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A protein histidine kinase induced in rat liver by peroxisome proliferators

In vitro activation by Ras protein and guanine nucleotides

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A novel protein kinase is induced in rat liver plasma membrane by the administration of peroxisome proliferators. A 36 kDa protein (P36) on the membrane was rapidly phosphorylated in vitro by the kinase and the phosphorylated amino acid was identified as phosphohistidine. Histidine phosphorylation of P36 was activated in vitro by recombinant Ras protein and GTP; both decreased Michaelis constant (K_m) for ATP from 1.25 to 0.25 μM. The novel histidine kinase, products of which have been overlooked due to their acid lability, may participate in cellular signaling and peroxisome proliferators may perturb the pathway.

Histidine kinase; Peroxisome proliferator; Ras protein; Signal transduction; Rat liver

1. INTRODUCTION

Peroxisomes are found in almost every eukaryotic cell and have diverse metabolic functions [1,2]. One of the most characteristic features of peroxisome biology is that the organelle is remarkably proliferated in response to structurally diverse xenobiotic agents, peroxisome proliferators [3]. Recent studies on a peroxisome proliferator receptor proposed a steroid hormone-like receptor-mediated mechanism for the transcriptional activation of several genes [4,5]. Remarkable information has also been learned about the targeting signal [6], peroxisomal assembly factors [7,8], and the cause of peroxisome-deficient Zellweger syndrome [9], whereas the mechanism for hepatomegaly and hepatocarcinogenesis caused by several of the non-genotoxic proliferators in rodent species has not been elucidated. Reddy and coworkers hypothesized that the main cause for carcinogenesis may be a leakage of excess oxidase-producing H_2O_2 from peroxisomes [10]. However, a definitive association between H_2O_2 leakage caused by peroxisome proliferation and hepatocarcinogenesis has not been demonstrated. We have been interested in the possibility that these agents may affect not only peroxisome-related cellular events but also other basic and/or liver-specific cellular functions unrelated to peroxisomes. Several such changes were found, including induction of elongation factor 2 of protein synthesis (K. Motojima et al., submitted) and down-regulation of BiP/GRP78 [11] and transthyretin [12].

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Abbreviations: P36, a 36 kDa protein from rat liver; PMS, post-mitochondrial supernatant; PM, plasma membrane; PIPES, piperazine-*N*, *N'*-bis(2-ethanesulfonic acid); SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TLC, thin layer chromatography; EGF, epidermal growth factor.

2. MATERIALS AND METHODS

2.1. *Animals and subcellular fractionation*

Male Fischer F-344 rats were fed a control diet (CE7, Clea Japan) or a diet containing 0.5% clofibrate. After 2 weeks, rats were killed and the livers were minced well with scissors, homogenized in 5 vols. of sucrose buffer (0.25 M sucrose, 1 mM EDTA, 10% ethanol, pH 7.4) by a Potter-Elvehjem homogenizer, and the homogenates were centrifuged for 5 min at 600 × *g*. The supernatant was centrifuged for 13 min at 2,000 × *g* to obtain post-mitochondrial supernatant (PMS) and the pellets. From the pellets, plasma membranes (PM) were purified according to the method of Hubbard et al. [13].

2.2. *In vitro phosphorylation*

Phosphorylation in subcellular fractions from livers of rats were assayed in kination buffer [14] (50 mM PIPES, pH 7.0, 0.1% Triton X-100, 1 mM $MnCl_2$, 2 mM $MgCl_2$, 10 μM sodium orthovanadate, 2 mM sodium fluoride, and 20 mM sodium molybdate] for phosphorylation in the presence of EGF (1 μg/ml). 10 μM Okadaic acid was also included in some experiments. The reactions were started by the addition of 1 μM (final concentration) [τ-^{32}P]ATP, and were terminated by adding two vols. of SDS-PAGE sample buffer (2% SDS, 8 M urea, 5% 2-mercaptoethanol, 125 mM Tris-HCl, pH 6.8). The phosphorylated proteins were analyzed by SDS-PAGE. The gels were fixed with 40% methanol and 3.7% formaldehyde, treated with alkali (1 N NaOH at 50°C for 1 h), dried and autoradiographed.

2.3. *Identification of phosphorylated amino acid residues*

For identification of phosphorylated amino acids in P36, ^{32}P-phosphorylated proteins in the PM fraction were separated by SDS-PAGE and transferred to an Immobilon filter (Millipore). One filter

was cut into strips and incubated at 50°C in 1 N NaOH or in 7% acetic acid (pH 2.2) for various periods to study the time-course of hydrolysis of phosphorylated residues in proteins. For phosphoamino acid analysis by thin-layer chromatography (TLC), ^{32}P-labeled P36 on an Immobilon filter was hydrolyzed in 2 N KOH at 105°C for 5 h. A portion of the hydrolysate was mixed with standard phosphoamino acids (phosphohistidine and phospholysine were synthesized as described [15] and others were obtained from Sigma) directly on a silica gel TLC plate (Merck) with two successive solvent (ethanol : 25% ammonia solution, 3.5 : 1.6) cycles [16].

2.4. *Quantitation of radioactivity*

The amounts of ^{32}P radioactivities were quantitated using a BAS2000 image analyzer (Fuji Film, Japan) for data acquisition and Bioimage (Millipore) data analyses. Autoradiograms on X-ray films were taken only for representative pictures.

2.5. *Recombinant Ras protein and anti-Ras antibody*

The recombinant EC *ras* (normal human c-Ha-*ras*-1) expressed in *Escherichia coli* and purified to homogeneity was the generous gift from J.B. Gibbs of Merck Sharp & Dohme Research Laboratories. TuMark monoclonal antibodies against *ras* p21 (pan) was obtained from NEN.

3. RESULTS

3.1. *Induction of P36 phosphorylation by clofibrate*

To assess the effects of peroxisome proliferators on cellular signaling, we studied the in vitro phosphorylation and dephosphorylation of endogenous proteins in subcellular fractions of liver. PMS fractions were used in the assay because they gave the most reproducible results under our conditions. A high level of autophosphorylation of the epidermal growth factor (EGF) receptor was also observed in these fractions and used as an internal control of in vitro phosphorylation. Apparently no proteins other than the receptor were phosphorylated in an EGF-dependent manner under the conditions used.

Feeding rats with a peroxisome proliferator, clofibrate, for 2 weeks induced hepatomegaly (nearly 2-fold weight) and proliferation of peroxisomes (more than 20-fold as assessed by the amounts of proliferation-associated enoyl-coenzyme A hydratase-dehydrogenase in PMS fractions). The pattern of phosphorylated proteins in PMS fractions changed when clofibrate was fed to old rats (27 month old) (Fig. 1). Phosphorylation of a protein having a molecular weight of 36 kDa (P36) was remarkably increased but the bands were detected only on those gels without acid treatment, such as occurs with destaining with acetic acid (see below). In addition to P36, phosphorylation of a protein of 100 kDa (P100) was also increased in young rats (5 month old) fed clofibrate (not shown; K. Motojima et al., submitted). In vitro phosphorylation of P36 was also enhanced in PMS fractions from rats administered other types of proliferators like bezafibrate and di(2-ethylhexyl)phthalate (not shown).

3.2. *Characterization of P36 phosphorylation*

To characterize P36 phosphorylation, we first examined effects of protein kinase inhibitors. The inhibitors and concentrations used were H-7 at 20 μM, H-8 at 5 μM, and ML-7 at 5 μM to inhibit protein kinase C, cyclic nucleotide-dependent protein kinase, and myosin light chain kinase, respectively [17,18]. None inhibited the reaction, suggesting that P36 is not phosphorylated by these kinases.

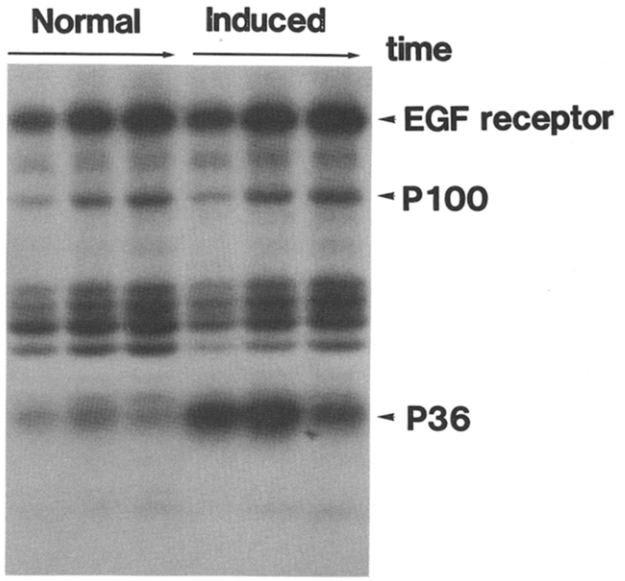

Fig. 1. In vitro phosphorylation of endogenous proteins in rat liver PMS fractions. Phosphorylation in PMS fractions from livers of control (Normal) and clofibrate (0.5%)-fed (Induced) (for 2 weeks) rats were assayed for phosphorylation in the presence of EGF (1 μg/ml). The phosphorylated proteins were analyzed by SDS-PAGE (10%). The gels were fixed with 40% methanol and 3.7% formaldehyde, treated with alkali (1 N NaOH at 50°C for 1 h), dried and autoradiographed.

Next, we analyzed the ^{32}P linkage to P36. It was as resistant in alkali as that in the EGF receptor in which tyrosine residues were phosphorylated. Acid hydrolysis of [^{32}P]P36 in 6 N HCl for 3 h at 100°C to identify the phosphorylated residues removed all ^{32}P from P36 and no P-Thr, P-Ser or P-Tyr was detected. A time-course study of the hydrolysis showed that the linkage of [^{32}P]P36 is resistant to alkali but labile even to mild acid (Fig. 2A). The first order rate constant for [^{32}P]P36 hydrolysis at 50°C in 7% acetic acid (about pH 2.2) was 0.031 min^{-1}. These results strongly suggest the phosphorylated residue in P36 is phosphohistidine. Similar values have been reported for the hydrolysis of 3-phosphohistidine in phospho-Enzyme I of the PTS (0.025 min^{-1}) [19] and in phospho-CheA (0.021 min^{-1}) [20] under similar conditions. To confirm that the phosphoamino acid in P36 is phosphohistidine, [^{32}P]P36 was alkali hydrolyzed and the products were analyzed by TLC (Fig. 2B). Most of the ^{32}P stayed at the origin, probably as inorganic phosphate liberated under the conditions employed, but a significant amount of phosphoamino acid co-migrated with the standard

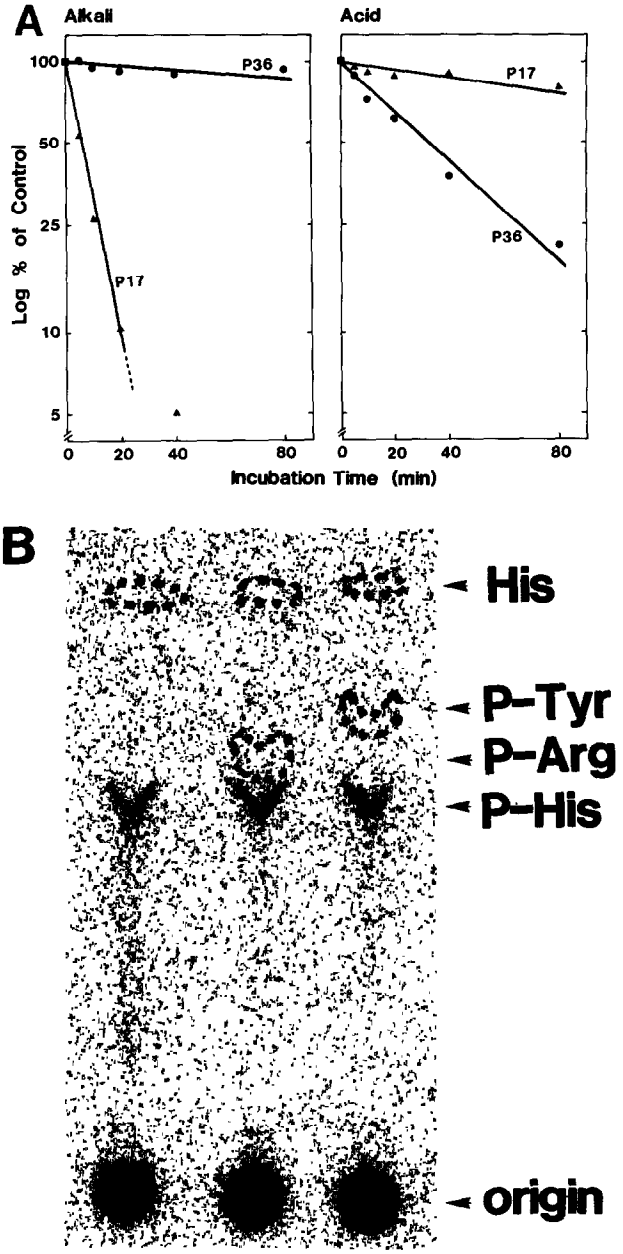

Fig. 2. Identification of the phosphorylated amino acid residues in P36. (A) Time-course of hydrolysis of phosphorylated amino acids in proteins, P36 and P17 (control) on Immobilon filter strips under alkaline or acidic conditions. The control amounts on the untreated strips were taken as 100 and relative amounts are shown. (B) Phosphoamino acid analysis by TLC. ^{32}P-labeled P36 was transferred to an Immobilon filter and hydrolyzed in 2 N KOH at 105°C for 5 h. A portion of the hydrolysate was mixed with standard phosphoamino acids and analyzed directly on a silica gel TLC plate (Merck) with two successive solvent (ethanol:25% ammonia solution, 3.5:1.6) cycles. A BAS2000 image of the plate is shown, as are the migrations of the standard phosphoamino acids.

phosphohistidine. Phospholysine migrated faster than phosphoarginine on the plate under our conditions (not shown). Thus the novel kinase induced by peroxisome proliferators in rat liver phosphorylates P36 at histidine residue(s).

3.3. *Activation of P36 phosphorylation by Ras protein and guanine nucleotides*

Hedge and Das [21,22] reported a similar protein in rat liver plasma membrane having a molecular weight of 38 kDa (P38), although they suggested that phosphorylation in P38 is probably not on histidine, as they had thought earlier [21], but on an acyl moiety [22]. More interestingly, they showed enhancement of P38 phosphorylation by p21 ras and GDP in vitro. To learn whether our P36 and their P38 were identical and, if so, to confirm the activation, we carried out kinetic studies of P36 phosphorylation with Ras protein, GTP, or GDP using PM fractions (see below) at 0°C. The K_m of the kinase for ATP was estimated as 1.25 μM (Fig. 3); this is significantly lower than that of recently purified yeast histidine kinase (60 μM) [23] and may explain the efficient incorporation of ^{32}P into P36 under the conditions used in which the concentration of ATP was 1 μM. Upon addition of the saturable level of Ras protein, there was a large enhancement of P36 phosphorylation and the affinity of the kinase for ATP increased to a K_m value of 0.25 μM. Essentially the same enhancement was obtained by addition of 1 μM GTP. In both cases there was also inhibition of the phosphorylation at high concentrations of ATP. In contrast, 1 μM GDP activated the phosphorylation without changing the affinity for the substrate. In these assays, we preincubated PM fractions with these activators for 60 min on ice. During preincubation, the GDP-bound Ras might have converted to the GTP-bound active Ras, probably by a GDP/GTP exchange reaction [24]. Added GTP could have promoted the exchange in endogenous Ras protein. This would explain how the addition of Ras protein and GTP to PM fractions could have exerted the same type of activation, whereas excess GDP might have prevented the exchange and the GDP-bound endogenous Ras activated P36 phosphorylation without changing the affinity for ATP. Phosphorylation reactions using PM fractions, however, can be affected by various modulating protein factors and there are many other possibile explanations of the present data. Resolution of these questions will require purification of the kinase and its substrate, P36, and the use of specific inhibitors. Although the mechanism of the activation is not yet known, an intimate, if not direct, interaction between Ras and the kinase was suggested by the kinetic analysis, and a possible role of Ras protein in P36 phosphorylation may be the switching of the affinity of the kinase for ATP.

3.4. *Dephosphorylation of [^{32}P]P36*

With PMS fractions, P36 was very rapidly phosphorylated with maximum values at 15 s at 0°C and at 30 s at 20°C, followed by immediate dephosphorylation especially at 20°C even though the reaction mixture contained phosphatase inhibitors (10 μM sodium vanadate, 2 mM sodium fluoride, and 20

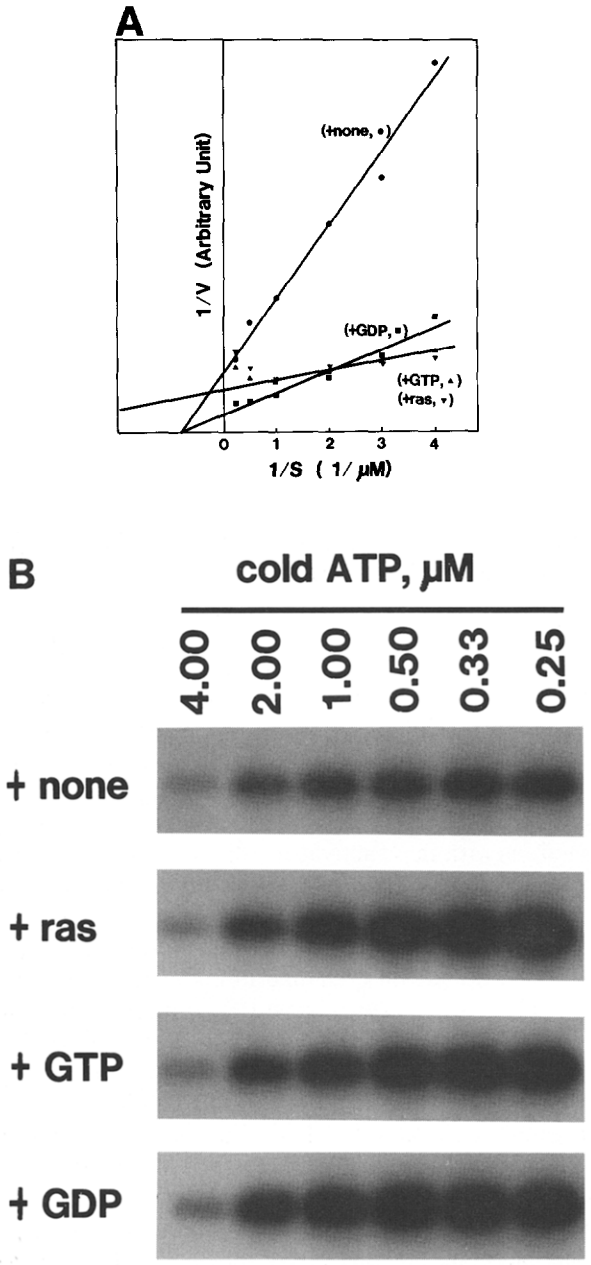

Fig. 3. Lineweaver–Burk plot of P36 phosphorylation kinetics and effects of Ras protein and guanine nucleotides. PM fractions (1 mg protein/ml) were preincubated in kination buffer with Ras protein (50 μg/ml), GTP (1 μM), GDP (1 μM) or none for 1 h on ice. P36 phosphorylation in PM fractions with various concentrations of ATP (adjusted with cold ATP) was carried out at 0°C for 60 s. The phosphorylated proteins were separated by SDS-PAGE and amounts of phosphorylated P36 were quantitated using BAS2000 and Bioimage (A). Representative autoradiograms on X-ray films are shown in (B).

mM sodium molybdate). Rapid dephosphorylation was also not inhibited by 10 μM okadaic acid. With PM fractions, however, only a relatively slow phosphorylation was observed at either temperature, suggesting that P36 and its kinase, which is not necessarily different from P36, are located in the plasma membrane, and that some modulating factor(s), such as a phosphatase or a transferase, is present in the cytoplasm.

4. DISCUSSION

Our studies seem to point to the histidine phosphorylation of P36 possibly being involved in a cellular signaling pathway in a novel manner, probably downstream of Ras. The reasons that the histidine phosphorylation has been overlooked could be its transient linkage, as suggested by our in vitro study, and acid lability of the linkage between histidine and phosphate. In bacteria, a signal transduction mechanism including histidine phosphorylation and transfer of the phosphoryl group to an aspartic acid side chain, followed by dephosphorylation by associated phosphatase activities has been demonstrated in several systems (for review see [25]). Interestingly, Huang et al. reported purification of a protein histidine kinase from yeast [23]; the presence of protein phosphohistidine in regenerating rat liver has also been reported [26]. Thus a similar mechanism including histidine phosphorylation may be operating in eukaryotic cells, and P36 and its kinase may be involved in the pathway.

Peroxisome proliferators changed the level of P36 phosphorylation activity in rat livers. Immunoblot analysis using anti-H-*ras* monoclonal antibody (against *ras* p21(pan); NEN) revealed that the level of Ras protein in PMS fractions did not change significantly during induction of peroxisome proliferation (not shown); so the level of the histidine kinase, its substrate P36, or another modulating factor(s) is thought to change in response to a proliferator. The function of P36 and its kinase is not known at present but such change could be the cause of perturbation of cellular signaling. Further in vitro and in vivo characterization of the kinase is obviously necessary and is in progress in our laboratory, both to elucidate its role in signal transduction and pursuant to our interest in the possibility that proliferator perturbation of the pathway in the liver may be a major cause of the hepatomegaly and hepatocarcinogenesis these proliferators induce.

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