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PHOSPHORYLATION OF ISOLATED PLASMA MEMBRANES OF AH-66 HEPATOMA ASCITES CELLS BY CASEIN KINASE 1

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SUMMARY: The isolated plasma membranes of AH-66 hepatoma cells were phosphorylated by casein kinase 1 purified from the cytosol fraction of AH-66 cells. Casein kinase 2 purified from the same source had little effect on the phosphorylation of the plasma membranes. Two-dimensional gel electrophoresis and autoradiography showed that casein kinase 1 enhanced the phosphorylation of approx. 10 plasma membrane proteins that are phosphorylated only faintly in the isolated plasma membranes by endogenous protein kinase. Among these phosphoproteins, tubulin was identified as judged from their molecular weights and isoelectric points. These results suggest that one of the physiological functions of casein kinase 1 is phosphorylation of plasma membrane and plasma membrane-associated proteins.

Plasma membranes are involved in various cellular functions such as control of cell growth, neoplastic transformation, and cell differentiation. Phosphorylation of plasma membrane proteins is one of the regulatory system for these cellular functions. Previously we found that a number of plasma membrane proteins of AH-66 hepatom ascite cells were phosphorylated by cyclic AMP-independent protein kinase(1). We also observed that casein kinase 1, but not casein kinase 2, purified from the cytosol of AH-66 cells can catalyze the phosphorylation of the plasma membrane proteins(2). In present study, we further studied the phosphorylation of plasma membrane proteins of AH-66 cells by casein kinase 1 and proposed that membrane

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phosphorylation is one of physiological functions of casein kinase 1.

MATERIALS AND METHODS

Materials [γ-^{32}P]ATP was purchased from the Radiochemical Center, Amersham. Casein kinases 1 and 2 were purified from the cytosol of AH-66 cells as described previously(2, 3). Protein kinase C was partially purified from rat brain according to Kikkawa et al.(4) through the stage of Sephadex G-150 column chromatography. Microtubule was prepared from porcine brain according to the method of Murphy(5). Actin from rabbit skeletal muscle was obtained from Sigma.

Phosphorylation of plasma membranes

The plasma membranes of AH-66 cells were isolated as described previously(6) by a modification of the method of Warren, Glick, and Nass(7). The characteristics and purity of the membrane preparation were described previously(8). The phosphorylation of plasma membranes were carried out in the reaction mixture in 100 µl containing 80 µg of plasma membrane proteins, 50 mM Tris-HCl(pH 7.5), 0.1 M NaCl, 5 mM $MgCl_2$, and 0.1 mM ATP containing [γ-^{32}P]ATP(26 cpm/pmol), and 0.2 µg of either casein kinase 1 or 2. In the case of phosphorylation of plasma membranes by protein kinase C, 0.5 mM $CaCl_2$, 5 µg phosphatidylserine, 1 µg 1,3-diolein, and 0.2 mM EGTA and 5 µg of the enzyme were added to the reaction mixture. The phosphorylation of tubulin and actin was conducted under the same reaction conditions except that 20 µg of tubulin and actin were used. The reaction mixture was incubated for 5 min at 37°C in a shaking water bath and the reaction was stopped by adding 10% cold trichloroacetic acid. The radioactivity was determined as described previously(2).

Polyacrylamide gel electrophoresis

Slab gel electrophoresis was conducted in 10% gels in SDS according to the method of Laemmli(9). Two-dimensional gel electrophoresis of plasma membranes was performed essentially according to O'Farrell(10) except that sodium dodecyl sulfate was added to both the sample solution and the disk gel in the first dimension. The first dimension was run in 10-cm gels of 2-mm internal diameter for 15 h at 400 V. Electrode solutions were 0.01 M phosphoric acid(anode) and 0.04 M NaOH(cathode). The second dimension was run at 100 V for 8 h in a 2-mm slot in a slab gel containing 10% acrylamide and 0.1% sodium dodecyl sulfate using Laemmli's buffer system. Gels were stained with Coomassie blue R-250. For autoradiography, the stained gel was dried, placed on an X-ray film(Kodak X-Omat AR) with Dupont Cronex Lightening-Plus intensifying screens, and exposed for 2 days at -80°C.

Other methods Protein determinations were made by the method of Lowry et al.(11) and by dye binding using Coomassie blue G-250(12), with BSA as a standard. The phosphorylated amino acid residues of the plasma membrane proteins, tubulin or actin were identified by the method of Hunter and Sefton(13). Cellulose thin-layer electrophoresis was conducted at pH 1.9(acetic acid:formic acid:H_2O, 78:25:897, v/v) for 120 min at 1,100 V.

RESULTS

Table 1 shows the effect of casein kinases 1 and 2 on the incorporation of ^{32}P into isolated plasma membranes of AH-66

Table 1. Uptake of radioactive phosphate from [γ-^{32}P]ATP into plasma membranes of AH-66 cells

Sample	Activity (pmol/min/mg membrane protein)
Plasma membrane (endogenous phosphorylation)	39 ± 2.8
Plasma membrane + casein kinase 1	189 ± 7.8
Plasma membrane + casein kinase 2	64 ± 11.0
Plasma membrane + protein kinase C	454 ± 23.0

The plasma membranes(80 μg) isolated from AH-66 cells were phosphorylated under the standard reaction conditions as described in the text.

cells. While casein kinase 2 had little effect on the incorporation of ^{32}P into plasma membranes, casein kinase 1 caused marked increase in the phosphorylation of the plasma membranes. Protein kinase C, which is known to phosphorylates cardiac plasma membranes(14), were also effective for the phosphorylation of the plasma membranes of AH-66 cells. In order to further clarify the effect of casein kinases 1 and 2 on the phosphorylation of plasma membrane proteins, the phosphorylated plasma membranes were analyzed by two-dimensional gel electrophoresis and autoradiography(Fig. 1). Part C in Fig. 1 shows endogenous pohosphorylation of the isolated plasma membranes. Autophosphorylation of casein kinase 1 was undetectable under these conditions(result not shown). When casein kinase 1 was added to the plasma membranes, it markedly catalyzed the phosphorylation of about 10 plasma membrane proteins(part D in the same figure). It is to be noted that casein kinase 1 enhanced the phosphorylation of the same membrane proteins that are endogenously but only faintly phosphorylated in isolated plasma membranes by endogenous protein kinase. As demonstrated previously(2), casein kinase 2 had practically no effect on the phosphorylation of plasma

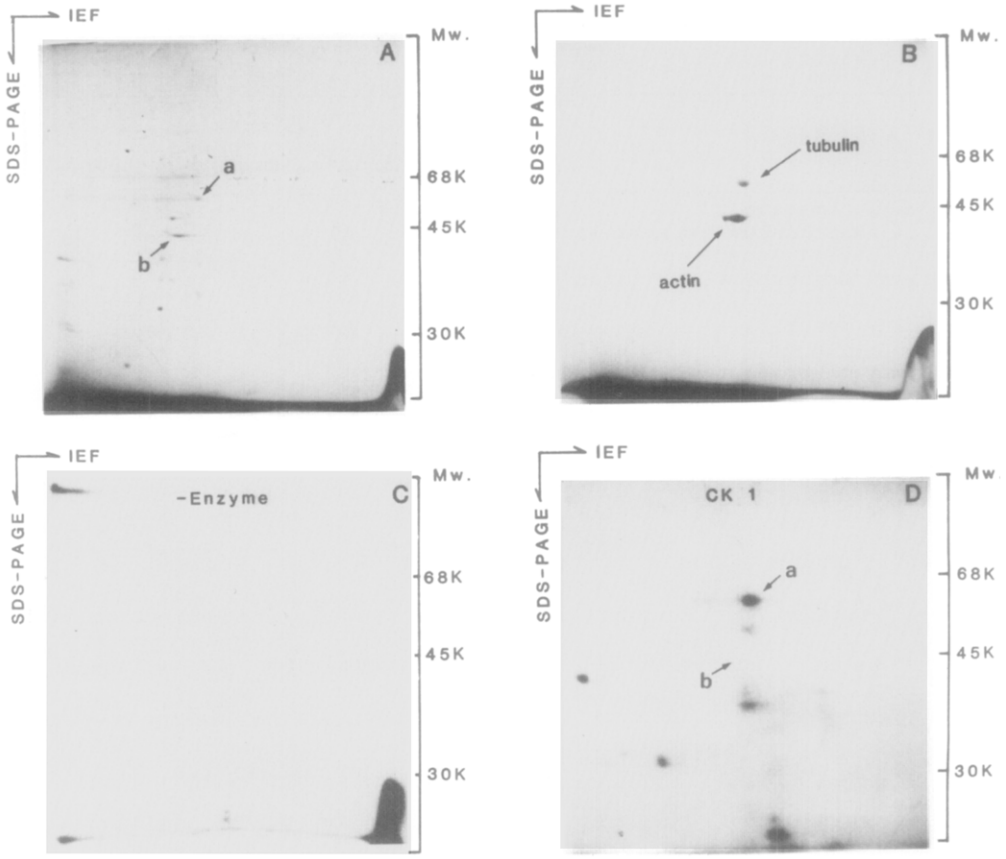

Fig. 1. Phosphorylation of the plasma membranes of AH-66 cells by casein kinase 1. Plasma membranes(80 µg protein) were phosphorylated with 0.1 mM [γ-^{32}]ATP(625 cpm/pmol) as in the text in the absence(A, B, and C) and in the presence of casein kinase 1(D) and analyzed by two-dimensional gel electrophoresis and autoradiography. Electrophoresis was carried out by a slight modification of the method of O'Farrell(10) as described in the text. The gel was stained with Coomassie blue(A and B) and then autoradiographed(C and D). Part B is the Coomassie blue staining pattern of the mixture of tubulin and actin electrophoresed under the same conditions. The spots corresponding to tubulin and actin are indicated by letters a and b, respectively.

membranes. Casein kinase 1 could not utilize GTP as phosphate donor(result not shown). The molecular weights and isoelectric points of proteins marked by a and b coincided with those of tubulin and actin, respectively(compare part A with part B in Fig. 1). As is evident from part D in Fig. 1, tubulin(marked by a) was markedly phosphorylated by casein kinase 1. Actin(marked by b in part D) was only weakly phosphorylated by the same

enzyme, although the radioactive spot was hardly visible on the photograph.

In order to ascertain that tubulin and actin are phosphorylated by casein kinase 1, isolated microtuble preparation and actin were treated with casein kinase 1 under the same conditions as those for the phosphorylation of plasma membranes. Microtuble-associated protein-2(MAP-2) of molecular weight of 270,000 was phosphorylated markedly by an endogenous prein kinase(s) contaminated in the microtuble preparation, while tubulin of molecular weight of 55,000 was only faintly phosphorylated(lane B in Fig. 2). When casein kinase 1 was added to the tubulin preparation, tubulin was strongly phophorylated, whereas the phosphorylation of MAP-2 was stimulated to a smaller extent(lane C in fig. 2). Isolated actin was also strongly phosphorylated by casein kinae 1(result is shown elsewhere).

Phosphorylated amino acid analysis indicated that the amounts of phosphoserine and phosphothreonine of plasma membrane

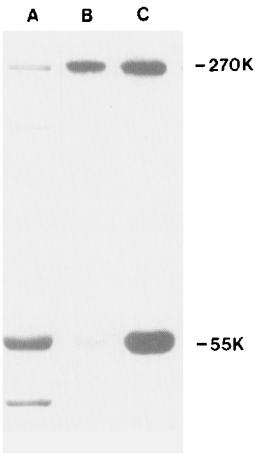

Fig. 2. Phosphorylation of microtuble protein by casein kinase 1. Microtuble protein(20 μg) from porcine brain was phosphorylated in the absence(A and B) and in the presence of casein kinase 1(C) under the standard reaction conditions as described in the text and analyzed by slab gel electrophoresis and autoradiography. Electrophoretic conditions were the same as in Fig. 1; A, Coomassie blue staining; B and C, autoradiograms.

proteins were enhanced about 2.3-fold and 3.8-fold, respectively, by treatment of the plasma membranes with casein kinase 1(result not shown). By contrast, phosphorylation of tubulin and actin by casein kinase 1 occurred almost exclusively on serine residues.

DISCUSSION

Protein phosphorylation by casein kinase 1 is known to be involved in the regulation of a wide variety of cellular functions such as protein synthesis(15-18), glycogen metabolism (19-21), and fatty acid synthesis(22). Muscle proteins such as troponin T(23), myosin light chain and myosin light chain kinase from both smooth and skeletal muscle(24) are also substrates of casein kinase 1. Singh et al.(25) are the first to report that microtuble proteins such as tubulin and MAP-2 are phosphorylated by casein kinae 1. The present report demomnstrated that plasma membrane proteins of AH-66 cells were phosphorylated by casein kinase 1 and that one of the phosphoproteins is tubulin. Yahara and Edelman(26) suggested that tubulin is associated with plasma membranes in vivo. Probably, a part of microtubules associated with plasma membranes of AH-66 cells were fractionated into the plasma membrane fraction and were phosphorylated by casein kinase 1. From the results presented in this report, we proposed that the phosphorylation of plasma membrane and plasma membrane-asssociated proteins is one of the physiological functions of this enzyme. This proposal is in agreement with the recent work of Singh abnd Huang(27), who showed that the activity of casein kinase 1 is very high in microsomal fraction of rat liver. The physiological significance of the membrane phosphorylation by casein kianse 1 in tumor cells deserves further study.

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