Detection of histidine-phospho-proteins in animal tissues

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Abstract In this report we outline a protocol for rapid detection of histidine phosphoproteins in cellular crude extracts prepared from different tissues. The nature of the phosphorylated amino acid residues was confirmed by determination of their stability under different pH conditions and by direct phospho-amino acid analysis. Furthermore, DEPC treatment that can selectively modify the histidine residues blocks the phosphorylation. Interestingly, the phosphoprotein pattern detected under these conditions in four different tissues is very similar, suggesting that these proteins play important roles in biochemical pathways shared by many cells and tissues.

Key words: Histidine kinase; Nucleoside diphosphate kinase; Phosphohistidine protein; EDTA dependent phosphorylation

1. Introduction

Histidine kinases are histidine phosphoproteins known to play a major role in signal transduction in bacteria. In this pathway a sensor component usually acts as a histidine phosphorylation autokinase. The phosphoryl group is then transferred to a response regulatory protein [1]. Recently, sequence similarities between two eukaryotic proteins and the transmitter/receiver domains of the bacterial two component regulator were identified [2,3], at least one of which was found to regulate an osmosensing MAP kinase cascade [4]. In yeast, a histidine kinase protein which phosphorylates histidine residues in histone H4 has been isolated [5]. In mammals, our knowledge of histidine kinases is very limited; rat liver mitochondrial β-ketoacid dehydrogenase [6] and pyruvate dehydrogenase kinase [7], have weak homology to the bacterial histidine kinases, however, in neither case has autophosphorylation been demonstrated. Protein histidine phosphorylation, which had been reported only in rat extracts [8,9], may play an important role in mammalian cellular pathways but had probably been overlooked because of its instability. Previously, we have reported that in the presence of EDTA the X protein of the hepatitis B virus (HBV) is autophosphorylated [10]. Interestingly, under these conditions the cellular enzyme nucleoside diphosphate kinase (NDPK) is phosphorylated [11]; [12] at a histidine residue [13], suggesting that EDTA may selectively permit histidine phosphorylation. Here, taking advantage of this rationale, we were able to detect phospho-histidine proteins in four different rat tissues.

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2. Materials and methods

2.1. Preparation of cytoplasmic extracts

Brain, liver, kidney and lung dissected from a 6-day-old rat were lysed in hypotonic buffer (5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES pH 7.1) containing 1 μg/ml each of leupeptin and antipain and 1 mM phenylmethyl sulfonyl fluoride (PMSF), homogenized using a Daunce homogenizer and centrifuged for 2 min at 15,000 × g. Supernatants were used for phosphorylation reactions.

2.2. Phosphorylation reaction

30 μg of cellular extracts prepared from the indicated rat tissues, were incubated for 1 h in buffer containing 50 mM HEPES, pH 8, 10 μM ATP, 100 mM NaCl, 5 mM EDTA or 10 μM $MgCl_2$ and 10 μCi [γ-^{32}P]ATP (5000 Ci/mmol) at 4°C or 30°C. The reactions were stopped by adding sample buffer, separated by 12% SDS-PAGE, blotted onto Hybond N (Amersham) membrane and submitted to 16 h of autoradiography. For DEPC (diethylpyrocarbonate) treatment, the cellular extracts were incubated for 15 min at room temperature, prior to the phosphorylation reaction, with 20 mM DEPC dissolved in 2% ethanol in Bis-Tris buffer, pH 6.5, as reported [14].

2.3. pH treatments

Cellular extracts were phosphorylated, separated by SDS-PAGE and transferred to Hybond membrane. After autoradiography, the membrane containing the phospho-proteins was cut out, incubated for 2 h at 45°C in 50 mM KCl-HCl (pH 1) or 1 M KOH (pH 14) and was autoradiographed again.

2.4. Phosphoamino acid analysis

^{32}P-Labeled proteins were transferred to a Hybond N (Amersham) membrane and hydrolyzed in 3 N KOH or 6 N HCl at 105°C for 4 h. A portion of the hydrolysate (10%) was mixed with standard phosphoamino acids and analyzed directly on a K6 silica gel 60A° TLC plate (Whatman) with two successive solvent (ethanol:25% ammonia solution, 3.5:1.6) cycles [8].

The position of the standard phosphoamino acids was detected by ninhydrin. Phosphohistidine was synthesized by mixing 10 mg of histidine with 100 mg of potassium phosphoramidate in 1 ml water [15].

3. Results

To develop a rapid method for detection of phosphohistidine proteins, cytoplasmic extracts prepared from four different rat tissues were incubated with [γ-^{32}P]ATP in the presence of EDTA (Fig. 1). Interestingly, in addition to the NDPK, several proteins were phosphorylated in all the tested tissues. In general, the phosphoprotein pattern obtained with the distinct tissues is similar. However, in the brain extract the level of phosphorylation (p43, p38 and p37) is very high, but p85 phosphoprotein, which is seen in all the other tissues, is not detectable. Furthermore, the p32 phosphoprotein is seen only in the brain extract. To test the nature of the phosphate link, we determined the phosphoproteins' pH stability. It is well documented that phosphotyrosine is stable at both pH 1 and 14 while other O-linked phospho-amino acids (phosphoserine and phosphothreonine) are stable at pH 1 but not at pH 14; *N*-phosphate amino acids (phosphohistidine, phospholysine

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and phosphoarginine) are known to be stable at pH 14 but not pH 1. On the other hand, acyl-phosphate amino acids (phosphoaspartate and phosphoglutamate) are labile at both pHs 1 and 14 [16]. The membrane containing the phosphoproteins was first treated by alkaline conditions, and it was found that p43 selectively disappeared in all the examined tissues and that the level of p85 sharply reduced (Fig. 1). Successive treatment with pH 1 resulted in the total loss of all radioactivity (data not shown). These data clearly suggest that p43 and a fraction of p85 contain O-linked phosphoprotein while the others are of the N-linked type. Thus under the employed phosphorylation reaction conditions both N- and O-linked phosphoproteins are formed. Note that the NDPK phosphorylation is mostly, as expected [13], of the N-linked type.

One possibility is that the N-linked phosphoproteins are generated by phosphorylation of histidine residues, and to address this issue we repeated the experiment using DEPC-pretreated brain extract. DEPC is known to selectively chemically modify histidine residues, preventing the N-linked phosphorylation [5]. Unexpectedly, no protein was phosphorylated (Fig. 2), suggesting that all the phosphorylation detected under EDTA conditions, including the O-linked phosphorylation, is phospho-histidine dependent. A control lane in which the DEPC-treated extract was subjected to conventional phosphorylation reaction in the presence of Mg^{2+} is shown to indicate that the effect of DEPC was specific. To reconfirm that p43 generated in the presence of EDTA and that the majority of the phosphoprotein

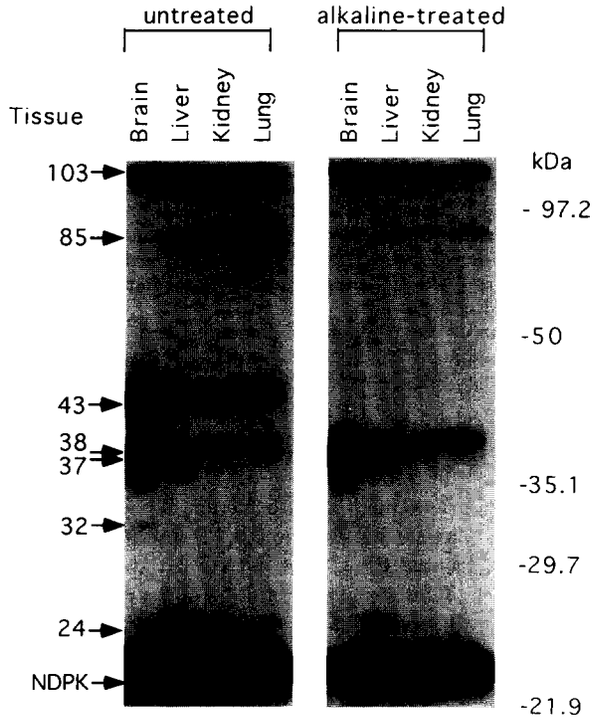

Fig. 1. Phosphorylation of cellular proteins in the presence of EDTA in different rat tissues. Extracts prepared from rat tissues (Brain, Liver, Kidney, Lung) were incubated with [γ-^{32}P]ATP in the presence of EDTA at 4°C. The phosphorylated proteins were resolved by SDS-PAGE, blotted onto Hybond N (Amersham) and exposed to X-Ray film at −70°C. After the first exposure the membrane was treated with pH 14 (alkaline treated) and exposed again under the same conditions. Molecular weight markers, and sizes of the phosphorylated proteins are indicated.

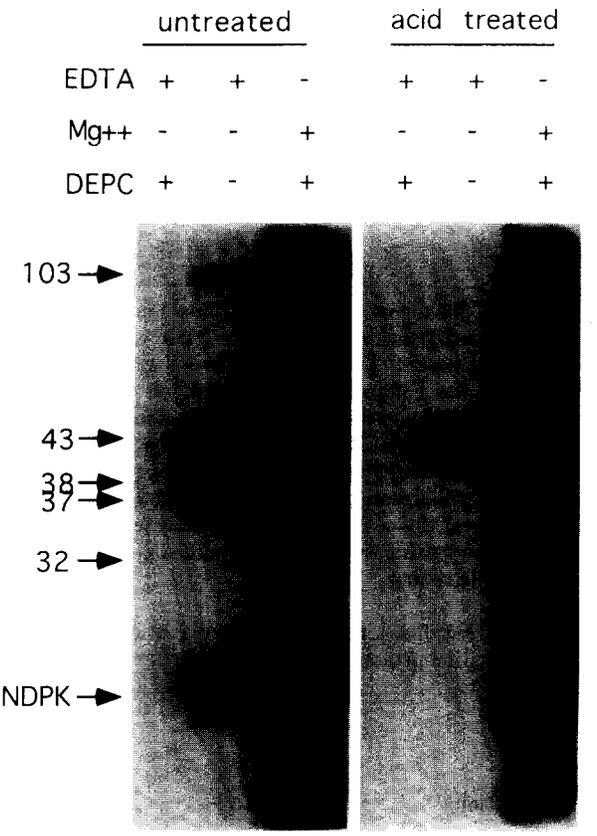

Fig. 2. DEPC abolishes cellular phosphorylation. Cytoplasmic brain extracts, either untreated or treated with DEPC, were phosphorylated in the presence of EDTA at 4°C or $MgCl_2$ at 30°C, separated, blotted and exposed as described in Fig. 1. The membrane was then treated with 50 mM KCl-HCl (pH 1) (acid treated) and re-exposed.

formed in the presence of Mg ions are of O-linked type, the phosphorylated proteins were treated with pH 1 (Fig. 2).

To further verify the nature of the phosphorylated amino acids we performed phospho-amino acid analysis of the major proteins; p43 that is alkaline labile and a mixture of p38 plus p37 that are acid labile. The corresponding bands were eluted from the filter and the proteins were hydrolyzed either by acid or alkaline buffers and the products were analyzed by TLC with internal phosphohistidine or phosphoserine and phosphothreonine unlabeled markers (Fig. 3). As expected, the majority of the alkaline-labile p43 phospho-amino acid is phosphoserine or phosphothreonine whereas of acid labile p37–p38 is phosphohistidine.

Clearly, the phosphorylation conditions employed by us preferentially generates phosphohistidine proteins. However, it is possible that the labeled proteins are NDPK-like enzymes that are artificially stabilized in the presence of EDTA, forming phosphohistidine-transient intermediates. To rule out this possibility we performed a two-step reaction (Fig. 4). The extract was first phosphorylated in the presence of EDTA, the excess [γ-^{32}P]ATP was removed by dialysis and then MgCl was added. As expected, the NDPK as well as the p43 and p37–38 proteins were highly phosphorylated in the presence of EDTA. However, in the presence of Mg ions NDPK transferred the phosphate presumably to NDP to complete the nucleotide

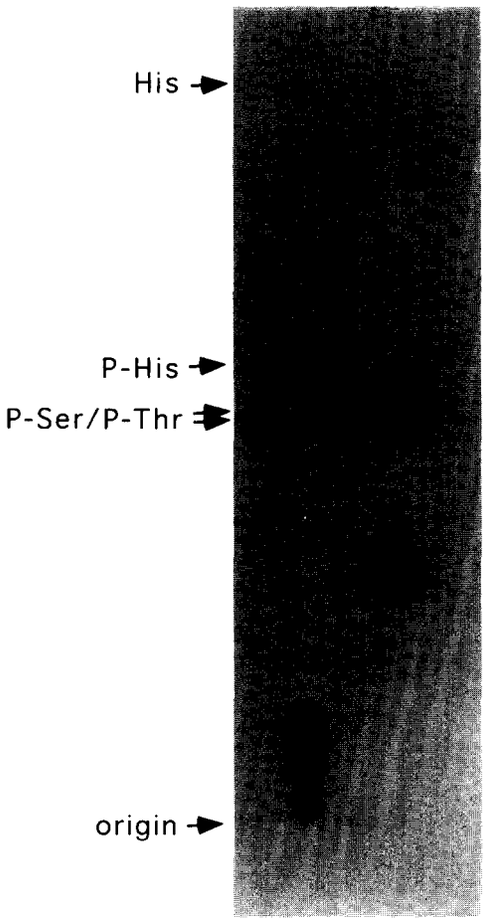

Fig. 3. Phospho-amino acid analysis of the phosphproteins p43 and p37–38. ^{32}P-Labeled p43 and a mixture of both p37 and p38 proteins, were transferred to a Hybond N membrane. p43 was hydrolyzed in 50 μl 6 N HCl, and p37 and p38 were hydrolyzed in 50 μl 3 N KOH at 105°C for 4 h. 5 μl of the hydrolysate was mixed with standard phospho-amino acids and analyzed directly on a silica TLC plate (Whatman) with two successive solvent (ethanol:25% ammonia solution, 3.5:1.6) cycles. The hydrolysate of p37/38 was applied to the left track while that of p43 was applied to the right track. A Fujix BAS 1000 Bioimager image of the plate is shown, as are the migration positions of the standard phospho-amino acids.

diphosphate kinase reaction and already within 20 s no labeled NDPK could be detected. In sharp contrast, p43 and p37–38 remained strongly labeled, arguing against these proteins being NDPK-like transient intermediates.

The de-phosphorylation of these proteins occurred in the presence of higher concentrations of Mg ions (data not shown). The nature of the phosphorylated proteins in the presence of Mg^{2+} was confirmed by examining their pH stability (Fig. 4). p43 was stable at pH 1 but not at pH 14, and p37–38 were stable at pH 14 but not at pH 1, verifying that p43 and p37–38 are phosphorylated at serine/threonine and histidine residues, respectively.

4. Discussion

In this study using EDTA and performing the phosphorylation reactions on ice, we developed a protocol for detection of phosphohistidine proteins in any animal tissue and possibly cell line. This protocol is simple and highly reproducible. The nature of the phosphoproteins was confirmed by pH stability and DEPC treatment as well as by direct phospho-amino acid analysis. Under these conditions we expected to identify only phosphohistidine proteins. However, an exceptional protein (p43) was phosphorylated at O-linked phospho residues (serine or threonine). There are two lines of evidence for p43 not being the substrate of regular serine/threonine protein kinases. First, it is well documented that serine/threonine kinases require divalent cations to phosphorylate any protein substrate. Second, we have clearly demonstrated here that the phosphorylation of p43 is abolished by DEPC treatment, under conditions in which Mg^{2+}-dependent phosphorylation was not affected. In bacteria, it has been reported that N-linked phosphate is transferred to an intra- or intermolecular aspartate residue [1], however, in rat, a histidine kinase phosphorylates its target on a serine residue [17]. Thus, given the fact that the serine/threonine phosphorylation of p43 is phosphohistidine dependent, it is very likely that p43 is a substrate of a yet unidentified histidine kinase.

Previously, it has been reported that NDPK can be detected as a phosphohistidine intermediate protein when EDTA is included in the reaction [12]. This behavior is the direct outcome of the mechanism of action of the enzyme. NDPK acts to transfer the phosphate residue from NTP to NDP by two steps, first transferring the phosphate residue to a histidine found in the active site and then from this histidine to NDP. EDTA blocks the second step. Consistent with this scenario, we found

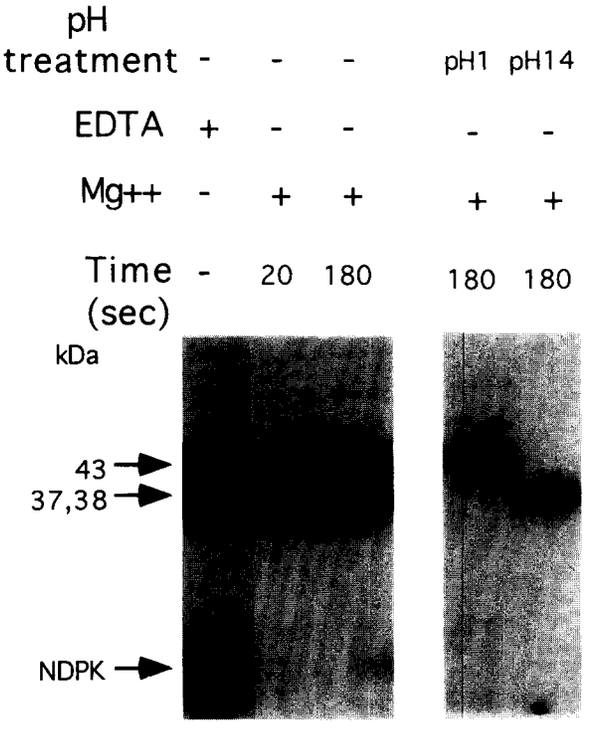

Fig. 4. Time-course analysis of the phosphohistidine proteins in brain extracts. Brain extracts were phosphorylated in the presence of EDTA as described in Fig. 1. The sample was dialyzed against the same buffer to remove free ATP. Next Mg^{2+} was added to the dialyzed mixture to a final concentration of 10 mM and, following incubation for 20 and 180 s, the samples were resolved by SDS-PAGE, blotted, autoradiographed and treated at different pHs.

that once the Mg ions are added, the phosphorylated NDPK loses the phosphate residue, within the minimum time that we measured (20 s). The other phosphoproteins identified by us remained phosphorylated, suggesting that they are not likely to be NDPK-like transient intermediates of catalytic reactions. We therefore infer that the experimental system described here can selectively detect histidine phosphoproteins that are the most likely candidates for histidine kinases.

The finding that the pattern of the phosphorylated proteins is very similar among the different tissues is informative. As the brain shows the strongest signals it is very unlikely that the labeled proteins originated from blood cells or vessels. Furthermore, a similar assay done with a T-cell line showed a different pattern (data not shown). Definitely purification and further characterization of these proteins should shed light on their origin and role in living organisms.

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