Dephosphorylation of Phosphoproteins of Human Liver Plasma Membranes by Endogenous and Purified Liver Alkaline Phosphatases*

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Purified alkaline phosphatase and plasma membranes from human liver were shown to dephosphorylate phosphohistones and plasma membrane phosphoproteins. The protein phosphatase activity of the liver plasma membranes was inhibited by levamisole, a specific inhibitor of alkaline phosphatase, and by phenyl phosphonate and orthovanadate, but was relatively insensitive to fluoride (50 μM). Evidence (9) that the enzyme was a specific inhibitor of alkaline phosphatase activity was optimal at pH 8.0, compared to pH 7.8 for purified liver alkaline phosphatase. Plasma membranes also exhibited protein kinase activity using exogenous histone or endogenous membrane proteins (autophosphorylation) as substrates; this activity was cAMP-dependent. Autophosphorylation of plasma membrane proteins was apparently enhanced by phenyl phosphonate, levamisole, or orthovanadate. The dephosphorylation of phosphohistones by protein phosphatase 1 was not inhibited by levamisole but was inhibited by fluoride. Inhibition of endogenous protein phosphatase activity by orthovanadate during autophosphorylation of plasma membranes could be reversed by complexation of the inhibitor with (R)-(−)-epinephrine, and the dephosphorylation that followed was levamisole-sensitive. Neither plasma membranes nor purified liver alkaline phosphatase dephosphorylated glycogen phosphorylase a. These results suggest that the increased [32P]phosphate incorporation by endogenous protein kinases into the membrane proteins is due to inhibition of alkaline phosphatase and that the major protein phosphatase of these plasma membranes is alkaline phosphatase.

We have shown previously (10) that the plasma membrane-bound form of alkaline phosphatase has significantly different kinetic and molecular properties compared to the soluble, purified enzyme. The membrane-bound form of alkaline phosphatase is probably tetrameric (11), and this may account for the differences in its enzymatic properties. It is likely that alkaline phosphatase carried out its physiological and biochemical roles as an integral membrane protein.

Protein phosphatases are involved in enzyme regulation by reversible phosphorylation reactions (12–14). Purified alkaline phosphatase is a protein phosphatase (3–7), and plasma membrane-bound alkaline phosphatase may also exhibit the activity. The plasma membranes of various eukaryotic cells have both endogenous protein kinase (15–19) and alkaline phosphatase activities (20, 21). These two activities could work in concert to regulate the degree of phosphorylation of plasma membrane-associated proteins and hence to control some aspects of plasma membrane function in much the same fashion as has been postulated for cytoplasmic systems (12–14).

In this study, we showed the presence of a protein phosphatase activity in the plasma membranes from human liver. In characterizing this phosphatase and that of purified liver alkaline phosphatase, we used phosphohistones, glycogen phosphorylase a, and phosphorylated plasma membrane proteins as substrates. The protein kinase activity of the plasma membrane was also studied, and the effects of levamisole (1L−)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b](thiazole) and phenyl phosphonate, specific inhibitors of alkaline phosphatase, and other inhibitors on the protein kinase and phosphatase activities were determined.

EXPERIMENTAL PROCEDURES

Materials

[γ-32P]ATP (7.8 Ci/mmol, tetra-triethylammonium salt) was obtained from New England Nuclear, and ATP (disodium salt) and cAMP were from Calbiochem Behring. Human serum albumin, (R)-(−)-epinephrine, bovine protein kinase (cAMP-dependent), glycogen phosphorylase b, MOPS, Tris, PNP, and calf thymus histone (type II A) were purchased from Sigma. Whatman GF/F filters and scintillation fluid (ScintiVerse II) were from Fisher, phenylphosphonic acid from Aldrich, and KP from J.T. Baker Chemical Co. Acrylamide, N,N′-methylenebisacrylamide, SDS, and SDS molecular weight protein markers were from Bio-Rad. Other chemicals were obtained from Fisher.

Purified alkaline phosphatase from human liver (22) and plasma membranes from human liver (10 h post-mortem) (10), and phosphohistones (50 nmol of 32P/mg) (23) were prepared as described. The membranes were used at a protein concentration of 10 mg/mL, con-

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Containing 0.03 unit² of alkaline phosphatase activity/mg of total membrane protein. Protein determination was performed as described previously (24). Protein phosphatase 1 was a gift from Dr. P. Cohen (Department of Biochemistry, University of Dundee, Scotland).

The preparations of purified alkaline phosphatase and plasma membranes were adjusted to contain the same alkaline phosphatase activity, as measured by pNPP hydrolysis.

**Methods**

**Alkaline Phosphatase Activity**—Alkaline phosphatase activity was determined as described (25). For the assay of the plasma membranes, a lag period of 5 min was allowed prior to activity determination. The effects of KF (50 mM), phenyl phosphonate (10 mM), and orthovanadate (1 mM), and orthovanadate (1 mM) on the alkaline phosphatase activity of the plasma membranes and of purified alkaline phosphatase were also determined.

**Effect of Inhibitors on Protein Phosphatase Activity**—The effects of orthovanadate (1 mM), levamisole (10 mM), phenyl phosphonate (10 mM), and orthovanadate (1 mM), and orthovanadate (1 mM) were phosphatase or liver plasma membranes was determined. A lag period of 5 min was allowed prior to activity determination. The effects of orthovanadate (1 mM), phenyl phosphonate (10 mM), levamisole (10 mM), and orthovanadate (1 mM) on the alkaline phosphatase activity of the plasma membranes and of purified alkaline phosphatase were also determined.

**Effect of Inhibitors on Protein Phosphatase Activity**—The effect of orthovanadate (1 mM), levamisole (10 mM), phenyl phosphonate (10 mM), and orthovanadate (1 mM), and orthovanadate (1 mM) were used to initiate the reaction. A reaction mixture was used to determine the percent inhibition of the phosphatase activity. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 15% (w/v), and the suspension was centrifuged at 5000 × g for 10 min. Supernatant was added to 3 ml of scintillation fluid to determine [32P]phosphate release using an isocap/300 6668 Liquid Scintillation counter (Searle Analytical). [32P]Phosphate-labeled glycogen phosphorylase a was also used as a substrate although the effects of the inhibitors were not determined. The assay was performed as described for the phosphohistone activity, but reaction time was 10 min.

**Effect of pH on Protein Phosphatase Activity**—The release of [32P]phosphate from 32P-labeled phosphohistones by purified alkaline phosphatase or plasma liver membranes was determined in 4 mM MgCl₂, 100 mM Tris-HCl at pH 7.5. [32P]-labeled phosphohistones (6 mg/ml) were added to start the reaction. A reaction mixture was used to determine the percent inhibition of the phosphatase activity. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 15% (w/v), and the suspension was centrifuged at 5000 × g for 10 min. Supernatant was added to 3 ml of scintillation fluid to determine [32P]phosphate release using an isocap/300 6668 Liquid Scintillation counter (Searle Analytical). [32P]Phosphate-labeled glycogen phosphorylase a was also used as a substrate although the effects of the inhibitors were not determined. The assay was performed as described for the phosphohistone activity, but reaction time was 10 min.

**Effect of Inhibitors on the Histone Kinase Activity of cAMP-dependent Bovine Protein Kinase**—Histones (2 mg/ml) were phosphorylated by cAMP-dependent bovine protein kinase (13 mg/ml), with and without inhibitors in a reaction medium containing 6 mM MgCl₂, 0.6 mM [32P]ATP, (0.1 Ci/mmol), 5 mM CAMP, and 150 mM Tris-HCl at pH 7.5 (15). Incubation was at 30 °C for 30 min, and the reaction was terminated by the addition of trichloroacetic acid to a final concentration of 15% (w/v). The precipitate was filtered onto a Whatman GF/F filter, washed five times with 1-ml volume of 10% (w/v) trichloroacetic acid, and dried (15). The filter was placed in a vial with 3 ml of scintillation fluid for [32P]phosphate determination.

**Histone Kinase Activity of Plasma Membranes**—The activity of endogenous plasma membrane protein kinases was determined using histones as the protein substrate. The reaction was performed in 100 μl of reaction buffer, 1.0 mM orthovanadate, and 20 mg/ml histone with or without 5 μM cAMP; plasma membranes (10 μl, containing 10 mg of protein/ml) were added to start the reaction. Incubation was at 30 °C for 0–90 min. The reaction was terminated by cooling the mixture on ice and immediately centrifuging it at 20,000 × g for 10 min to pellet the plasma membranes from the soluble histones. The supernatant was removed, the histones were precipitated by trichloroacetic acid, the precipitate was filtered and washed, and [32P]phosphate was determined as described above.

The histone kinase activity of bovine protein kinase was determined similarly as described for the plasma membranes, in the presence of 5 μM CAMP. Reaction volumes were 100 μl of buffer and 10 μl of bovine protein kinase (2 mg/ml).

**Plasma Membrane Protein Phosphorylation by cAMP-dependent Bovine Protein Kinase**—Liver plasma membranes (5 mg/ml) were phosphorylated by cAMP-dependent bovine protein kinase (0.2 mg/ml) as described, with some modification (15). The phosphorylation buffer used was 0.2 mM [32P]ATP, (0.1 Ci/mmol), 4 mM MgCl₂, and 100 mM Tris-HCl, pH 7.5. Incubation was at 30 °C for 0–60 min. Incorporation of [32P]phosphate into the plasma membrane proteins was determined by trichloroacetic acid precipitation as described above. The effects of inhibitors on the bovine protein kinase activity were also determined.

**Autophosphorylation of Liver Plasma Membrane Proteins**—Plasma membranes (10 mg of protein/ml) were autophosphorylated using the phosphorylation buffer, with and without cAMP. The effects of inhibitors on this activity were also determined. Incubation was at 30 °C for 0–120 min. [32P]Phosphate incorporation was determined by trichloroacetic acid precipitation as described above.

**SDS-Polyacrylamide Gel Electrophoresis**—Plasma membrane proteins were resolved by polyacrylamide gel electrophoresis in the presence of SDS by the method of Laemmli (26). The support consisted of a stacking gel (4.8% T, 2.7% C) and a resolving gel (10% T, 2.7% C). Native and [32P]phosphate-labeled plasma membrane proteins (autophosphorylation of the membrane proteins was performed as described above) were solubilized with an equal volume of a buffer containing 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol in 62.5 mM Tris-HCl, pH 6.8. The protein samples were heated in boiling water for 3 min before tracking dye (0.1% (w/v) bromphenol blue, final concentration) was added. The samples (50–75 μl) were applied to the gel, and electrophoresis was carried out at a constant current of 30 mA in a Bio-Rad Protein II apparatus. The gels were either stained with Coomassie Blue R-250 (25) or dried for autoradiography as described (27).

**Dephosphorylation of [32P]Phosphate-labeled Liver Plasma Membrane Proteins**—Plasma membranes (10 mg of protein/ml) were autophosphorylated in the phosphorylation buffer described above except that 0.2 mM [32P]ATP with a specific activity of 2.6 mCi/μmol was used. The mixture was incubated for 15 min at 30 °C and then non-radioislated ATP plus MgCl₂ were added to 10 mM (final concentration). Equal volumes of the mixture were removed at appropriate times, and plasma membrane proteins were precipitated with trichloroacetic acid (final concentration of 15% (w/v)); [32P]phosphate was quantitated as described above.

In another procedure, plasma membranes were autophosphorylated using the phosphorylation buffer with 1 mM orthovanadate present. After incubation for 30 min at 30 °C, the plasma membranes were washed three times in 4 mM MgCl₂, 1 mM orthovanadate, and 100 mM Tris-HCl, pH 7.5, at 4 °C and resuspended to a final protein concentration of 5 mg/ml. Dephosphorylation of the [32P]phosphate-labeled plasma membrane proteins was initiated by the addition of 5 mM (R)-(-)-epinephrine (final concentration) which complexes the orthovanadate. Equal volumes of the mixture were removed and treated as described above to determine [32P]phosphate remaining on the membrane proteins.

**RESULTS AND DISCUSSION**

The effects of fluoride, phenyl phosphonate, levamisole, and orthovanadate on the protein phosphatase activity of purified alkaline phosphatase, protein phosphatase 1, and liver plasma membranes are summarized in Table I. To calculate the inhibition values, the experiments were done under conditions as close to initial rate inhibition as possible, using an incubation period of 5 min. The protein phosphatase activity of these plasma membranes is inhibited by fluoride, phenyl phosphonate, and orthovanadate, known protein phosphatase inhibitors (28–31). Levamisole, a specific inhibitor of alkaline phosphatase (32, 33), and orthovanadate are potent inhibitors of the pNPP hydrolysis activity of alkaline phosphatase, but less so of its protein phosphatase activity. The reverse is true of KF and phenyl phosphonate. Levamisole had little effect on protein phosphatase 1. None of these compounds were good inhibitors of bovine protein kinase (Table 1).

Using 32P-labeled histone as substrate, the inhibition profiles of the protein phosphatase activity of purified alkaline phosphatase and of the liver plasma membranes were similar, although the pure enzyme was more susceptible to all inhibitors (1 and Table 1). Particularly in the case of the pure enzyme, the dephosphorylation reactions appeared to be biphasic, with a rapid initial rate of protein phosphatase activity.

² One unit of alkaline phosphatase releases 1 μmol of p-nitrophenol from pNPP in 1 min under assay conditions defined under "Experimental Procedures."
TABLE 1
Effect of inhibitors on protein kinase and phosphatase activities
Protein kinase activity was determined in 0.6 mM [32P]ATP, 6 mM MgCl₂, 2 mg/ml histones, 13 μg/ml bovine kinase, 5 μM cAMP, and 150 nM Tris-HCl, pH 7.5. Protein phosphatase activity was determined using 5 mg/ml 32P-labeled histones, 4 mM MgCl₂ in 100 mM Tris-HCl, pH 7.5. Reactions were initiated by addition of either enzyme or liver plasma membrane. The reaction time was 5 min.

<table>
<thead>
<tr>
<th>Activities determined</th>
<th>KF (50 mM)</th>
<th>Phenyl phosphonate (10 mM)</th>
<th>Levamisole (10 mM)</th>
<th>Orthovanadate (10 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis of pNPP by alkaline phosphatase</td>
<td>13</td>
<td>47</td>
<td>94</td>
<td>97</td>
</tr>
<tr>
<td>Hydrolysis of pNPP by liver plasma membranes</td>
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<td>45</td>
<td>96</td>
<td>98</td>
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<tr>
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<td>82</td>
<td>84</td>
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<tr>
<td>Dephosphorylation of 32P-labeled histones by liver plasma membranes</td>
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<td>61</td>
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<td>Dephosphorylation of 32P-labeled histones by protein phosphatase</td>
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<td>15</td>
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<tr>
<td>Phosphorylation of histones by bovine protein kinase</td>
<td>38</td>
<td>27</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of inhibitors on histone phosphatase activity of purified alkaline phosphatase (panel A) and liver plasma membranes (panel B). The reaction buffer was 4 mM MgCl₂ and 5 mg/ml 32P-labeled histones in 100 mM Tris-HCl, pH 7.5. Additions were: none (O), 50 mM fluoride (■), 10 mM phenyl phosphonate (○), 10 mM levamisole (▲), or 1 mM orthovanadate (△).

Fig. 2. Effect of pH on histone phosphatase activity of purified alkaline phosphatase (panel A) and liver plasma membranes (panel B). The medium was 4 mM MgCl₂, 5 mg/ml 32P-labeled histone, and 100 mM buffer salt. Buffers were: MES-HCl (O), MOPS-HCl (▲), Tris-HCl (■), and bicarbonate (△). No inhibitor (— — —) or 10 mM levamisole (---) was added.

Protein phosphatase activity was determined in 0.6 mM [32P]ATP, 6 mM MgCl₂, 2 mg/ml histones, 13 μg/ml bovine kinase, 5 μM cAMP, and 150 nM Tris-HCl, pH 7.5. Protein phosphatase activity was determined using 5 mg/ml 32P-labeled histones, 4 mM MgCl₂ in 100 mM Tris-HCl, pH 7.5. Reactions were initiated by addition of either enzyme or liver plasma membrane. The reaction time was 5 min.

Activity. This may reflect the accessibility of the phosphoamino acid residues on the histones to the phosphatase and/or a degree of substrate heterogeneity. Nevertheless, it is clear that alkaline phosphatase in its native plasma membrane environment is still capable of dephosphorylating histone. Mg²⁺, present for these experiments at 4 mM, is an activator of the protein phosphatase activity as well as the pNPP hydrolase activity (24).

The pH profiles of the histone phosphatase activity of purified alkaline phosphatase and of the liver plasma membranes were similar (Fig. 2); both activities were optimal at pH 7.5-8.0. Addition of 10 mM levamisole to the reaction reduced the protein phosphatase activity of the plasma membranes to less than 22% at all pH values and that of the purified alkaline phosphatase to less than 8%. This difference may be a reflection of the histone phosphatase activity in the membranes that is levamisole-insensitive. The histone phosphatase activity of membrane alkaline phosphatase is less sensitive to pH than is the pure enzyme; at pH 5.0, the membranes still have 50% of the activity they had at the optimum, whereas the pure enzyme had only 15%. The same is true at pH 10.0, although the difference is less. In contrast to the constant alkaline pH optimum that alkaline phosphatase displays with respect to its phosphomonoesterase activity, the pH optimum of the protein phosphatase activity is apparently substrate-dependent (4).

In the presence of orthovanadate to inhibit all phosphatases (Table I), the liver plasma membranes exhibited a cAMP-dependent protein kinase activity capable of phosphorylating exogenous histone (Fig. 3). Alkaline phosphatase, although capable of hydrolyzing free ATP, will not use Mg-ATP as a substrate, and indeed Mg-ATP is not even an inhibitor of the enzyme (1). Membrane proteins can also be phosphorylated by the cAMP-dependent catalytic subunit of bovine protein kinase (Fig. 4). Both the rate and degree of phosphorylation were enhanced by levamisole, orthovanadate, and phenyl phosphonate, an effect that could have resulted from either an activation of the kinase or an inhibition of the protein phosphatases. Since the phosphorylation of histones by bovine protein kinase is inhibited (not activated) by these compounds (Table I), it is likely that inhibition of endogenous membrane phosphatases is responsible. Endogenous kinases can also catalyze the incorporation of [32P]phosphate from...
Protein Phosphatase Activity of Liver Alkaline Phosphatase

FIG. 3. Histone kinase activity of liver plasma membranes. The medium was 1 mM orthovanadate, 4 mM MgCl₂, 20 mg/ml histones, 0.2 mM [³²P]ATP in 100 mM Tris-Cl, pH 7.5. Additions were: none (○), plasma membranes only (△), plasma membranes and 5 μM cAMP (●), and bovine protein kinase and 5 μM cAMP (▲).

FIG. 4. Effect of inhibitors on the phosphorylation of plasma membranes by cAMP-dependent bovine protein kinase. The buffer was 4 mM MgCl₂, 8 mg/ml plasma membranes, 5 μM cAMP, and 0.2 mM [³²P]ATP in 100 mM Tris-Cl, pH 7.5 (○). Kinase (0.2 mg/ml) was added to initiate reaction (●). The inhibitors were: 50 mM fluoride (●), 10 mM phenyl phosphonate (▲), 10 mM levamisole (△), and 1 mM orthovanadate (Δ).

FIG. 5. Autophosphorylation of liver plasma membrane proteins. The medium was 100 mM Tris-Cl, 4 mM MgCl₂, 10 mg/ml membrane protein, and 0.2 mM [³²P]ATP, pH 7.5. Additions were: none (○), 5 μM cAMP (●), 5 μM cAMP and 10 mM levamisole (▲), and 5 μM cAMP and 1 mM orthovanadate (Δ).

[³²P]ATP into the plasma membranes (Fig. 5); this autophosphorylation is also enhanced in the presence of levamisole and orthovanadate. Many membrane proteins are phosphorylated, but three bands with molecular masses of 74, 45 and 36 kDa were most prominent (Fig. 6). Although ATP will phosphorylate alkaline phosphatase at its active site serine, this membrane enzyme does not contribute significantly to the [³²P]phosphate incorporated as judged by the lack of an 81-kDa band in the autoradiogram (25).

To further investigate the nature of the inhibitor-enhanced phosphorylation of the plasma membranes, it was necessary to assess separately the protein kinase and phosphatase activities. This was achieved with orthovanadate, an inhibitor of all phosphatases but not of bovine protein kinase (Table I). The inhibitory effect of orthovanadate on protein phosphatase activity can be reversed by (R)-(−)-epinephrine; this is accomplished through chelation of the inhibitor by the amine (33). Plasma membrane proteins, phosphorylated by endogenous kinase in the presence of orthovanadate, sedimented and washed, were not dephosphorylated to any appreciable extent until (R)-(−)-epinephrine was added (Fig. 7). Furthermore, this dephosphorylation could be inhibited by levamisole. The inhibitors were: 50 mM fluoride (●), 10 mM phenyl phosphonate (▲), 10 mM levamisole (△), and 1 mM orthovanadate (Δ).
sole, the specific inhibitor of alkaline phosphatase, or accelerated by the addition of purified alkaline phosphatase. Kinase and phosphatase activities were also "resolved" by carrying out the phosphorylation of plasma membranes with [32P]ATP and then after 15 min adding 10 mM non-radio-labeled Mg-ATP. Phosphorylation with [32P]ATP was essentially abolished, and protein phosphatase activity can be measured by [32P]phosphate release; this activity was sensitive to levamisole (not shown).

Neither purified alkaline phosphatase nor plasma membranes had any activity toward glycogen phosphorylase, but not pNPP.

That the two activities are one and the same is supported by the biological activity of the membranous proteins. Phosphorylation of protein at tyrosine residues has been observed in cell proliferation, differentiation, and transformation (1). However, alkaline phosphatase did not have a greater specificity just as aspects of cellular metabolism are regulated through control of the phosphorylation state of cytoplasmic enzymes. Phosphorylation of protein at tyrosine residues has been observed in cell proliferation, differentiation, and transformation (35-40). Reversible tyrosine phosphorylation was found during meiotic cell division in Xenopus laevis oocytes, and alkaline phosphatase appeared to be involved in the process (41). It has been reported that alkaline phosphatase was more specific for phosphotyrosyl proteins (4), although other studies have indicated that acid phosphatase will also dephosphorylate phosphotyrosyl proteins (38) and that alkaline phosphatase did not have a greater specificity for phosphotyrosine than for either phosphoserine or phosphothreonine (10).

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REFERENCES