17,18]. The probes used for detection of histidine phosphatase activity were different: a peptide in one case [17], the bacterial autophosphorylated histidine kinase cheA in the other [18]. A physiological substrate for protein

☆ *Abbreviations:* ACL, ATP-citrate lyase; BSA, bovine serum albumin; PHP, protein histidine phosphatase; PP1, PP2A, PP2B, PP2C, types 1, 2A, 2B, 2C eukaryotic serine/threonine-specific protein phosphatases; PVDF, polyvinyliden difluoride; TEA, triethanolamine.

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histidine phosphatase (PHP) was missing. Therefore, we set out to search for a vertebrate substrate protein of PHP. Here, we describe ATP-citrate lyase (ACL) as a substrate for PHP, suggesting a crucial role of this phosphatase for metabolic pathways.

Materials and methods

Preparation of the soluble extract from rabbit liver. Rabbit liver was homogenized at 4 °C using an ultraturrax (CATx620). The buffer (2 ml/g tissue) consisted of 30 mM TEA (pH 7.5), 1 mM EDTA, 300 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, and 0.02% NaN_3. After spinning at 48,000*g* (1 h), the supernatant was passed through cheesecloth, and subsequently centrifuged at 100,000*g* (1 h). The resulting supernatant fraction (soluble extract) was stored frozen at −80 °C.

Phosphorylation and dephosphorylation reactions. Phosphorylation of rabbit liver soluble extracts was carried out at 37 °C for 10–15 min as described [19]. Reactions were performed in a volume of 100 μl containing 1 mg soluble extract, 25 mM Tris–HCl (pH 7.5), 5 mM EDTA, and 1 μM ATP including 30 μCi [γ-^{32}P]ATP (185 TBq/mmol). Assays were stopped by placing the tubes on ice. In addition, unincorporated ATP was removed using Centrisep spin columns (Princeton Separations). Dephosphorylation reactions (15 μl) of the rabbit liver phosphorylated proteins were performed immediately thereafter at 37 °C for 30 min. The assays contained 10 μl of the terminated phosphorylation free of ATP, 25 mM TEA (pH 7.5), 0.1% 2-mercaptoethanol, and PHP as indicated. Reactions were stopped by addition of 5 μl sample buffer, not heated, and run on 15% SDS–PAGE minigels followed by autoradiography. The sample buffer consisted of 15 mM Tris–HCl (pH 6.8), 4% SDS, 2% 2-mercaptoethanol, 8 M urea, 10% sucrose, 10 mM EDTA, and 0.01% bromphenol blue.

Phosphorylation of the bacterial histidine kinase cheA was performed in the presence of 1 μM ATP containing 100 μCi [γ-^{32}P]ATP and 0.1 mM Mg^{2+}, otherwise as described [20]. Unincorporated [γ-^{32}P]ATP was removed by Centrisep spin columns. For dephosphorylation of [^{32}P]his-cheA, PHP was incubated for 30 min at 37 °C in a 40 μl reaction mixture containing 0.6 ng [^{32}P]his-cheA (0.21 pmol [^{32}P]/ml), 25 mM TEA (pH 7.5), 10 mM $MgCl_2$, and 0.1% 2-mercaptoethanol.

Acid and alkaline treatment of phosphoproteins. Phosphorylated proteins separated on 15% SDS–PAGE minigels were electroblotted onto polyvinylidene difluoride membranes. A modified version of a protocol developed to discriminate O-phosphates from N-phosphates via different stability to acid- and alkaline solutions was employed [21]. The membranes were incubated in either 6 N HCl or 1 M KOH or 0.1 M Tris–HCl (pH 7.5), at 45 °C for 2 h, respectively, then air-dried and autoradiographed. CheA undergoing autophosphorylation at his-48 was used as a positive control.

Purification of the substrate protein of histidine phosphatase. A purification procedure was developed to isolate the 110 kDa histidine phosphoprotein following its phosphorylation in the presence of EDTA and detection by autoradiography. This protein later on turned out to be ACL. The soluble extract from rabbit liver was used as starting material. Enzyme preparations were carried out at 4 °C. The column buffers used consisted of 20 mM Tris–HCl (pH 7.5), 1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, and 0.02% NaN_3 with supplements as indicated.

Ten milliliters of the soluble extract from rabbit liver (0.5 g protein) was loaded on a 12 ml column of Source 30Q equilibrated in column buffer and run at 1 ml/min. The 110 kDa protein eluted with 150 mM NaCl added to the buffer. Active fractions were pooled, diluted 1/5, and applied to a 1 ml column of Blue Sepharose 6 Fast Flow run at 0.5 ml/min. Buffer supplemented with 15 mM ATP was used for elution. The first 10 ml of the eluate was concentrated using Centriprep centrifugal filter devices (Millipore) and further purified on a HiLoad 26/60 Superdex 200 prep grade column of 320 ml run at 1 ml/min with the column buffer in the presence of 5% glycerol. Fractions containing the 110 kDa histidine phosphoprotein (elution volume 130–140 ml corresponding to a molecular mass of 400–450 kDa) were pooled and stored frozen in aliquots at −80 °C.

Miscellaneous. PHP was obtained from lysates of SF9 insect cells co-transfecting BaculoGold and pVL1392-php (pST118) [18]. CheA was expressed in *Escherichia coli* [20]. Both proteins were purified by affinity chromatography on Ni^{2+}-NTA agarose using imidazole for elution. Protein concentrations were determined according to Lowry (homogenate) or Bio-Rad (soluble extract, recombinant, and purified proteins) with bovine serum albumin as a standard.

Results and discussion

Detection of a substrate for histidine phosphatase

PHP had been identified in various vertebrate tissues just recently [17,18]. We began searching for a physiological substrate using rabbit liver as starting material. Conventional phosphorylation with [γ-^{32}P]ATP in the presence of Mg^{2+} resulted in hundreds of phosphorylated proteins as expected, implicating that the probability to detect only few signals after adding PHP would be low. Therefore, we took advantage of a method developed for showing histidine phosphoproteins exclusively [19]. Phosphorylation of rabbit liver with [γ-^{32}P]ATP in the presence of EDTA labeled three proteins only corresponding to molecular masses of about 110, 40, and 20 kDa under denaturing electrophoretic conditions. Addition of PHP resulted in dephosphorylation of the 110 kDa protein (Fig. 1). In contrast, histidine phosphoproteins of 40 and 20 kDa were not affected by PHP (Fig. 1). Dephosphorylation of the 110 kDa protein increased with increasing PHP concentration (Fig. 1A) and incubation time (Fig. 1B).

Purification of the substrate protein

The 110 kDa protein was purified following its phosphorylation in the presence of EDTA and detection by autoradiography. It soon became apparent that we were dealing with autophosphorylation of the 110 kDa protein. Coelution of a kinase and its corresponding substrate is unlikely to occur with three different chromatographic criteria: anion exchange, affinity chromatography, and gel filtration. Phosphorylation of the 110 kDa protein was not recovered in the breakthrough of Source 30Q, but eluted with 150 mM NaCl (Figs. 2A and B). The autophosphorylating protein was retained by Blue Sepharose and eluted upon addition of 15 mM ATP (data not shown). Gelfiltration revealed the surprising result that we were dealing with a protein of ~450 kDa (Fig. 2C). The discrepancy in its molecular mass depending on native conditions (gelfiltration:

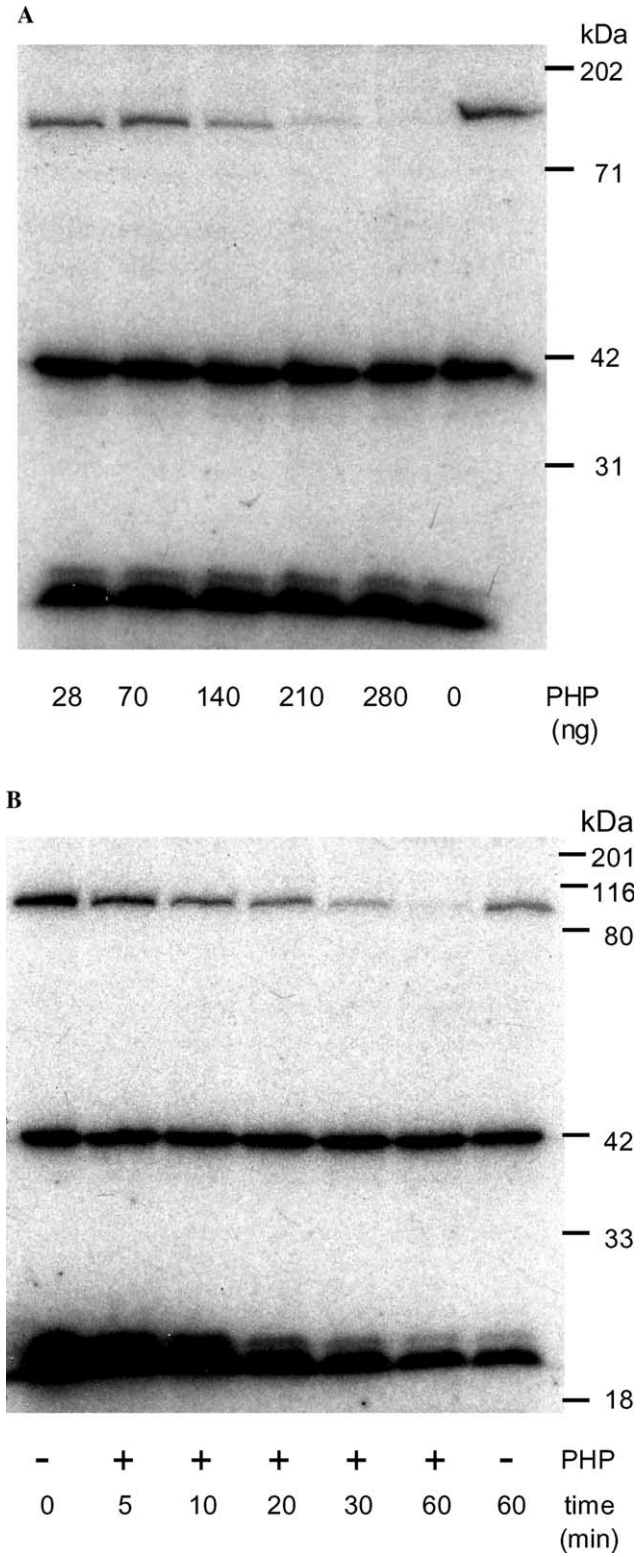

Fig. 1. Effect of PHP on histidine phosphoproteins. The soluble extract from rabbit liver was phosphorylated with [γ-^{32}P]ATP in the presence of 5 mM EDTA. Labeled proteins were subsequently treated with PHP, separated on 12.5% SDS–PAGE, and autoradiographed. (A) Protein dependence. PHP was increased from 0 to 280 ng. (B) Time dependence. Incubation of histidine phosphoproteins in the presence of 140 ng PHP was carried from 0 to 60 min.

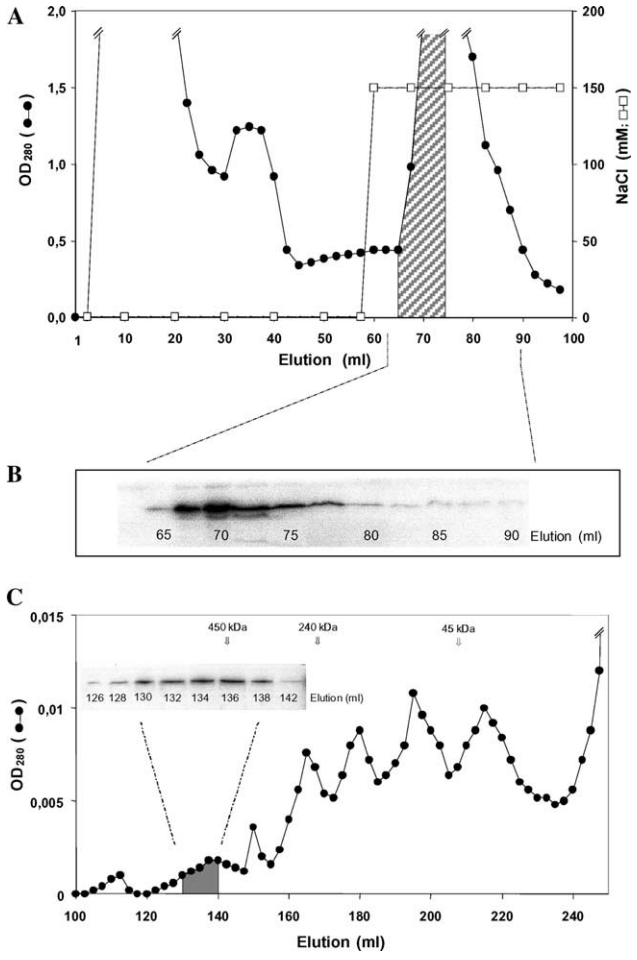

Fig. 2. Purification of the PHP substrate running at 110 kDa on SDS–PAGE. (A,B) Anion-exchange chromatography (Source 30Q). The phosphohistidine-containing protein eluted at 150 mM NaCl (shaded area in A). (B) An autoradiogram after phosphorylation of the fractions in the presence of EDTA. (C) Gelfiltration (Superdex 200). The autoradiogram (inset) demonstrates elution of the phosphohistidine-containing protein corresponding to a molecular mass of about 450 kDa. Marker proteins used for calibration were ovalbumin (45 kDa), catalase (240 kDa), and ferritin (450 kDa).

450 kDa) versus denaturing conditions (SDS–PAGE: 110 kDa) was indicative of a multimeric enzyme. The purity of the protein isolated as described above is shown on a colloidal Coomassie blue stained gel (Fig. 3A) and the phosphorylation potency of the purified protein is demonstrated in an autoradiogram (Fig. 3B).

Identity of the substrate protein

In order to identify this protein, the stained 110 kDa band was excised from the gel and cleaved with trypsin and CNBr. Sequence information after Edman degradation and post-source decay MALDI mass spectrometry revealed peptides of ACL; WGDIEFPPPFGR and TIAIIAEGIPEALTR correspond to ACL amino acids 233–244 and 593–607, respectively. Indeed, ACL is a

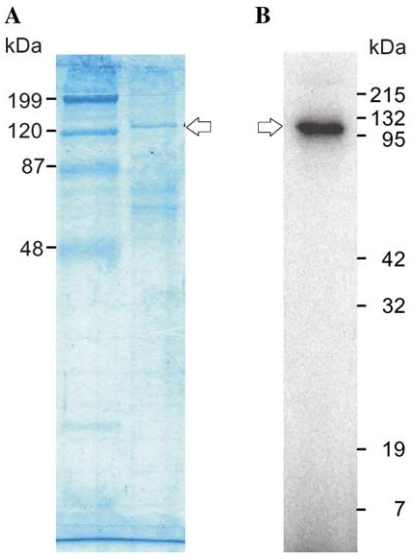

Fig. 3. Purity and autophosphorylation of the protein running at 110 kDa on SDS–PAGE. For calibration, prestained markers were used. (A) Purity was examined on a 12.5% SDS–PAGE minigel using colloidal Coomassie blue for staining. Left: Marker proteins. Right: 0.1 μg of the protein purified by Source 30Q, Blue Sepharose, and Superdex 200 as described in Materials and methods. (B) The purified protein was phosphorylated as described in Materials and methods, run on a 15% SDS–PAGE minigel, and autoradiographed.

tetramer composed of 110 kDa subunits [22] and phosphorylates at histidine-760 as an intermediate in the catalysis [23,24]. In addition to this autophosphorylation by ATP, histidine-760 of ACL more recently was shown to be a substrate for nucleoside diphosphate kinase [25]. Such dual phosphorylation mechanisms—intermediate *and* kinase substrate—are rare but do exist in nature, and are best studied in bacterial phosphoenolpyruvate:sugar phosphotransferase systems [26].

The identity of ACL was further verified taking advantage of the fact that phosphate bound to ACL can be removed by ADP [27]. Addition of 1 mM ADP to the soluble extract of rabbit liver or to purified ACL after phosphorylation in the presence of EDTA eliminated the 110 kDa signal immediately (Fig. 4). In contrast, AMP, ATP, or GTP did not cause hydrolysis. Finally, we were using acid- and alkaline-treatment of the phosphoproteins to make sure that we were dealing with phosphohistidine and to exclude phosphoserine, -threonine, and -tyrosine (data not shown), confirming the results by Noiman and Shaul [19].

Substrate specificity of histidine phosphatase

It was known that PHP is capable of dephosphorylating the phosphohistidine-containing peptide succinyl-ala-his(P)-pro-phe-*p*-nitroanilide [17] and the bacterial protein cheA [18]. Furthermore, here we discovered ACL as a vertebrate substrate protein for PHP. Next,

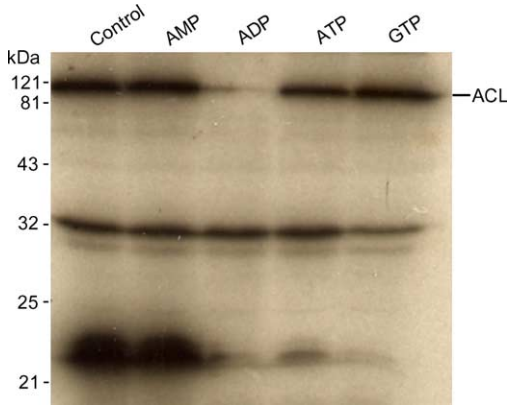

Fig. 4. Removal of phosphate bound to ACL by ADP (autoradiogram). Proteins in the soluble extract from rabbit liver were phosphorylated in the presence of EDTA as described in Materials and methods. One millimolar of nucleoside mono-, di-, or triphosphates was added after 15 min of phosphorylation and the reactions stopped by addition of sample buffer after an additional 5 min at 37 °C.

we addressed the questions about substrate specificity of PHP in general and also vice versa, whether PHP substrates would be subject to dephosphorylation by other phosphatases.

PHP did not hydrolyze phosphohistidine-containing proteins unspecifically, e.g., 20 kDa [^{32}P]his-nucleoside diphosphate kinases were not a substrate for PHP

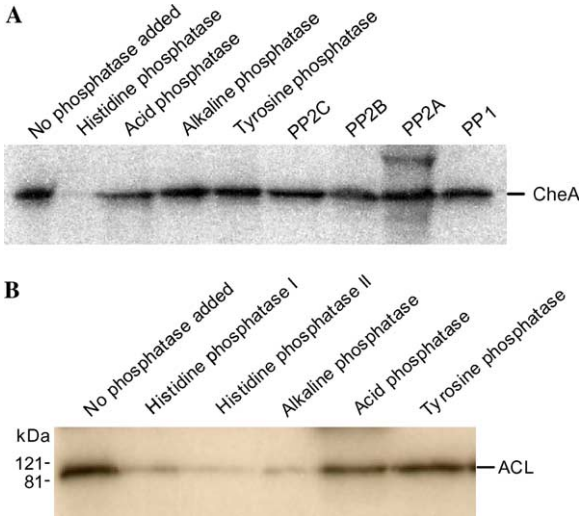

Fig. 5. Substrate specificity (autoradiograms). PHP assay, see Materials and methods; acid phosphatase, 50 mM sodium acetate, pH 5; alkaline phosphatase, 50 mM Tris pH 7.5; tyrosine phosphatase, 50 mM Tris–HCl, pH 7.5, 2.5 mM EDTA, and 100 μg/ml BSA; PP2B, 50 mM Tris–HCl, pH 7.5, 0.1 mM Ca^{2+}, 10 μg/ml calmodulin, and 100 μg/ml BSA; PP1 and PP2A, 50 mM Tris–HCl, pH 7.5, 5 mM Mn^{2+}; and PP2C, 50 mM Tris–HCl, pH 7.5, 20 mM Mg^{2+}. All buffers in addition contained 1% glycerol and 0.1% 2-mercaptoethanol. (A) Dephosphorylation of [^{32}P]his-cheA using 0.2 μg of phosphatases, respectively. (B) Dephosphorylation of phosphohistidine-containing proteins in the soluble extract from rabbit liver. Phosphatases (0.3 μg) was added, respectively, except for the lane histidine phosphatase II containing 0.6 μg PHP.

(Fig. 1). Furthermore, [^{32}P]tyr-EGF receptor and [^{32}P]ser/thr-casein could not be dephosphorylated by PHP, suggesting specificity of PHP for phosphohistidine. *p*-Nitrophenyl phosphate, the widely used low molecular mass compound for photometric phosphatase assays also did not undergo dephosphorylation by PHP (data not shown).

Dephosphorylation of [^{32}P]his-cheA turned out to be remarkably specific for PHP (Fig. 5A). Neither acid- or alkaline phosphatases, nor the main representatives of ser/thr and tyr-phosphatases were capable of dephosphorylating [^{32}P]his-cheA (Fig. 5A). In contrast, dephosphorylation of [^{32}P]his-ACL was not exclusive for PHP. Similar to what was described for the dephosphorylation of [^{32}P]his-histone by some of the classical ser/thr phosphatases [16], alkaline phosphatase (Fig. 5B) and PP2A (data not shown) were also capable to act on ACL as a substrate. In contrast, acid phosphatase and tyr-phosphatase did not dephosphorylate [^{32}P]his-ACL (Fig. 5B).

Conclusions

Here, we have shown that [^{32}P]his-ACL is a substrate of the 14 kDa PHP identified recently. According to what is known about histidine phosphorylation of ACL by ATP, and its properties, we suggest that PHP acts on histidine-760 of ACL. The physiological significance of this reversible phosphorylation of ACL at histidine is unclear at present. Having identified PHP acting on ACL, the tools are available now to examine whether there is an influence on ACL enzyme activity. The results may vary depending on the phosphate source, e.g., glucose and hexokinase are known to prevent phosphorylation of ACL by [γ-^{32}P]ATP, but not its phosphorylation by [^{32}P]nucleoside diphosphate kinase [25].

With the data presented in this paper it is intriguing to recognize that the complex regulation of ACL allosterically by phosphorylated sugars and covalently by phosphorylation of serine- and threonine residues [28] may not be the entire story.

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