Histidyl Phosphorylation and Dephosphorylation of P36 in Rat Liver Extract*

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Protein histidine kinase (Motojima, K., and Goto, S. (1993) FEBS Lett. 319, 75-79) and phosphatase in rat liver extract were characterized. The histidine kinase was recovered mostly in the membrane and the phosphatase in the soluble fraction. The kinase and its substrate 36-kDa protein (P36) were co-solubilized from the membrane under conditions in which most of the other kinases, and their substrate proteins were not solubilized. The solubilized kinase and P36 were co-eluted after high pressure liquid chromatography gel filtration, showing an apparent molecular mass of 70-75 kDa. They were also co-eluted after ion exchange chromatography. These characteristics, together with its complete resistance to genistein, indicate that the rat liver histidine kinase is not cognate to the yeast enzyme (Huang, J., Nasr, M., Kim, Y., and Matthews, H. R. (1992) J. Biol. Chem. 267, 15511-15515). The phosphatase that dephosphorylates histidyl-phosphorylated P36 was also studied using rat liver subcellular fractions and in vitro phosphorylated P36 as the substrate. The characteristics of the phosphatase, that is, 1) Mg requirement for activity, 2) apparent molecular mass of 45 kDa by high performance liquid chromatography gel filtration, and 3) resistance to 100 μM okadaic acid, suggest that the primary phosphatase active in vitro is protein phosphatase 2C.

Biological activities of various proteins may be modulated by phosphorylation and dephosphorylation (see Refs. 1-3 for reviews), and the effects may be diversified to various processes and dedicated to a specific response. Complex regulatory cascades of phosphorylation-dephosphorylation systems, cross-talks among them, and their major roles in signal transduction pathways have been extensively studied and elucidated in both prokaryotes (see Refs. 6-11 for reviews) and eukaryotes (see Refs. 12-15 for reviews). The phosphorylated amino acids studied most to date are Ser, Thr, and Tyr, all of which form acid-stable linkages with phosphate and are readily identifiable after acid hydrolysis of the phosphorylated proteins. Assay procedures for kinases and phosphatases also usually include acid treatment of the phosphorylated proteins such as trichloroacetic acid precipitation. In addition to acid-stable phosphoester linkage in Ser(P), Thr(P), and Tyr(P), however, acid-labile phosphoamidate linkage has been reported in His(P) and Lys(P) (12, 13). The number of these reports is limited, probably because the linkage has been overlooked after acid hydrolysis of the proteins (14).

In bacteria, a signal transduction mechanism including histidine phosphorylation and transfer of the phosphoryl group to an Asp side chain, followed by dephosphorylation and associated phosphatase activities has been demonstrated in several systems (15). A similar signal transduction mechanism including histidine phosphorylation has not been demonstrated in eukaryotes, although the presence of protein phosphohistidine in a few proteins including A-kinase proteins in regenerating rat liver has been shown (12). A protein histidine kinase has also been purified from yeast (16), but the role(s) of histidyl phosphorylation in eukaryotes has not been elucidated.

We recently found that several proteins in rat liver extract were phosphorylated in vitro with an acid-labile linkage when we refrained from treating the phosphorylated proteins with acid (17). One such protein having a molecular mass of 36 kDa, P36,1 was shown to be phosphorylated at histidine residue(s) identified by the time course study of acid hydrolysis of 32P-phosphorylated amino acids in P36 and by thin-layer chromatography of the amino acids obtained after alkali hydrolysis of 32P-labeled P36 (17). The P36 phosphorylation activity was induced in rat liver by the administration of peroxisome proliferators, such as clofibrate, and was activated in vitro by Ras protein and GTP, suggesting its involvement in a signal transduction pathway (17).

This report describes characterization of P36 kinase and phosphatase with emphasis on the effect of genistein on the histidine kinase, because yeast protein histidine kinase has recently been reported to be inhibited by this drug (18). Genistein, an isoflavonoid compound, was first isolated as an inhibitor of tyrosine kinases (19). It has been shown that the drug specifically inhibited protein tyrosine kinase activity of growth factor receptors and oncogenes but scarcely affected serine and threonine kinase activities (20). Because of their proposed specificity, genistein and other isoflavonoids have been used to elucidate the role of tyrosine phosphorylation in signal transduction pathways (e.g., see Refs. 21-23). If genistein inhibits both mammalian protein tyrosine and histidine kinases at low concentrations, some of the reported observations obtained using genistein must be due to the inhibition of not (only) tyrosine but histidine kinase (19). Thus it is important to determine whether mammalian protein histidine kinase is also inhibited by genistein to interpret previous data and establish conditions for its use.

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1 The abbreviations used are: P36, a 36-kDa protein; PP36, phosphorylated P36; P130, a 130-kDa protein; Pp, protein phosphatase; EGF, epidermal growth factor; FNS, post-nuclear fraction; FMS, post-mitochondrial supernatant; PM, plasma membrane; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid).
EXPERIMENTAL PROCEDURES

Materials

Clofibrate (2-p-chlorophenoxyisobutyric acid ethyl ester) was purchased from Tokyo-Kasei (Tokyo, Japan), EGF (receptor grade) and the molecular mass marker proteins were obtained from Sigma. Okadaic acid was from Wako Pure Chemical Co. (Osaka, Japan). DE52 cellulose and Sephadex G-50 (fine) were purchased from Whatman and Pharmacia LKB Biotechnology Inc., respectively. The TSK G3000SWXL gel filtration columns were from Tosoh Co. (Osaka, Japan). Immobilon membranes and x-ray films were from Millipore (Bedford, MA) and Eastman, respectively. [γ-32P]ATP (4000 Ci/mmol) was obtained from ICN (Irvine, CA). All other reagents were of analytical grade.

Methods

Preparation of the Subcellular Fractions—Male Fischer F-344 rats were fed a diet (CET, Clea Japan) containing 0.5% clofibrate (24). After 2 weeks, rats were killed, and the livers were minced well with scissors, homogenized in 5 volumes of sucrose buffer (0.25 M sucrose, 1 mM EDTA, 0.1% ethanol, pH 7.4) by Potter-Evvelhjem homogenizer (25), and the homogenates were centrifuged for 5 min at 600 × g. The supernatant, post-nuclear fraction (PNS) was centrifuged for 13 min at 20,000 × g to obtain the post-nuclear supernatant (PNS) and pellets (24). From the pellets, plasma membranes (PM) were purified according to the method of Habbard et al. (26).

In Vitro Phosphorylation Assay—Two assay conditions were used. The PMS and PM fractions were assayed for in vitro phosphorylation activity by adding 200,000 cpm [γ-32P]ATP (4000 Ci/mmol) to 2 μg of protein of the solubilized fraction (see above) with 0.5% methanol and 3.7% formaldehyde for 20 min on ice. For the solubilized P36 kinase, 20-μl samples containing 5 μg of protein were added for phosphorylation in phosphorylation buffer B containing 50 mM Tris-HCl, pH 7.5, and 10 mM MgCl₂ in a total volume of 30 μl. The reactions were started by the addition of 1 μl (final concentration) [γ-32P]ATP and were terminated by adding 2 volumes of the sodium dodecyl sulfate-acrylamide gel electrophoresis (SDS-PAGE) sample buffer (below).

Partial Purification of the Histidine Kinase—The crude membrane fraction was obtained from PMS by centrifugation at 100,000 × g for 60 min. The pellet was homogenized in buffer C (20 mM Tris-Cl, pH 7.5, 10 mM EDTA) with a Potter-Evvelhjem homogenizer. The homogenate (about 20 μg of protein/ml) was centrifuged at 100,000 × g for 60 min, and the solubilized proteins were recovered. For HPLC gel filtration, 0.11 volume of 5 mM NaCl was added to the extract, and the mixture (about 2 mg of protein in 200 μl) was injected into TSK G3000SWXL columns (7.8 × 30 cm) equilibrated with buffer D (20 mM Tris-Cl, pH 7.9, 1 mM EDTA, 150 mM NaCl) as above. 20 μl of each fraction was mixed with 10 μl of NaOAc at pH 5.0 for 20 min and incubated at room temperature for the PP36 phosphatase activity. The remaining 32P of P36 was determined after SDS-PAGE.

RESULTS

Rapid Phosphorylation and Dephosphorylation of P36—Liver subcellular fractions obtained from clofibrate-fed rats were incubated with [γ-32P]ATP to study the time course of phosphorylation and dephosphorylation of endogenous proteins. PMS and PM fractions were used, and incubation temperatures of 0 and 20°C were compared (Fig. 1). In these fractions histidyl phosphorylation of P30 and tyrosyl phosphorylation of the EGF receptor were reproducibly detected when compared with their phosphorylation in the total homogenate (see above) and the PNS fraction. With the PMS fraction, P30 was very rapidly phosphorylated with maximum values at 30 s after the addition of ATP at 0°C and at 15 s at 20°C, followed by immediate dephosphorylation, especially at 20°C. Tyrosyl phosphorylation of the EGF receptor (identified by immunoprecipitation using monoclonal antibodies and by phosphoamino acid analysis; not shown) and phosphorylation of P100 were somewhat slow, followed by slight dephosphorylation under the same conditions. With the PM fraction, in contrast, P36 was phosphorylated at a similar rate at 0°C and at 20°C, but dephosphorylated at 0°C much more rapidly at 20°C. With the PM fraction, P30 quickly dephosphorylated at both 0°C and 20°C.
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Fig. 1. Time course of in vitro phosphorylation of the endogenous proteins in PMS and PM fractions. Phosphorylation in PMS or PM fractions from the livers of clofibrate (0.5%)-fed (for 2 weeks) rats was assayed in the presence of EGF (1 μg/ml) on ice or at 20°C. Aliquots were removed at 7.5, 15, 30, 60, 120, and 240 s after the addition of [γ-32P]ATP (1 μM, final concentration), and the reactions were terminated by adding 2 volumes of the SDS-PAGE sample buffer containing 8 M urea. The proteins were separated by SDS-PAGE (8%). The gels were fixed with 49% methanol and 3.7% formaldehyde, treated with alkali (1 N NaOH at 50°C for 1 h), and autoradiographed. A, autoradiogram showing the time course of in vitro phosphorylation in PMS and PM fractions on ice (0°C) or at 20°C. On the right, the positions of the major alkali-resistant phosphorylated proteins, the EGF receptor (EGF.R), a 190-kDa protein (P100) and a 36-kDa protein (P36), are indicated. B, quantitative changes in the amount of 32P bound to P36 during the incubation period. The amount of radioactivity in P36 at 0°C (△) or at 20°C (○) in each lane was quantitated using BAS2000 (Fuji) before taking an autoradiogram (A). The relative values were plotted as percentage of the maximum value in each reaction.

was efficiently phosphorylated without apparent dephosphorylation. These results suggest that P36 and its kinase are associated with the membrane and that its phosphatase or transferase is present in the soluble fraction.

Solubilization of P36 and Its Kinase—To study the relationship among P36, its kinase, and other membrane-associated kinases, the PM fraction was treated with several reagents, and the remaining phosphorylation activities either in the supernatants or pellets were assayed (Fig. 2). The autoradiogram was taken after drying the gel with heating during which [32P]phosphate was partially released from P36 and showed blotches around the P36 bands. Only the gel in Fig. 2 was heated; other gels were processed without heating. Among various treatments including homogenization with detergents, EDTA most efficiently released practically all of the P36 phosphorylation activity from the membrane, leaving most of the other kinases and their substrates intact (compare lanes 3 and 4 with lanes 6 and 7). Some phosphorylated proteins detected with intact membranes were recovered in neither fraction nor mixed preparation (see thick phosphorylated bands at the top and bottom of the gel in lanes 1 and 2, for example).

Selective solubilization with EDTA of the P36 phosphorylation activity from the membrane indicates that P36 phosphorylation did not require a membrane structure and that P36 kinase is different from most other major membrane-bound kinases. P36 and its kinase may be hydrophilic proteins with peripheral association with the membranes. Their loose association was also suggested by the result that a minor part of the P36 phosphorylation activity could be recovered in the soluble fraction after high speed centrifugation of PNS and PMS fractions, although a quantitative estimation of the recovery is impossible at present as the phosphatase exists in the same fraction (see Fig. 1 and below).

Partial Purification of P36 Histidine Kinase—To examine whether P36 and its kinase are different molecules and separable, the active solubilized fraction was subjected to gel filtration by HPLC, and each fraction was assayed for phosphorylation with or without the solubilized P36 (Fig. 3). The applied sample was obtained by high speed centrifugation of the EDTA-treated crude membrane fraction instead of the PM, and additional phosphorylation activities of P36 (see high molecular region) were also detected after gel filtration (compare Figs. 2 and 3B). Part of these results may be due to the separation of some kinases and their product-preferential phosphatases. P36 phosphorylation activity was recovered in the fractions corresponding to those containing the 70–75-kDa molecules (Fig. 3, A and B). To examine the possibility that P36 and its kinase were dissociated and the major portion of both was fractionated into overlapping but separate fractions, the solubilized P36 and its kinase from PM were added to each fraction, and phosphorylation activities were reassayed (Fig. 3C). No other peak of the activity than that obtained by solo incubation was detected.
These results indicated that P36 and its kinase were fractionated into the same tubes after HPLC gel filtration, suggesting two possibilities: 1) P36 itself has autophosphorylation activity and forms a complex of homodimer or with other molecules having a total molecular mass of 70–75 kDa and 2) P36 and its kinase are different molecules and may make a complex with each other or with other molecules. Further purification by anion exchange chromatography could not separate P36 and its kinase, although the recovery of the activity was low (less than 10%; not shown), supporting the first possibility.

Retention of the P36 phosphorylation activity without addition of any substrate other than ATP after these partial purification steps indicates that the histidyl phosphorylation of P36 is not the formation of a substrate-dependent intermediate as in catalysis of mitochondrial succinyl-CoA synthetase (29). In addition to the difference in molecular masses (36 kDa versus 32 kDa), immunoblot analysis of the partially purified fraction using anti-succinyl-CoA synthetase antibodies excluded the possibility that P36 is a phosphorylatable small subunit of the enzyme (not shown).

To determine whether P36 or another protein having the same molecular mass on SDS-PAGE has an activity to phosphorylate the histidyl residue(s) of P36, the proteins separated by SDS-PAGE were blotted to a membrane, and the renatured proteins were assayed for protein kinases (30). We could not detect any histidyl phosphorylation on the filter, although several kinases, most products of which were alkali-labile, were reproducibly detected (not shown). Thus a conclusive result showing the autophosphorylation activity of P36 has not yet been obtained.

Effect of Genistein on Protein Histidine Phosphorylation—We next examined the effect of genistein on P36 histidyl phosphorylation, because yeast histidine kinase has recently been reported to be inhibited by the drug (18). The drug was dissolved in dimethyl sulfoxide and added to the in vitro phosphorylation mixtures with a constant concentration (10%) of the solvent. The reactions were terminated at 20 s for PMS fractions and at 2 min for PM fractions after the addition of [γ-32P]ATP to detect transiently phosphorylated products. Autoradiograms are shown in Fig. 4A, and quantitated radioactivities in protein bands are graphed in Fig. 4B and C. Alkali or acid labilities of phosphoamo acids in each band are shown in Fig. 5.

Tyrosine autophosphorylation of the EGF receptor in both PMS and PM fractions was inhibited by genistein at low concentrations (more than 50% inhibition occurred at 0.3 μg/ml (about 1.2 μM)) (Fig. 4). This is comparable to the reported observation (20) that the half-maximal inhibition of autophosphorylation of the receptor in A431 cell membrane fraction occurred at 0.7 μg/ml genistein. Alkali-resistant phosphorylation of three unidentified proteins, in the PM fraction having molecular masses around 50 kDa, was 50% inhibited at about 30 μg/ml.

In contrast, histidyl phosphorylation of P36 was not inhibited even at very high concentrations (less than 50% at 300 μg/ml) by genistein (Fig. 4). Acid labile (possibly histidyl but not yet identified) phosphorylation of two other proteins having molecular masses of 130 and 32 kDa (Fig. 5) was also resistant to genistein. Thus the histidine kinase activities in rat liver detectable under our conditions are not inhibited by genistein.

Dephosphorylation of Histidyl Phosphorylated P36—To characterize the rapid dephosphorylation reaction of the phosphorylated P36 (PP36) in PMS fraction (see Fig. 1), the PNS was fractionated by centrifugation, and the dephosphorylation activity in each fraction was assayed with or without MgCl₂ (Fig. 6). For this study, P36 was in vitro phosphorylated in the EDTA extract from the PM fraction in which little PP36 phosphatase activity was present (see Figs. 1 and 2), and then small molecules such as Mg²⁺ and ATP were removed by gel filtration (see "Experimental Procedures"). All of the 32P of P36 prepared in this way could be removed by acid treatment, and the phosphorylated residues were assumed to be histidines.

Most PP36 phosphatase activities present in the low speed supernatant, PNS (1.5 kS) was recovered in the soluble fraction (10 kS) after centrifugation at 10,000 rpm. All of the PP36
phosphatase activities absolutely required Mg$^{2+}$.

Characterization of PP36 Phosphatase—PP36 phosphatase activities were recovered mostly in the soluble fraction (see Fig. 4), and the apparent molecular mass of the phosphatase was determined by HPLC gel filtration. The soluble proteins were separated, and each fraction was assayed for PP36 phosphatase with or without Mg$^{2+}$ (Figs. 7, A and B). The decreases in $^{32}$P of P36 were not proportional to the activities of the phosphatase partly because of the prolonged incubation time, and a broad elution profile of the phosphatase activity was obtained. From the peak of the activity, however, the apparent molecular mass of the phosphatase was estimated to be about 45 kDa.

The substrate specificity and an inhibitor sensitivity of the phosphatase were next examined using the peak fraction of the gel filtration eluate (Fr. 25) as the PP36 phosphatase (Fig. 8). For the study of the substrate specificity, in vitro phosphorylated P130, which is a soluble protein and probably autophosphorylates histidyl residues (see Fig. 5), was mixed with PP36 and used as the substrate. The specific radioactivities of PP36 and P130 were comparable (not shown). Acid labilities of the $^{32}$P linkage to P36 and to P130 can be seen by the decreases (about 30% of control) of $^{32}$P of both protein bands after brief acid treatment (compare lanes 6 and 7 in Fig. 8).

Depending on Mg$^{2+}$, the PP36 phosphatase almost completely dephosphorylated PP36 in a time-dependent manner, whereas it did not promptly dephosphorylate the phosphorylated P130 and some of the other co-fractionated phospho-

\[ 2 \text{K. Motojima and S. Goto, unpublished results.} \]
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Fig. 6. Dephosphorylation of in vitro phosphorylated P36 and effect of Mg²⁺. In vitro phosphorylated P36 was incubated with various fractions obtained by differential centrifugation of the rat liver homogenate in the presence (solid lines) or absence (broken lines) of MgCl₂. The PNS fraction was fractionated by centrifugation at 10,000 rpm for 15 min to obtain the supernatant (10kS) and the pellet (10kP). 50 μg of protein of the 1.5kS fraction (●), its equivalent 10kS (▲), or 10kP (▲) fractions were added to the phosphorylated P36 containing fraction (about 3 μg) in a total volume of 100 μl containing 10 mM MgCl₂ or 5 mM EDTA. The incubation was continued at room temperature, and 20-μl aliquots were removed at 1, 3, 10, and 30 min after mixing to determine the remaining ³²P of P36. The proteins were separated by SDS-PAGE, and the gels were processed as described in the legend for Fig. 1. The amount of ³²P remaining in P36 was quantitated using BAS2000 (Fu-jix), and the values were plotted as a percentage of the control (time 0). The inset shows the relevant portions of the representative autoradiographic patterns of the dephosphorylation of PP36.

Discussion

Histidine phosphorylation and dephosphorylation may play an important role in reversible post-translational modification of proteins to modulate their functions in mammalian cells. A key role of histidine phosphorylation in several bacterial signal transduction systems has been established (4, 5, 15), and yeast protein histidine kinase has been purified (16). In this report, we have described the characteristics of rat liver protein histidine kinase (17) and protein histidine phosphatase.

Histidine phosphorylation in a mammalian cell has been overlooked for at least two reasons: one is the acid-labile phosphoamide linkage in His(P) and the other reason is the rapid dephosphorylation reaction in crude extracts (see Fig. 1). In addition to avoiding the treatment of phosphorylated proteins with acid, short time course monitoring on ice and the use of subcellular fractions were essential to detect the activity. Rat liver histidine kinase, P36 kinase, was co-fractionated with the plasma membrane, and most of the phosphatase was recovered in the soluble fraction, making it possible to analyze quantitatively the phosphorylation reaction when the PM fraction was used as the kinase and the products were separated by SDS-PAGE (17). Co-fractionation with PM, however, does not always mean that P36 kinase is exclusively located on the plasma membrane, despite being enriched more than 20-fold over the starting PNS fraction (32). Further analysis using specific antibodies, for example, will be necessary to determine its localization in the cell.

P36 and its kinase could easily be removed from the membrane by EDTA treatment. They may be peripherally bound to the membrane through divalent cations with unidentified molecules, the function of which should be most interesting. Although association with the membrane is not necessary for the in vitro activity (Fig. 2), its role in modulation of the activity is

Fig. 7. Partial purification of PP36 phosphatase by HPLC gel filtration. The rat liver homogenate was centrifuged at 100,000 × g for 60 min, and the supernatant containing 0.5 v NaCl was injected in TSK G3000SWXL column as described in the legend for Fig. 3. A, protein elution profile. B, fractions (13–28) were assayed for dephosphorylation of PP36 with 10 mM MgCl₂ (EDTA (●)) or with 5 mM EDTA (EDTA (▲)) for 30 min at room temperature. The remaining ³²P of P36 was quantitated and plotted as described in the legend for Fig. 4. The representative autoradiographic patterns are shown at the top. PP36 incubated with the applied sample under the same conditions was analyzed in far left lane.
plausible but not yet studied. The possibility that phosphorylation of P36 induces translocation of the protein from the membrane to the cytoplasm may be ruled out because the phosphorylated P36 in the PM fraction was recovered in the pellet after high speed centrifugation of the reaction mixture (not shown).

The solubilized P36 and its kinase were fractionated by HPLC gel filtration into the region of an apparent molecular mass of 70–75 kDa. They were not separated after anion exchange column chromatography. The structure of P36 kinase will be of great interest for understanding its function. In several bacterial signaling systems, "sensor" proteins, often located in the cytoplasmic membrane, contain transmitter modules that have an autophosphorylation activity that attaches phosphoryl groups from ATP to histidine residues (5). In contrast, yeast protein histidine kinase is a soluble protein, and its autophosphorylation activity has not been reported (16). These differences must be the reflection of the involvement of histidyl phosphorylation in various reactions. In rat liver, two other possible histidyl phosphorylated proteins, in addition to PP36, were detected by in vitro studies, and one of them was in the soluble fraction. All of the histidine kinases including yeast enzyme cannot be involved in signaling systems. A phosphoramide in His(P) in contrast to phosphoric esters in Tyr(P), Ser(P), or Thr(P), has a high free energy of hydrolysis (33, 34), playing an important role as an intermediate in catalysis of several enzyme reactions including those of mitochondrial succinyl-CoA reductase (35) and a bacterial phosphoenolpyruvate-sugar phosphotransferase system (36, 37). His(P) in the histone of regenerating rat liver (38) would be another example as it is not apparently involved in a signaling system or in a catalytic reaction.

Huang et al. (18) reported that yeast histidine kinase was inhibited by genistein and discussed the possibility that genistein routinely used as a specific inhibitor for tyrosine kinases may inhibit mammalian histidine kinase in the cellular signaling pathway. If so, the interpretation of much previous data obtained using genistein should be reconsidered. Our present data on the effect of genistein on rat liver histidine kinase, however, showed that the kinase was not inhibited by the drug. Probable histidyl phosphorylation of two other proteins (P130 and P32 in Fig. 5) was also resistant to high concentrations of genistein. These data suggest that rat liver histidine kinase detected under our conditions is significantly different from yeast enzyme (16) and that mammalian histidine kinase may not be inhibited by genistein. The present study, however, cannot exclude the possibility that mammalian cells may have another histidine kinase that is homologous to yeast enzyme and is inhibited by genistein, although no information on its role in the cellular signaling pathway has yet been obtained.

PP36 was very rapidly dephosphorylated in the crude extract, and the characteristics of the phosphatase, namely, absolute dependency on Mg$^{2+}$ for the activity, the apparent molecular mass of 45 kDa as a soluble form, and its resistance to high concentrations of okadac acid, all suggest that PP36 phosphatase is PrP-2C (31). Partial recovery of the Mg$^{2+}$-dependent activity in the membrane fraction and broad elution by gel filtration suggesting loose association with other molecules also coincide with the properties of PrP-2C (39). This was an unexpected result as we had thought that a phosphatase to hydrolyze phosphoamidase in His(P) should be different from phosphatases for phosphoric esters. In accordance with our present result, Kim and Matthews (40) reported, during preparation of this report, that purified mammalian Ser(P), Thr(P) PrP-1, 2A, and 2C all had His(P) phosphatase activity when histidyl-phosphorylated histone H4 of yeast kinase was used as the substrate. PP36, in contrast, was dephosphorylated only by prob- able PrP-2C under our conditions. PrP-2C has been reported from in vitro studies to preferentially dephosphorylate several substrate proteins including hydroxymethylglutaryl-CoA reductase (41, 42), myosin light chain (43), glycogen synthase (39), Ca$^{2+}$/calmodulin-dependent protein kinase II (44), and elongation factor 2 (45), but it may not dephosphorylate these substrates in vivo because of its supposed very low activity under physiological conditions as discussed by Cohen (31). Strict preference of PrP-2C for PP36 but not for PP130, however, may provide a clue to the in vivo role of the generally disregarded phosphatase. Identification of the phosphatase to dephosphorylate PP36 in vivo must await further analysis considering the fact that P36 is associated with the membrane whether it is phosphorylated or not.

Furthermore, dephosphorylation of His(P) in PP36 in vivo may not involve phosphatase, but the phosphate may be transferred to another residue of the same or another protein as in the bacterial sensory signaling system. It therefore appears that further characterization of P36 kinase and its phosphatase or transferase is essential in elucidating their in vivo function and may shed light on a new signaling pathway in mammalian cells.

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Fig. 8. Characterization of PP36 phosphatase. As the partially purified PP36 phosphatase, the fraction 25 sample described in Fig. 7 was used. In vitro phosphorylated P36 and P130 fractions were mixed and used as substrates. The substrate mixture was incubated with the fraction 25 phosphatase alone (Fr. 25), with 10 μM okadic acid (Fr. 25 + OA), or with 5 mM EDTA (Fr. 25 + EDTA). The reaction was carried out at room temperature, and aliquots were removed at 1, 3, 10, and 30 min after mixing. The proteins were separated by SDS-FAGE and autoradiographed as described in the legend for Fig. 2 without drying the gel. In the two far left lanes, untreated substrate mixture (0) and briefly acid-treated (with trichloroacetic acid on ice for 5 min) mixture (T) were separated. Positions of P130 and P36 are indicated at the right.
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