Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 899-904

Histidyl Phosphorylation of P36 in Rat Hepatoma Fao Cells *In Vitro* and *In Vivo*

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Received October 20, 1994

We have previously shown that a membrane-associated P36 from rat liver was *in vitro* phosphorylated at His residue(s) with a phosphoric amide bond (*FEBS Lett.*, 319:75-79, 1993), and the activity was solubilized and partially purified (*J. Biol. Chem.*, 269:9030-9037, 1994). The present study demonstrates that the P36 histidyl phosphorylation occurs in rat hepatoma cells under normal conditions. Phosphorylation and dephosphorylation of histidine as well as those of serine, threonine and tyrosine residues may also play an important role in animal cells.

We found that several proteins in rat liver extract were phosphorylated *in vitro* with an acid-labile linkage when we used partially purified fractions and refrained from treating the phosphorylated proteins with acid (1,2). A membrane-associated protein, P36 is most predominantly phosphorylated at histidine residue(s), and it was identified by a time course study of acid hydrolysis and by thin-layer chromatography of the ^{32}P-phosphorylated amino acids in P36 (1). A phosphoric amide His(P) has been found in the histone of regenerating rat liver (3) in addition to the intermediate in a catalysis of mitochondrial succinyl-CoA synthetase (4) and NDP kinase (5). However, the presence and the function of protein histidine kinases in animal cells has not been established. P36 histidyl phosphorylation activity

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0006-291X/94 $5.00

was induced in the rat liver by the administration of peroxisome proliferators, such as clofibrate, and activated *in vitro* by Ras protein and GTP (1). These *in vitro* results suggest the involvement of P36 in signal transduction, but there are no data regarding the *in vivo* reaction of P36. To examine whether P36 is histidyl phosphorylated *in vivo* as well as *in vitro*, we performed *in vivo* labeling using cultured rat hepatoma Fao cells (6).

EXPERIMENTAL PROCEDURES

Preparation of the crude membrane fraction: Rat hepatoma Fao cells cultured in Ham F12 medium (see below) were collected and homogenized in sucrose buffer A, pH 7.5 (0.25 M sucrose, 10 mM EDTA, 4 mM EGTA, 0.2 mM PMSF, 5 μM pepstatin A, 25 mM NaF, 15 mM $Na_4P_2O_7$, 50 μM sodium orthovanadate, 25 mM β-glycerophosphate) with a Potter-Elvehjem homogenizer. The post-nuclear supernatant obtained by centrifugation of the homogenate at 900 x g for 10 min was centrifuged at 20,000 x g for 30 min and the pellet was suspended in sucrose buffer B (0.25 M sucrose, 1 mM EDTA, 0.1% ethanol).

In vitro phosphorylation assay: The membrane fractions were assayed for *in vitro* phosphorylation in phosphorylation buffer (pH 7.9) containing 20 mM Tris-Cl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM DTT, 0.1 % Triton X-100, and phosphatase inhibitors (5 mM NaF, 3 mM $Na_4P_2O_7$,10 μM sodium orthovanadate, 5 mM β-glycerophosphate). The reactions were started by adding 1 μM (final concentration) [γ-^{32}P]ATP and were terminated by adding 2 volumes of SDS-PAGE sample buffer.

Solubilization of P36 phosphorylation activity and gel filtration: The membrane fraction prepared as above was sonicated in buffer A and the solubilized proteins were obtained after centrifugation at 20,000 x g for 30 min. For gel filtration, 0.11 volumes of 5 M NaCl was added to the extract, and the mixture was loaded onto a Sephacryl S-200 column (7 mm x 20 cm) equilibrated with column buffer (200 mM Tris-Cl, pH 7.6, 100 mM NaCl, 0.5 mM EDTA, phosphatase inhibitors as above). Fractions (0.3 ml) were collected in microtiter plates and stocked at -70 °C until the phosphorylation assay.

Cell culture and in vivo labeling: The Fao cell line that is a subclone of rat hepatoma HII4E, was maintained in Ham F12 medium containing 146 mg/l glutamine, 125 IU/ml specillin G, 125 μg/ml streptomycin, and 5 % fetal calf serum. Confluent cells cultured overnight in the medium containing half the concentration of phosphate were labeled with ^{32} P orthophosphate (200 μCi/5 cm^2 dish) in phosphate-free medium for 2 hours. After labeling, the cells were washed with MOPS buffer (pH 7.4) (6), collected, disrupted by sonication, then solubilized with SDS-PAGE sample buffer.

SDS-PAGE and densitometry: SDS-PAGE proceeded according to the standard method (7). The sample buffer contained 8 M urea and the samples were incubated at about 45 °C for 10 min before loading (1). The gels were fixed with 40% ethanol and 3.7% formaldehyde for 30 min, dried

and autoradiographed. In several experiments, the fixed gels were treated with acid (7% acetic acid at 45 °C to 50 °C for 30 to 60 min) or alkali (1 N NaOH at 50 °C for 1 hr) (2). For desitometric analysis, the X-ray film was scanned using an XRS 3cx/6cx scanner and the data were processed using the VISAGE Electrophoresis Gel Analysis System (Millipore).

RESULTS

In vitro phosphorylation of P36 in Fao membrane fractions

The crude membrane fraction was prepared from cultured rat hepatoma Fao cells and *in vitro* phosphorylation proceeded as described for rat liver subfractions (1,2) to examine whether P36 histidyl phosphorylation occurs in this cell extract. As shown in Fig.1, a 36 kDa protein was as efficiently phosphorylated as P36 in the rat liver extract. Acid treatment of the gel removed most of ^{32}P from the 36 kDa protein. Phosphorylation of the 36 kDa protein with an acid-labile linkage occurred in the membrane fraction but to a low level in the soluble fraction (not shown). The phosphorylation activity was solubilized by sonication in a buffer containing 10 mM EDTA and the solubilized activity was fractionated by Sephacryl S-200 gel filtration

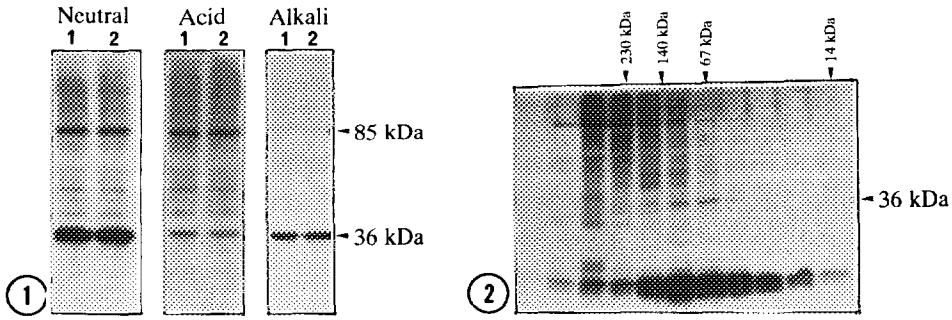

Fig. 1. The effect of alkali and acid treatment on *in vitro* phosphorylated endogenous proteins in the membrane fractions from Fao cells. The proteins were phosphorylated *in vitro* (in duplicate) and separated by SDS-PAGE (8 %) in triplicate. The gels were fixed and treated with 7 % acetic acid at 45 °C for 30 min (acid), 1 N NaOH at 50 °C for 1 hr (alkali), or kept in SDS-PAGE running buffer (neutral, control). The remaining radioactivity levels were detected by autoradiography.

Fig. 2. Gel filtration and *in vitro* phosphorylation of the solubilized proteins from the Fao membranes. The crude membrane extract with an EDTA buffer was applied to a Sephacryl S-200 column and the separated fractions were assayed for *in vitro* phosphorylation. After SDS-PAGE (11 %), the phosphorylated proteins were detected by autoradiography. The elution positions of molecular weight marker proteins (top) and the position of the migrated 36 kDa protein (right) are indicated.

into the region of an apparent molecular weight of 60-100 kDa (Fig.2) as is rat liver P36 and its kinase (2). The *in vitro* phosphorylated 36 kDa protein in the Fao membrane fraction was indistinguishable from the histidyl phosphorylated P36 that is a membrane associated protein in the rat liver (2).

In vivo labeling

As the presence of P36 in Fao cells was confirmed, we performed *in vivo* labeling with ^{32}P-orthophosphate. The total proteins were prepared from the ^{32}P-labeled Fao cells and analyzed by SDS-PAGE, followed by autoradiography (Fig.3). One of two sets of sample lanes on the same gel was treated with acid (2) and the autoradiograms were compared. The ^{32}P was removed from the lower band of the 36 kDa doublet by acid treatment and densitometric scanning of the autoradiograms confirmed this (Fig .3B). To confirm the lower band of the 36kDa doublet is P36, *in vitro* and *in vivo* phosphorylated proteins were analyzed on the same gel in parallel (Fig. 4).Though the profiles of the ^{32}P-labeled proteins were dissimilar because of wide differences between the *in vitro* and *in vivo* protein phosphorylation conditions, the migration of the *in vitro* phosphorylated P36 exactly corresponded to that of the *in vivo* labeled lower band, indicating that the 36 kDa protein phosphorylated in *vivo* with an acid labile linkage is P36. Thus P36 is histidyl phosphorylated in the cell.

DISCUSSION

We detected the *in vitro* phosphorylation of P36 under non-physiological conditions, namely on ice with a substrate ATP concentration of 1 μM (1,2). Reactions at higher temperatures activated other kinases and phosphatases more efficiently, making it difficult to detect P36 phosphorylation on one-dimensional gels. Thus, there has been a need to examine the *in vivo* reaction of P36. In this study, we confirmed that P36 is histidyl phosphorylated in rat hepatoma cells under normal conditions.

In animal cells, the modification of Ser, Thr, and Tyr residues of the proteins with phosphoric ester bonds and its importance in the modulation of biological activities has been emphasized. In bacteria (see Ref. 8 for review) and in plants (9) as well as in yeast (10,11), genetic approaches have shown that modification of His residues with a phosphoric amide bond is involved in signal transduction. These histidine kinases are thought to be involved in two-component systems, in which an environmental stimulus is recognized by the first component, the histidine kinase autophosphorylates at a His residue, followed by the transfer of the phosphoryl group to an Asp residue of the second component (8). The biochemical approaches have identified (12) and characterized (13) possibly another yeast histidine kinase

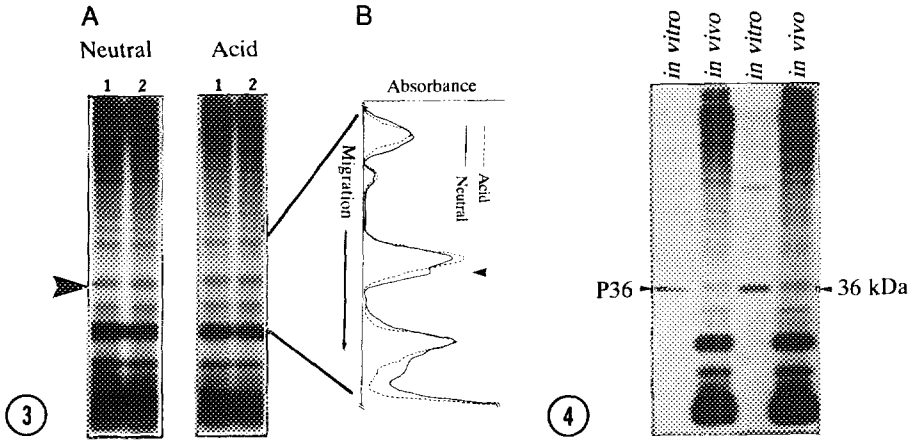

Fig. 3. The effect of acid treatment on *in vivo* phosphorylated proteins in Fao cells. Fao cells in two culture wells were independently labeled with ^{32}P-orthophosphate, and the total proteins were analyzed by SDS-PAGE (11 %) for phosphorylation in duplicate. One gel was treated with acid (7 % acetic acid at 50 °C for 1 hr). Autoradiograms (A) and the scanning patterns (B) were shown. Arrowheads indicate the positions of the migrated 36 kDa protein.

Fig. 4. A comparison of *in vitro* and *in vivo* phosphorylated proteins. The *in vitro* and *in vivo* phosphorylated proteins obtained as in Fig. 1 and in Fig. 3, respectively, were separated on the same gel (10 %) in adjacent lanes. The positions of the phosphorylated P36 and the 36 kDa protein are indicated.

that may not be involved in this system. Branched-chain α-ketoacid dehydrogenase in rat has a similar domain to that in bacterial histidine kinase, but the target protein of the dehydrogenase is phosphorylated at a Ser residue (14). Thus, further investigation is necessary to determine whether the P36 histidyl phosphorylation in animal cells is involved in a histidine kinase-containing two-component system or whethr it is a different type histidine kinase.

REFERENCES

1. Motojima, K. and Goto, S. (1993) FEBS Lett. **319**, 75-79.
2. Motojima, K. and Goto, S. (1994) J. Biol. Chem. **269**, 9030-9037.
3. Chen, C. -C., Smith, D. L., Bruegger, B. B., Halpern, R. M., and Smith, R. A. (1974) Biochemistry **21**, 4867-4873.
4. Kreil, G. and Boyer, P. D. (1964) Biochem. Biophys. Res. Commun. **16**, 551-555.
5. 5.Gilles, A-M., Presecan, E. Vonica and Lascu, I. (1991) *J. Biol. Chem.* **266**, 8784-8789.

6. Brocard, C., Es-Souni, M., Ramirez, L. C., Latruffe, N. and Bournot, P. (1993) Biol. Cell **77**, 37-41.
7. Laemmli, U. K. (1970) Nature **227**, 680-685.
8. Parkinson, J. S. and Kofoid, E. C. (1992) Ann. Rev. Genet. **26**, 71-112.
9. Chang, C., Kwok, S. F., Bleecker, A. B., and Meyerowitz, E. M. (1993) Science **262**, 539-544.
10. Ota, I M. and Varshavsky, A. (1993) Science **262**, 566-569.
11. Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994) Nature **369**, 242-245.
12. Huang, J., Wei, Y., Kim, Y., Osterberg, L. and Matthews, H. R. (1991) J. Biol. Che. **266**, 9023-9031.
13. Huang, J., Nasr, M., Kim, Y. and Matthews, H. R. (1992) J. Biol. Chem. **267**, 15511-15515.
14. Popov, K. M., Zhao, Y., Shimomura, Y., Kuntz, M. J. and Harris, R. A. (1992) J. Biol. Chem. **267**, 13127-13130.