Microsomal Ca^{2+}- and phospholipid-dependent protein kinase

Identification and in vitro binding studies

Marianne Sommarin and Bengt Jergil*

Biochemistry, Chemical Centre, POB 740, S-220 07 Lund, Sweden

Received 19 April 1983

A Ca^{2+}- and phospholipid-dependent protein kinase (CaPK) has been identified in rat liver microsomes. CaPK isolated from liver cytosol bound to smooth microsomes in the presence of 100 μM $CaCl_2$. A saturation in binding was observed when a 5-fold excess of enzyme over that present in microsomes had become bound. The microsomal CaPK and 50% of the enzyme bound in vitro was not removed by EGTA treatment. This suggests that Ca^{2+} is required for the binding of CaPK to microsomes, but not for the retention of the enzyme on the membrane.

Ca^{2+} *Liver microsome* *Membrane binding* *Phospholipid* *Protein kinase*

1. INTRODUCTION

The Ca^{2+}- and phospholipid-dependent protein kinase [1] (CaPK) is present in particulate and soluble fractions of various types of cells [2]. In [3] the enzyme was activated by membrane fractions in the presence of Ca^{2+}, and it was inferred, but not directly shown, that the enzyme would bind to membranes. Later work suggests that CaPK bound to membranes cannot be detected unless the enzyme is solubilized first [2,4].

In [4] we have devised a method for measuring CaPK in membrane fractions. About 2/3rds of the total enzyme activity in rat liver was found in particulate material, and >90% of the bound enzyme was present in microsomes [4]. It was not shown conclusively, however, that the bound enzyme was phospholipid-dependent, since phospholipids were present in the membrane extracts analyzed. Here we show that the microsomal enzyme is indeed CaPK. We have also examined some factors required for the enzyme to bind to microsomal membranes in vitro. Such model studies may be of interest in view of the rapid redistribution of CaPK between membranes and the soluble fraction detected in [5], suggesting a strict control of CaPK–membrane interactions.

* To whom correspondence should be addressed

2. EXPERIMENTAL

2.1. *Preparation of microsomes*

Rat liver smooth microsomes were prepared as in [6] with the following modifications: the buffer used was 10 mM Tris–HCl (pH 7.4); $MgCl_2$ was omitted; 1 mM EDTA and 0.5 mM EGTA were present in the homogenization medium and the sucrose gradient. Smooth microsomes for DEAE–Sephacel chromatography were used immediately, whereas those to be used for binding studies were stored at −70°C.

2.2. *DEAE–Sephacel chromatography*

Smooth microsomes were solubilized in 1% Triton X-100 and chromatographed on DEAE–Sephacel (Pharmacia) in the presence of 0.25% Triton X-100 [6].

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00 © Federation of European Biochemical Societies

2.3. *Preparation of CaPK*

CaPK was partially purified from the cytosol by DEAE–Sephacel chromatography [6] performed in the presence of 10^{-4} M cyclic AMP. The CaPK obtained was virtually free from cyclic AMP-dependent protein kinase as judged by its very slow phosphorylation of the peptide Leu–Arg–Arg–Ala–Ser–Val–Ala (provided by Dr Ö. Zetterqvist, Uppsala). The specific activity with histone H1 as substrate was 1–2 nmol phosphate incorporated.min^{-1}.mg $protein^{-1}$ in the presence of $CaCl_2$ and phosphatidylserine. The enzyme was stimulated >20-fold by $CaCl_2$ in the presence of phosphatidylserine.

2.4. *Analyses*

CaPK was analyzed in the presence of octylglucoside [4]. When Triton was present (DEAE–Sephacel eluate) it was ⩽0.0125% in the analysis mixture, the concentration of phosphatidylserine was doubled and octylglycoside omitted. Protamine kinase was analyzed as in [7]. Protein was determined [8] with bovine serum albumin as standard.

2.5. *Binding studies*

Smooth microsomes (~0.5 mg microsomal protein) were incubated at 0°C with CaPK (~0.15 mg) and $CaCl_2$ (or other additions) in 250 μl total vol. After 45 min, 750 μl 10 mM Tris–HCl (pH 7.4) was added and the samples centrifuged at 105000 × *g* for 60 min. Pellets were suspended in 300 μl Tris buffer. The protease inhibitors leupeptin (2.5 μg/ml), pepstatin (1 μg/ml) and phenylmethylsulfonyl fluoride (350 μg/ml) were present in all solutions.

3. RESULTS

3.1. *Identification of CaPK in microsomes*

A microsomal extract was chromatographed on DEAE–Sephacel, and the effluent was analyzed for protamine kinase and CaPK activities (fig.1). One peak of CaPK activity, coincident with the major protamine kinase peak, eluted at 0.14 M KCl. This enzyme was stimulated 8–10-times by $CaCl_2$ in the presence of phosphatidylserine using histone H1 as substrate, whereas it was virtually inactive in the absence of phospholipid. The specific activity was 4 nmol phosphate incorporated/mg protein. The recovery of CaPK on chromatography was quantitative, indicating that the Ca^{2+}-dependent activity of the extract represented CaPK rather than other Ca^{2+}-dependent protein kinases.

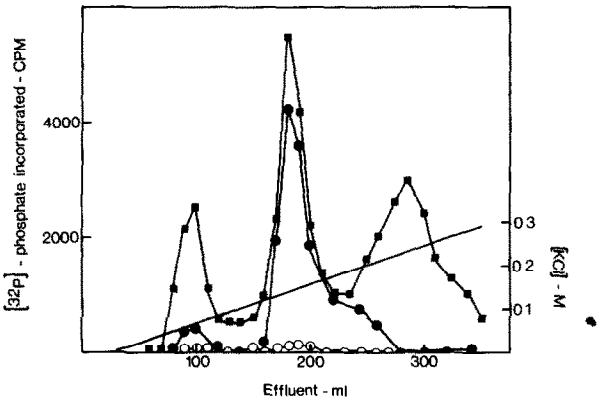

Fig.1. DEAE–Sephacel chromatography of a Triton X-100 extract of smooth microsomes. The phosphorylation of histone H1 was measured in the presence (●) and absence (○) of $CaCl_2$ and phosphatidylserine: (■) protamine phosphorylation; the specific activities of [γ-^{32}P]ATP were 235000 and 23000 cpm/nmol in the histone H1 and protamine phosphorylations, respectively.

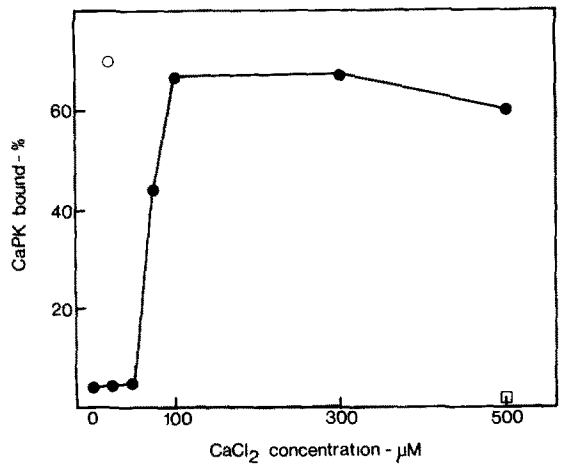

Fig.2. The binding of CaPK to smooth microsomes at different [$CaCl_2$]. CaPK bound is expressed as % of recovered enzyme activity. The recovery of CaPK was around 140% up to 75 μM $CaCl_2$, 100% at 100 μM $CaCl_2$, and 50–60% above 100 μM $CaCl_2$: (○) 25 μM $CaCl_2$ in a 4-fold increased volume of the enzyme–membrane binding mixture; (□) 500 μM $MgCl_2$ instead of $CaCl_2$.

The two protamine kinase peaks eluting before and after CaPK constitute the catalytic subunit and the type II holoenzyme of cyclic AMP-dependent protein kinase, respectively [6].

3.2. *Binding of CaPK to microsomes*

The binding of partially purified CaPK to smooth microsomes was tested at various [$CaCl_2$] (fig.2). Microsomes prepared in the presence of chelators to remove bound Ca^{2+} did not bind CaPK unless $CaCl_2$ was added. Maximum binding occurred at 100 μM $CaCl_2$. About 70% of the recovered kinase activity (in other experiments up to 90%) then sedimented with the microsomes. The same high degree of binding was obtained at 25 μM $CaCl_2$ when the volume of the incubation mixture for binding was increased 4-fold keeping the amount of microsomes constant (○). No binding of CaPK occurred at 500 μM $MgCl_2$ (□) unless $CaCl_2$ was present. Smooth microsomes prepared in the absence of chelators bound CaPK to a certain extent (20–30%) without addition of $CaCl_2$.

Diolein enhances the stimulatory effect of phospholipids on CaPK activity at low [Ca^{2+}] [9] and has therefore been invoked in

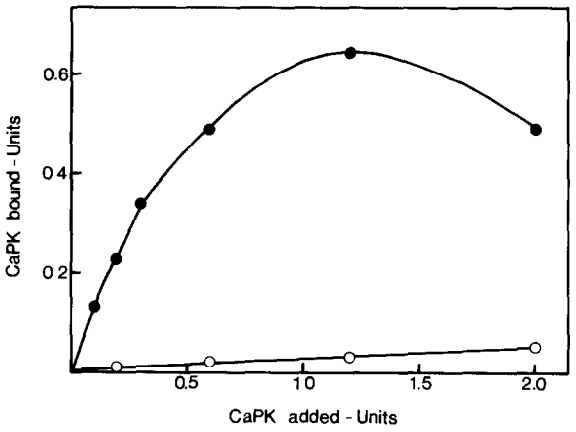

Fig.3. Maximum binding of CaPK to smooth microsomes. Various amounts of CaPK were added to smooth microsomes (0.3 mg membrane protein) in presence (●) or absence (○) of 0.5 mM $CaCl_2$, and in 1.0 ml total vol. The mixture was incubated, centrifuged without further dilution, and analyzed for CaPK activity as in section 2. One unit is the amount of enzyme which catalyzes the incorporation of 1 nmol phosphate into histone H1/min at 22°C.

CaPK–membrane interactions [10]. Added diolein (40 μg/ml incubation mixture) or intrinsic diacylglycerol generated in the membrane by hydrolysis of exposed phosphatidylinositol by a phospholipase C [11,12] did not affect the binding of CaPK to smooth microsomes at various [Ca^{2+}]. Thus, diolein does not seem to influence the binding of CaPK to microsomes.

3.3. *Extent of CaPK binding to microsomes (fig.3)*

When smooth microsomes were incubated with progressively more CaPK in the presence of $CaCl_2$ a maximum of ~5-times more enzyme bound than is usually found in the membrane [4]. There was no appreciable binding in the absence of $CaCl_2$ (○).

4. DISCUSSION

Our results show that the Ca^{2+}-dependent protein kinase in microsomes also is phospholipid-dependent. CaPK has not been identified unequivocally in microsomes previously. In view of the abundance of the enzyme in microsomes [4], these are the major source of CaPK in rat liver cells.

We have earlier isolated and partly characterized both the microsomal and soluble CaPK as protamine-phosphorylating enzymes [6]. They had all properties examined in common, suggesting that CaPK does not undergo substantial changes when it becomes associated with membranes. The microsomal enzyme appeared located on the cytosolic side, and was released only by solubilization of the membrane.

The rapid translocation of CaPK from cytosol to plasma membranes in parietal yolk sac cells treated with phorbol esters [5] suggests that the binding of CaPK to membranes is under physiological control. However, nothing is known about membrane factors involved in this binding. The activation of soluble CaPK by Ca^{2+}, phospholipids and diolein [1–3,9] suggests that these factors may participate.

Our study shows that Ca^{2+} is required for binding in vitro, although this occurred at a much higher [$CaCl_2$] than that present in the cytosol in vivo. However, the [Ca^{2+}] at the membrane surface in vivo may be rather high. The binding of CaPK to smooth microsomes prepared without

chelators suggests that sites with high affinity for Ca^{2+} are involved. Removal of Ca^{2+} with EGTA does not cause the release of CaPK from microsomes [4]. Also about 50% of the enzyme bound in our studies was not released by EGTA. This indicates that the binding of CaPK to microsomes is Ca^{2+}-dependent, but that its retention in the membrane is Ca^{2+}-independent (presupposed Ca^{2+} was removed by EGTA).

If the binding in vitro occurs to specific sites (presumably involving proteins and/or phospholipids) it is expected to reach saturation at a level of CaPK not far exceeding that found in isolated membranes. The maximum amount of CaPK bound was 5-times that present in smooth microsomes. Since 50% of the bound CaPK was resistant to EGTA, a 2–3-fold excess of CaPK had become attached irreversibly. The binding of CaPK was not affected appreciably by prior treatment of microsomes with trypsin (10–20% decrease). Whether the CaPK attached irreversibly in vitro and the enzyme present in isolated membranes are bound in the same manner remains to be elucidated.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Natural Science Research Council. We are grateful to Ms Ann-Christin Nilsson for drawing the figures.

REFERENCES

[1] Takai, Y., Kishomoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695.
[2] Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) Proc. Natl. Acad. Sci. USA 77, 7039–7043.
[3] Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., Nishizuka, Y., Tamura, A. and Fujii, T. (1979) J. Biochem. 86, 575–578.
[4] Jergil, B. and Sommarin, M. (1983) Biochim. Biophys. Acta, in press.
[5] Kraft, A.S. and Anderson, W.B. (1983) Nature 301, 621–623.
[6] Sommarin, M. and Jergil, B. (1978) Eur. J. Biochem. 88, 49–60.
[7] Jergil, B. (1972) Eur. J. Biochem. 28, 546–554.
[8] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R. (1951) J. Biol. Chem. 193, 265–275.
[9] Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979) Biochem. Biophys. Res. Commun. 91, 1218–1224.
[10] Nishizuka, Y. (1983) Trends Biochem. Sci. 8, 13–16.
[11] Sundler, R., Alberts, A.W. and Vagelos, P.R. (1978) J. Biol. Chem. 253, 4175–4179.
[12] Jergil, B. and Sundler, R. (1983) J. Biol. Chem., in press.