Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 251-257

CHARACTERIZATION OF A PROTEIN KINASE-SUBSTRATE COMPLEX PRECIPITABLE WITH Ca^{2+} FROM THE CYTOSOL FRACTION OF AH-66 HEPATOMA CELLS

Shigeo Nakajo, Kazuyasu Nakaya, Takemi Yoshida and Yasuharu Nakamura

School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142 Japan

Received October 16, 1984

SUMMARY: A protein kinase-substrate complex was precipitated by adding Ca^{2+} to the cytosol fraction of AH-66 ascites hepatoma cells. The amount of the precipitated complex was increased with increasing concentrations of Ca^{2+} and reached a plateau at about 5 mM Ca^{2+}. In the presence of [γ-^{32}P]ATP, extensive uptake of radioactive phosphate into this complex occurred. The phosphorylation reaction was little affected by addition of cyclic nucleotides, Ca^{2+}-phospholipid, Ca^{2+}-calmodulin. When the complex after phosphorylation was analyzed by SDS-PAGE, a protein with molecular weight of 33,000 was most heavily phosphorylated. These phenomena were also observed for mouse myeloid leukemia cells(M1 cells). By contrast, the addition of Ca^{2+} to the cytosol fractions of regenerating rat liver, normal rat liver or brain caused little precipitation of the complex.

Phosphorylation of proteins plays an important role in the regulation of a variety of biological processes including transformation of normal cells. We have previously compared the endogenous phosphorylation of the cytosol fraction of AH-66 hepatoma cells with that of normal liver and found that there are several qualitative and quantitative differences in phosphoproteins between them(1,2). In the present study, we found that the proteins precipitated with Ca^{2+} from the cytosol fraction of AH-66 cells contain protein kinase complexed with its substrate. The composition of the precipitate was constant and quite different from that of the cytosol. We designated the precipitate as protein kinase-substrate complex and examined the properties of endogenous phosphorylation of this complex. The phosphorylation reaction and content of the complex in another fast growing cell line such as mouse myeloid leukemia cells (M1 cells) and regenerating rat liver were also compared with those of the complex in normal liver and brain.

0006-291X/84 $1.50

EXPERIMENTAL PROCEDURES

Preparation of protein kinase-substrate complex

AH-66 ascites hepatoma cells washed with phosphate-buffered saline were homogenized in 6 volumes of 10 mM imidazole-HCl(pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride(buffer A). The homogenate was centrifuged at 20,000 x g for 20 min, and then the supernatant was further centrifuged at 105,000 x g for 60 min. The supernatant was adjusted to 5 mM $CaCl_2$ by addition of 0.5 M $CaCl_2$. After 15 min, the solution was centrifuged at 20,000 x g for 40 min and the precipitate was collected. The precipitate was dissolved in buffer A and the solution was centrifuged at 20,000 x g for 40 min. The resulting supernatant was readjusted with $CaCl_2$ and the precipitate was redissolved in a minimum amount of buffer A as described above. The solution was centrifuged at 20,000 x g for 40 min and its supernatant was used as the protein kinase-substrate complex.

Protein kinase assay

The standard reaction mixture in a final volume of 0.1 ml contained, unless otherwise indicated, 50-80 μg of protein kinase-substrate complex, 50 mM Tris-HCl(pH 7.5), 5 mM $MgCl_2$ and [γ-^{32}P]ATP(8,000-15,000 cpm/nmol). For measuring casein kinase, 0.2 mg of casein and 0.1 M NaCl were added to the reaction mixture described above. Histone kinase was measured under the same reaction conditions as those of casein kinase except that 0.2 mg mixed histone was used as substrate without NaCl. Cyclic AMP-dependent protein kinase or cyclic GMP-dependent proterin kinase was assayed under the same assay conditions as those for measuring histone kinase except that 2 μM cyclic AMP or 2 μM cuclic GMP was added. Ca^{2+}-activated, phospholipid-dependent protein kinase was measured according to the method of Kikkawa et al.(3). Ca^{2+}-, calmodulin-dependent protein kinase was assayed in the reaction mixture containing 0.5 mM $CaCl_2$, 0.2 mM EGTA, 2 μg calmodulin and 0.2 mg mixed histone. All phosphorylation reactions were conducted for 5 min at 37°C in a shaking water bath and the reactions were stopped by adding 2 ml of 10% trichloroacetic acid. The radioactivity was determined as described previously(2,3).

Other methods

SDS-gel electrophoresis was carried out in 13% polyacrylamide slab gel containing 0.1% SDS(4). Protein concentration was measured by the method of Bradford using bovine serum albumin as standard(5). Calmodulin was purified from rat brain according to Dedman et al.(6). Phosphorylated amino acid analysis in the protein kinase-substrate complex was analyzed by the method of Hunter and Sefton(8). Cellulose thin-layer electrophoresis was conducted at pH 3.5(glacial acetic acid: pyridine: H_2O, 50: 5: 945, v/v) for 20 min at 1,600 V. Ml cells(clone T22) were cultured in Eagle's minimal essential medium as described previously(7).

RESULTS

When the cytosol fraction from AH-66 ascites hepatoma cells was adjusted to 5 mM Ca^{2+} by addition of $CaCl_2$, the solution became turbid. Fig. 1 shows the turbidity change of AH-66 cytosol as measured by addition of various concentrations of $CaCl_2$. The turbidity was increased with increasing concentrations of Ca^{2+} and reached a plateau at about 5 mM Ca^{2+}. Mg^{2+} was far less effective than Ca^{2+}. The turbidity caused by 5 mM Mg^{2+} was about 16% of that formed by the same concentration of Ca^{2+}. When the turbid solution resulting from the addition of 5 mM Ca^{2+} was centrifuged at 20,000 x g for 40

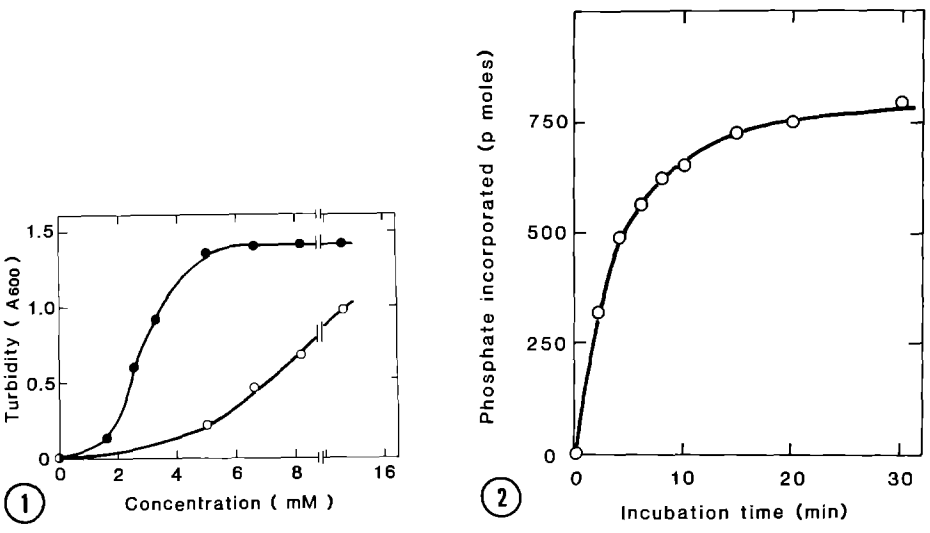

Fig. 1. Effects of varying concentrations of $CaCl_2$ and $MgCl_2$ on the turbidity of the cytosol fraction prepared from AH-66 cells. The cytosol fraction(3.8 mg protein/ml) of AH-66 cells was incubated for 13 min with various concentrations of $CaCl_2$ or $MgCl_2$ as indicated, and the turbidity was measured at 600 nm. —●— ; $CaCl_2$, —○— ; $MgCl_2$.

Fig. 2. Uptake of radioactive phosphate into the protein kinase-substrate complex precipitated with Ca^{2+} as a function of incubation time. The complex prepared from AH-66 cells was incubated with [γ-^{32}P]ATP and the complexes (65 μg protein) were periodically withdrawn at the times indicated.

min, a white precipitate was obtained. The precipitate thus formed was protein as judged from the Folin test. For further experiments, the precipitate formed in 5 mM Ca^{2+} was dissolved in buffer A.

Incubation of the dissolved precipitate with [γ-^{32}P]ATP and 5 mM $MgCl_2$ resulted in extensive uptake of radioactive phosphate into the protein precipitated with Ca^{2+}. The rate of incorporation of radioactive phosphate into the protein was linear up to 200 μg protein(result not shown). The time course of the phosphorylation reaction was linear for at least 5 min and reached a plateau at approximately 30 min(Fig. 2). These results indicate that the protein precipitated with Ca^{2+} from the cytosol fraction of AH-66 cells contains a protein kinase complexed with its substrate. To investigate the property of the protein kinase in the protein kinase-substrate complex, we tested the phosphorylation reaction of the complex under various conditions. When casein was used as exogenous substrate, the incorporation of radioactive phosphate was increased about 2-fold over that of the endogenous phosphory-

lation. Addition of heparin, which is known to be a potent inhibitor of casein kinase 2(9), caused a slight inhibition in the phosphorylation of casein as compared with that measured with casein alone(result not shown). In the absence of exogenous substrate, however, heparin had no effect on the endogenous phosphorylation. The phosphorylating activity with histone as exogenous substrate was practically unaffected by addition of either cyclic AMP, cyclic GMP, calmodulin and Ca^{2+}, or phospholipid plus Ca^{2+}. Neither histone nor Ca^{2+}-calmodulin affected on the endogenous phosphorylation. These results suggested that the protein kinase in the complex is neither cyclic nucleotide-dependent protein kinases, Ca^{2+}-, calmodulin-dependent nor Ca^{2+}-activated, phospholipid-dependent protein kinases.

We examined whether the protein kinase-substrate complex is present in normal liver and brain and in another fast growing cell line such as the mouse myeloid leukemia cells(Ml cells) and regenerating rat liver. When the soluble fractions of these cells and tissues were treated with 5 mM Ca^{2+} as described in "EXPERIMENTAL PROCEDURES", the protein kinase substrate complex was also precipitated. The yields of the complex with respect to total protein in each cytosol fraction were 8.5% for AH-66 cells and 3.4% for Ml cells(Table 1). In

Table 1. Incorporation of radioactive phosphate into the protein kinase-substrate complex and yield

	Endogenous phosphorylation (pmol/min/mg)	Total activity[1] (pmol/min)	Yield[2] (%)
AH-66 cells	1,496 ± 107	12,700	8.5 ± 0.9
Ml cells	3,028 ± 105	10,300	3.4
Rat brain	768 ± 17	576	0.75
Rat liver	202 ± 32	61	0.30
Regenerating rat liver(24 h)[3]	412 ± 47	140	0.34
Regenerating rat liver(48 h)[4]	448 ± 72	148	0.33

1) Calculated as activity of the complex obtained from 100 mg of each cytosolic protein.
2) Calculated on the basis of the amount of protein in protein kinase-substrate complex as compared with that of each cytosol fraction.
3) 24 h after partial hepatectomy, 4) 48 h after partial hepatectomy.

the case of normal cells such as normal liver and brain, the amount of the protein kinase-substrate complex was much lower than observed for tumor cells. The total incorporation of radioactive phosphate into the protein kinase-substrate complex in liver and brain cytosol were only 0.5% and 5%, respectively, as compared with that of AH-66 cytosol. Interestingly, both the amount of protein and total incorporation of radioactive phosphate of the protein kinase-substrate complex in regenerating rat liver was almost the same as in normal liver.

When the protein kinase-substrate complex after endogenous phosphorylation was analyzed by SDS-PAGE and autoradiography, several proteins were heavily phosphorylated. Among them, the protein with molecular weight of 33,000 was most strongly phosphorylated both in AH-66 cells and in M1 cells(lanes A' and B' in Fig. 3). Phosphorylation of the substrate proteins in the complex of AH-66 cytosol occurred almost exclusively on serine residues(Fig. 4).

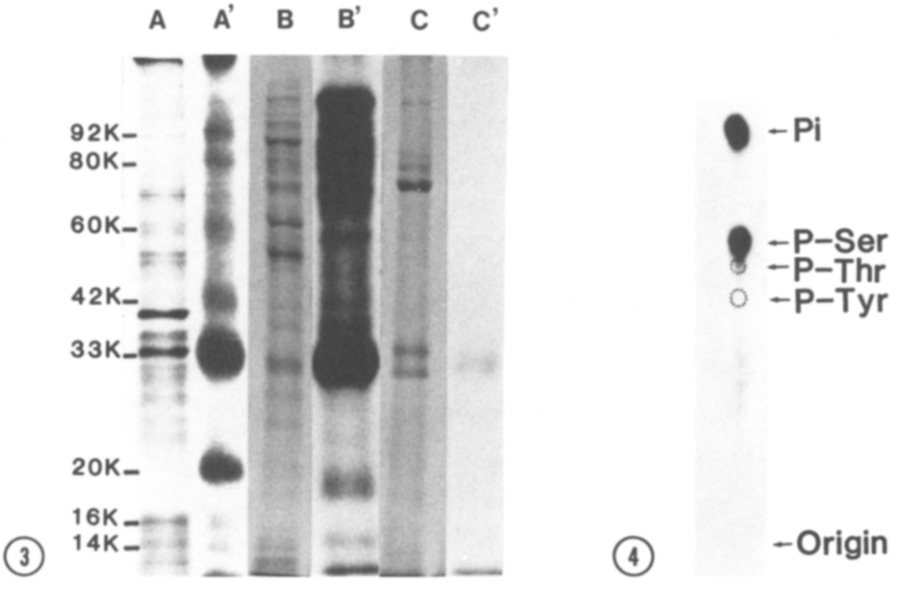

Fig. 3. Endogenous phosphorylation of the complex(60 μg protein) obtained from the cytosol fractions of AH-66 cells, M1 cells and normal liver. The gel was stained with Coomassie blue(A-C), and then autoradiographed(A'-C'): A and A', AH-66 cells; B and B', M1 cells; C and C', normal liver.

Fig. 4. Identification of the phosphorylated amino acid redidue in the protein kinase-substrate complex of AH-66 cytosol.

DISCUSSION

Protein kinases, which catalyze the phosphorylation of casein as exogenous substrate and are not stimulated either cyclic AMP, Ca^{2+}-calmodulin, or Ca^{2+}-phospholipid, are classified as casein kinases. Since the protein kinase in the complex precipitated with Ca^{2+} from AH-66 cytosol is able to phosphorylate casein as exogenous substrate without stimulation by cyclic nucleotides, Ca^{2+}-calmodulin, and Ca^{2+}-phospholipid, it seems to be either casein kinase 1 or 2. Although heparin had no effect on the endogenous phosphorylation of the complex, it gave a slight inhibition on the phosphorylation activity when casein was used as exogenous substrate. We interpret this result that heparin does not affect the phosphorylation of endogenous substrate of the protein kinase in the complex but inhibits the phosphorylation of casein. It seems, therefore, that the protein kinase has properties of casein kinase 2 rather than casein kinase 1. Purification of protein kinase from the complex is underway.

The physiological significance of the protein kinase-substrate complex precipitable with Ca^{2+} from cytosol is unknown at present. It is, however, remarkable that the content and phosphorylating activity of the protein kinase-substrate complex in tumor cells such as AH-66 cells and Ml cells were markedly higher than those of normal cells and regenerating cells. These results suggest the possibility that the protein kinase-substrate complex and its phosphorylation may be related to tumor formation. Further accumulation of data on the content and phosphorylating activity of the complex in various tumor cells other than those examined in the present study will be necessary to evaluate the physiological significance of the complex.

REFERENCES

1. Nakaya, K., Shimizu, T., Segawa, Y., Nakajo, S. and Nakamura, Y. (1983) Cancer Res. 43, 3778-3782.
2. Nakajo, S., Shimizu, T., Shinkawa, K., Nakaya, K. and Nakamura, Y.(1983) Biochem. Biophys. Res. Commun. 113, 1010-1017.
3. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341-13348.

4. Laemmli, U. K. (1970) Nature 227, 680-685.
5. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.
6. Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. and Means, A. R. (1977) J. Biol. Chem. 252, 8415-8422.
7. Nakaya, K., Sakagami, H., Takeda, M., Konno, K. and Nakamura, Y. (1984) Biochemistry Int. 8, 313-318.
8. Hunter, T. and Sefton, B. M. (1980) Proc. Natl. Acad. Sci. USA 77, 1311-1315.
9. Hathaway, G. M., Lubben, T. H. and Traugh, J. A. (1980) J. Biol. Chem. 255, 8038-8041.