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PROTEASE-ACTIVATED PROTEIN KINASE IN RAT LIVER PLASMA MEMBRANE*

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SUMMARY: Upon limited proteolysis with trypsin, a cAMP and Ca^{2+}-independent protein kinase was produced from rat liver plasma membrane. This enzyme showed a multifunctional capacity and phosphorylated calf thymus histone and rat liver ribosomal proteins. The molecular weight was estimated to be 5.0×10^4. When plasma membrane was treated with a buffer containing Triton X-100, a proenzyme with a molecular weight of 8.4×10^4 was extracted. By tryptic digestion, the proenzyme was converted to an active protein kinase which was similar to the enzyme obtained by the direct digestion of membrane. However, this proenzyme phosphorylated H1 histone in the presence of Ca^{2+} and phospholipid without proteolytic digestion. These results indicate the existence of a protease-activated protein kinase in rat liver plasma membrane and the proenzyme seems to be same as protein kinase C.

Since the first report on the insulin-dependent protein phosphorylation in adipose tissue by Benjamin and Singer (1), experimental results have accumulated indicating that the phosphorylation of acetyl CoA carboxylase, ATP-citrate lyase and ribosomal protein S-6 was increased by insulin (2,3). Although the involvement of casein kinase and protease-activated kinase was suggested by several investigators (4-6), the process between insulin binding and protein kinase activation has been remained obscure. In parallel with these reports, it was also shown that plasma membrane contained trypsin-like protease and its activity

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The abbreviations used are: EGTA, ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TPCK, L-1-tosylamide 2-phenylethyl chloromethyl ketone; SDS, sodium dodecyl sulfate.

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might be regulated by insulin (7,8). Based on these results, we were inclined to suppose that membrane-bound, protease-activated kinase is one of the candidates for the protein kinase operating in insulin-stimulated protein phosphorylating system. In spite of many reports on the protease-activated kinase, most of the enzymes were purified from cytosol of various types of tissues (9-15). This report showed the existence of a protease-activated kinase in rat liver plasma membrane and some properties of this enzyme were described.

EXPERIMENTAL PROCEDURES

Wistar rats were employed for the present studies. Rat liver plasma membrane was prepared as described by Prpić et al. (16) except that EGTA was omitted from the buffer. Calf thymus whole histone and H1 histone were prepared as specified earlier (17). Rat liver ribosomal proteins were obtained as described previously (18). Protein inhibitor of cAMP-dependent protein kinase was purified as described by Walsh et al. (19). TPCK-treated trypsin and soybean trypsin inhibitor were obtained from Sigma. (γ-^{32}P)ATP was prepared by the method of Walseth and Johnson (20). Phospholipid and diolein were kindly donated by Dr. Y. Nishizuka, Kobe University. Other materials were obtained from commercial sources.

Protein kinase (active form) was assayed as described previously (9) except that the specific activity of (γ-^{32}P)ATP was increased (8-20 x 10^4 cpm/nmol). After stopping the reaction, the mixture was boiled for 10 min to prevent non-specific binding of radioactive material. The acid-precipitable materials were collected on a glass filter as indicated previously (21) and radioactivity was determined with a Packard TriCarb liquid scintillation spectrometer, Model 300, by Cerenkov radiation. One unit of the enzyme was defined as indicated previously (9). Protein kinase C was assayed as described by Kikkawa et al(22).

For solubilization of membrane-bound, protease-activated kinase, rat liver plasma membrane containing 147 mg of protein was precipitated by centrifugation at 21,000 x g for 10 min and the membrane was treated with 7.8 ml of extraction buffer containing 20 mM Tris-HCl at pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA, 5 mM EGTA and 0.1 % (v/v) Triton X-100 with stirring for 1 h at 4 °C. After centrifugation at 21,000 x g for 10 min, 8 ml of the supernatant (35 mg of protein) was applied to a Sephadex G-100 column (2.6 x 86 cm) equilibrated with 20 mM Tris-HCl at pH 7.5, 50 mM 2-mercaptoethanol and 0.5 mM EGTA. Elution was performed downward with the same buffer at a flow rate of 35 ml/h. Fractions of 5 ml each were collected. Each fraction (0.19 ml) was incubated with or without 0.5 µg of trypsin for 4 min at 30°C. After addition of 10 µg of trypsin inhibitor, protein kinase activity was measured as described above except that Tris-HCl and 2-mercaptoethanol were omitted and incubation was performed for 15 min. Trypsin activated-protein kinase, appeared in Fractions 33 through 45, was collected and concentrated to 3.4 ml (4.9 mg of protein) using an Amicon ultrafiltration cell equipped with PM-10 filter membrane.

The enzyme was dialyzed overnight against a buffer containing 50 mM Tris-HCl at pH 8.0 and 250 mM sucrose.

Protein was determined by the method of Bradford (23) with bovine serum albumin as a standard. Phosphorylated amino acid was determined by the method described by Casnelli et al. (24). Before hydrolysis in 6.0 N HCl, the radioactive histone was treated as described previously (25). SDS-polyacrylamide slab gel electrophoresis was carried out by the method of Laemmli (26) utilizing a 10 % acrylamide running gel and a 4 % stacking gel. Molecular weight standards used in the slab gel electrophoresis were bovine serum albumin, ovalbumin, DNase I and cytochrome c. For autoradiography an X-ray film, JX(8x10), Fuji Photo Film Co. was exposed for 4 days at -80°C to the dried gel under test. Molecular weight of protein kinase was estimated by gel filtration procedure as described previously (9) using a Sephadex G-150 column (0.7 x 99 cm) equilibrated with buffer containing 20 mM Tris-HCl at pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA and 70 mM NaCl.

RESULTS

When rat liver plasma membrane was subjected to limited-proteolysis with trypsin, time-dependent increase in histone phosphorylating activity was observed as shown in Fig. 1. In the absence of trypsin, protein kinase activity was essentially unchanged. This trypsin-dependent kinase production was completely inhibited by addition of trypsin inhibitor. Previous report showed that Mg^{2+} ion influences various types of protein kinase activities (9). When the protease-activated kinase activity was

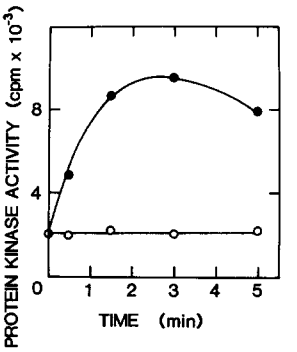

Fig. 1. Activation of membrane-bound protein kinase by trypsin. Rat liver plasma membrane (1.1 mg of protein) was incubated at 30° C with (●) or without (○) 3 µg of trypsin in a solution (0.6 ml) which contained 45 mM Tris-HCl at pH 8.0, 5 mM 2-mercaptoethanol and 175 mM sucrose. At the time indicated, an aliquot (0.1 ml) was transferred to a small plastic tube containing 10 µg of trypsin inhibitor. After centrifugation at 16,000 x g for 10 min, an aliquot of the supernatant (25 µl) was assayed for protein kinase under the conditions described under EXPERIMENTAL PROCEDURES.

compared between 5 mM and 75 mM Mg^{2+}, rapid reaction rate was observed at the higher concentration of this cation as indicated in Table I. Under the same condition, phosphorylated amino acid was identified as serine. The histone kinase activity at 75 mM Mg^{2+} was not affected by cAMP (1 μM) or EGTA (1 mM). Inhibitor protein of cAMP-dependent protein kinase was also ineffective. These results suggest that cAMP-dependent and Ca^{2+}-dependent protein kinases are not involved in this trypsin-activated protein kinase activity.

It has been reported that phosphorylation of ribosomal protein S-6 is increased in response to insulin (2,3). When ribosomal proteins were employed as phosphate acceptors, trypsin-stimulated phosphorylation was observed and this reaction was also more rapid at 75 mM Mg^{2+}[1] (Table I). Fig. 2 shows the autoradiogram of the phosphorylated ribosomal proteins analyzed by SDS-polyacrylamide slab gel electrophoresis.[2] Intensity was

TABLE I

Effect of Mg^{2+} on phosphorylation of calf thymus whole histone and rat liver ribosomal proteins by protease-activated protein kinase

	5 mM Mg^{2+}		75 mM Mg^{2+}	
	Before Proteolysis	After Proteolysis	Before Proteolysis	After Proteolysis
	cpm		cpm	
whole histone	830	1,200	1,840	6,790
ribosomal proteins	560	1,230	930	2,700

Enzymes were prepared as described in the legend to Fig. 1 except that incubation with or without trypsin was performed for 3 min. Each aliquot (25 μl) was assayed for protein kinase activity under the conditions described under EXPERIMENTAL PROCEDURES except that 0.1 mg of ribosomal proteins were employed and Mg^{2+} concentration was changed as indicated.

1 Although it is not clear whether the enzymes phosphorylating histone and ribosomal proteins were same or not, both activities were not separated by column chromatographies on DEAE-cellulose and Sephadex G-100 gel filtration.

2 The large amount of radioactivity remaining at the top of the gel may be due to highly insoluble nature of ribosomal proteins after extraction from ribosomal particle.

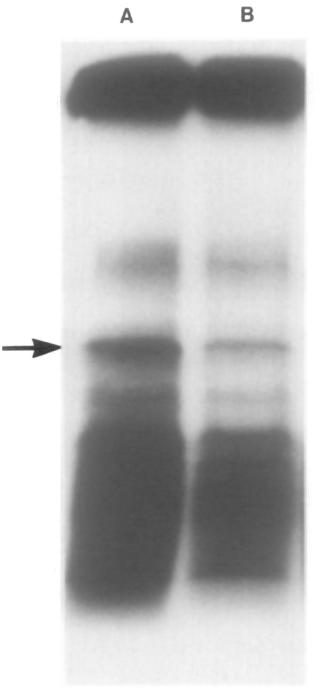

Fig. 2. SDS-polyacrylamide slab gel electrophoresis of ribosomal proteins phosphorylated by protease-activated protein kinase. Rat liver plasma membrane (4.2 mg of protein) was digested with 11 μg of trypsin for 2.5 min at 30°C. The reaction was stopped by the addition of 0.23 mg of trypsin inhibitor. In control sample trypsin inhibitor was added before starting the reaction by trypsin. The supernatants obtained after centrifugation at 22,000 x g were employed as enzyme source. Ribosomal proteins (0.5 mg) were phosphorylated under the standard condition described under EXPERIMENTAL PROCEDURES except that 1) the reaction mixture was scaled up by 5-fold (1.25 ml) 2) 31 μg of inhibitor protein of cAMP-dependent protein kinase, 0.1 mg of trypsin inhibitor and EGTA at a final concentration of 1 mM were included 3) specific activity of (γ-^{32}P)ATP was increased to 2.2 x 10^6 cpm/nmol. The enzyme activities employed were 47 units and 22 units for trypsin-treated and control enzymes, respectively. Ribosomal proteins were phosphorylated for 5 min at 30°C. The reactions were stopped by the addition of 50 % trichloroacetic acid to a final concentration of 10 %. In the absence of ribosomal proteins, acid precipitable radioactivity was negligible. The precipitated proteins were collected and washed as described previously (18). Each 31 μg of protein sample (8,000-12,000 cpm) was analyzed by SDS-polyacrylamide slab gel electrophoresis. An arrow shows the protein whose molecular weight was approximately 3 x 10^4. A, with trypsin-treated enzyme; B, with control enzyme.

increased on various proteins in trypsin-dependent manner. As indicated by an arrow, one of the heavily labelled proteins was detected at the position of approximate molecular weight of 3 x 10^4. Although this protein may correspond to the ribosomal protein

S-6 judging from the molecular weight, the exact identification must await further analysis.

In an attempt to identify a proenzyme, rat liver plasma membrane was treated with a buffer containing Triton X-100 and the extracted proteins were analyzed by gel filtration on Sephadex G-100. At first, the enzyme obtained from a direct digestion of plasma membrane by trypsin was applied to the column. As described in Fig. 3A, histone phosphorylating activity appeared in Fractions 23 through 30. The molecular weight was estimated to be 5.0×10^4. When the solubilized enzyme was applied to the same column, a

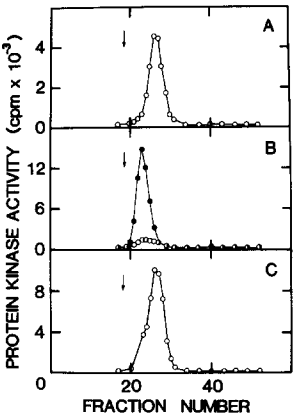

Fig. 3. Gel filtration of proenzyme and trypsin-activated protein kinases. In experiment A, rat liver plasma membrane (11 mg of protein) was digested with trypsin for 1 min under the same condition as described in the legend to Fig. 2. An aliquot of the supernatant (0.5 ml) was applied to a Sephadex G-100 column (0.8 x 105 cm) equilibrated with a buffer containing 20 mM Tris-HCl at pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA and 70 mM NaCl. In experiments B and C, the solubilized proteins from plasma membrane (2.1 mg of protein) were incubated with or without 21 µg of trypsin for 1 min at 30°C. After addition of 0.42 mg of trypsin inhibitor, 0.5 ml of each reaction mixture was applied to the same Sephadex G-100 column. In each experiment, elution was performed downward with the buffer described above at a flow rate of 6 ml/h. Fractions of 1 ml each were collected. An arrow shows the position of void volume. In experiments A and C, 205 µl of each fraction was assayed for protein kinase activity under the condition described under EXPERIMENTAL PROCEDURES except that Tris-HCl and 2-mercaptoethanol were omitted and incubation was performed for 10 min. In experiment B, 0.19 ml of each fraction was incubated with (●) or without (○) 0.5 µg of trypsin for 1 min at 30°C. After addition of trypsin inhibitor (10 µg), protein kinase activity was measured as described above. Molecular weight of each sample was estimated using a Sephadex G-150 column under similar conditions. A, trypsin-activated protein kinase obtained from membrane digestion; B, proenzyme; C, trypsin-activated protein kinase obtained from proenzyme.

trypsin-activated protein kinase was detected slightly ahead of the eluting position of the enzyme obtained by the membrane digestion. The molecular weight was estimated to be 8.4×10^4. After activation by trypsin, the digested enzyme was eluted in Fractions 22 to 30. The molecular weight was decreased to 4.9×10^4 (Fig. 3C). The activated enzyme also showed a high Mg^{2+} requirement for calf thymus histone phosphorylation. In contrast to the proteolytic activation, this enzyme fraction also showed an ability to phosphorylate H1 histone in the presence of Ca^{2+} and phospholipid. These results suggest that the enzyme detected in Fig. 3B corresponds to the proenzyme of the trypsin-activated, multifunctional protein kinase and is essentially same as protein kinase C.

DISCUSSION

Although there have been several reports on protease-activated kinase, most of these enzymes were purified and characterized from cytosol of various mammalian tissues (9-15). This report showed the existence of a protease-activated kinase in rat liver plasma membrane. Judging from the high Mg^{2+} requirement in histone phosphorylation, the active form of the enzyme may correspond to that first described in central nervous tissue (9,10). It is well known that further analysis of this type of protease-activated kinase was extended to the discovery of protein kinase C (27). This class of protein kinase was detected in particulate fraction including plasma membrane as well as cytosolic fraction (28,29). In addition to the proteolytic activation, the proenzyme described in this paper showed an ability to phosphorylate H1 histone in Ca^{2+}, phospholipid-dependent manner. Although the enzyme preparation employed was not homogeneous, these accumulated results suggest that the proenzyme corresponds to protein kinase C. In parallel with the analysis of protein kinase,

experiments have been also performed on solubilization and purification of membrane-bound, trypsin-like protease. The mechanism of activation of membrane-bound protein kinase by such a protease and substrate specificity of the protease-activated form of the enzyme have been currently studied in our laboratory.

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