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Identification of a 80 kDa calmodulin-binding protein as a new Ca^{2+}/calmodulin-dependent kinase by renaturation blotting assay (RBA)

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We surveyed rabbit brain cytosol for a new Ca^{2+}/calmodulin (CaM)-dependent kinase. The renaturation blotting assay (RBA) exploits the ability of blotted SDS-denatured proteins to regain enzymic activity after guanidine treatment. Using RBA, we found that the eluate of rabbit brain cytosol from a CaM affinity column contains at least four electrophoretically distinct protein kinase bands which were autophosphorylated in a Ca^{2+}/CaM-dependent manner. The 49 kDa band and the 60 kDa band were α and β subunit of CaM kinase II, and the 42 kDa band was presumed to be CaM kinase I, but the 80 kDa band could not be attributed to any reported Ca^{2+}/CaM-dependent protein kinases. The 80 kDa protein kinase was isolated by three-step chromatography. We examined the phosphorylation of exogenous substrates by 80 kDa protein kinase, and histone IIIs and myosin light chain were phosphorylated in a Ca^{2+}/CaM-dependent manner. W-7, a specific inhibitor for calmodulin, inhibited this kinase activity, but KN-62, a specific inhibitor for CaM kinase II, had no effect on this protein kinase activity. Autoradiography using boiled rabbit brain homogenate as substrate showed three intrinsic substrates (80 kDa, 60 kDa and 42 kDa), which were phosphorylated in a Ca^{2+}/CaM-dependent manner. These findings suggest that a new Ca^{2+}/CaM-dependent protein kinase could be identified by the RBA.

INTRODUCTION

Calmodulin (CaM) is the major Ca^{2+}-binding protein in most non-muscle cells and plays a central role in processing of the intracellular Ca^{2+} signal [1]. Several CaM-dependent protein kinases have been demonstrated to date, and protein phosphorylation is considered one of the important routes by which Ca^{2+}/CaM signal transduction regulates cellular function. It is known that a family of Ca^{2+}/CaM-dependent protein kinases includes Ca^{2+}/CaM-dependent protein kinase I [2], II [3] and III [4], myosin light-chain kinases (MLCK) [5] and phosphorylase kinase [6]. It has been reported that histone H3 kinase in the calf thymus is activated by Ca^{2+}/CaM [7], and there remains the possibility that there are more kinds of Ca^{2+}/CaM-dependent protein kinases which have not been identified. In 1989, Ferrell & Martin [8] observed 11 unidentified protein kinases in platelets using a renaturation blotting assay (RBA), which can detect the autophosphorylation of protein kinases from crude samples. We set out to determine whether this method is available for detection of new Ca^{2+}/CaM-dependent protein kinases, because most Ca^{2+}/CaM-dependent protein kinases except for MLCK are autophosphorylated.

In this paper, we report two unidentified protein kinases in the eluate from CaM affinity chromatography and partially characterize the 80 kDa Ca^{2+}/CaM-dependent protein kinase.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (6000 Ci/mmol) was purchased from ICN, Tokyo, Japan. Histone IIIs and phosvitin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Myosin light chain was prepared from chicken gizzard by the method of Perrie & Perry [9]. Myelin basic protein was prepared from rabbit brain [10]. Casein was purchased from Wako Pure Chemical Industries, Tokyo, Japan CaM kinase II was isolated from rat brain [11]. CaM was purified from bovine brain [12]. W-7 [13], KN-62 [14] and ML-9 [15,16] were synthesized as previously described. The prepared calmodulin (25 mg) was coupled to CNBr–Sepharose by the manufacturer's protocol (Pharmacia LKB Biotechnology, Uppsala, Sweden) (1.2 mg of CaM/ml of CNBr–Sepharose 4B suspension).

RBA

Electrophoresis. Sample buffer contains 11.5% (w/v) SDS, 25% (v/v) 2-mercaptoethanol, 25 mM-EDTA, 312.5 mM-Tris (pH 6.8) and 8 M-urea. Each sample was mixed with $\frac{1}{4}$ vol. of sample buffer and subjected to 10%-polyacrylamide-gel electrophoresis.

Blotting. Proteins were transferred to PVDF (Immobilon P; Millipore, MA, U.S.A.; 0.45 μm pore size) membranes in 192 mM-glycine/25 mM-Tris at 15 V for 60 min.

Guanidine treatment and renaturation. Blots were incubated for 1 h at room temperature with gentle rocking in 7 M-guanidine hydrochloride/50mM-Tris/50mM-dithiothreitol/2mM-EDTA/0.1% (v/v) Triton X-100 (pH 8.3). Enzymes on the guanidine-treated blots were allowed to renature in 50 mM-Tris/2 mM-dithiothreitol/2 mM-EDTA/0.1% Tween-20/1% casein (pH 7.5) for 12 h at 4 °C. Blots were then blocked with 5% casein in 30 mM-Tris (pH 7.5) at room temperature for 1 h. Ferrell & Martin [8] used bovine serum albumin for the blocking buffer, but in the present study casein was more effective for detection of CaM kinase II.

Phosphorylation on blotting membrane. Blocked blots were incubated in 35 mM-Hepes/10 mM-$MgCl_2$/1 mM-$CaCl_2$/0.1 μM-CaM (pH 8.0) plus 50 μCi of [γ-^{32}P]ATP/ml at room temperature for 30 min. Kinase reactions were terminated by soaking the blots in phosphate-buffered saline (PBS). Blots were washed three times with PBS, each time for 10 min at room temperature. Then the blots were incubated in 1 M-KOH for 10 min at room

Abbreviations used: CaM, calmodulin; RBA, renaturation blotting assay; MLCK, myosin light-chain kinase.
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temperature, and rinsed five times with PBS, dried and subjected to autoradiography.

Chromatography

All procedures were carried out at 4 °C unless otherwise indicated.

Buffers. Buffer A contained 40 mM-Tris/HCl, 0.1 mM-EGTA and 10 μg of leupeptin/ml (pH 7.5). Buffer B contained 40 mM-Tris/HCl, 0.2 M-NaCl, 1 mM-$CaCl_2$ and 10 μg of leupeptin/ml (pH 7.5). Buffer C contained 40 mM-Tris/HCl, 0.2 M-NaCl, 2 mM-EGTA, 1 mM-EDTA and 10 μg of leupeptin/ml (pH 7.5).

Isolation of 80 kDa Ca^{2+}/CaM-dependent protein kinase

Rabbit brain (50 g) was homogenized with 5 vol. of Buffer A in a Teflon/glass homogenizer at 1000 rev./min with 10 up-and-down strokes. The homogenate was centrifuged at 100000 *g* for 30 min.

As the preliminary study showed that these autophosphorylated bands were absorbed by the DEAE-cellulose column and eluted with higher concentrations than 0.2 M-NaCl, we applied the tissue extract to the DEAE-cellulose column (3 cm × 25 cm) and the proteins were eluted with a linear gradient of 0–0.4 M-NaCl in Buffer A. Then the fractions between 0.2 M- and 0.4 M-NaCl were collected (100 ml) and applied to the dialysis bag and incubated in polyethylene-glycol powder to concentrate the protein for 6 h. The concentrated eluate (10 ml) was subjected to gel-filtration column chromatography (CL-4B; 3 cm × 100 cm). $CaCl_2$ was added to the active fraction to give a final concentration of 2 mM, and the fraction was applied to CaM–Sepharose affinity resin that had been equilibrated in Buffer B. After washing the column with Buffer B, the kinase was eluted with Buffer C.

Kinase assay. Kinase activity was determined in a standard 5 min assay (200 μl), containing 35 mM-Hepes (pH 8.0), 10 mM-$MgCl_2$, 1 mM-$CaCl_2$, 0.1 μM-CaM and 10 μM-[γ-^{32}P]ATP (500–2000 c.p.m./pmol) at 30 °C. The kinase reaction was halted by adding 1 ml of 20% (v/v) trichloroacetic acid as described in [16].

Intrinsic substrate

The rabbit brain was homogenized in 10 vol. of Buffer A and boiled at 90 °C for 1 min to eliminate intrinsic kinase activities. Then the kinase assay was carried out with this preparation as a substrate. The kinase reaction was halted by adding 40 μl of SDS-sample buffer, and phosphorylated proteins were identified by autoradiography.

RESULTS

CaM-binding protein kinases in the rabbit brain cytosol

Firstly, we attempted to identify the CaM-binding protein kinases in the rabbit brain cytosol. The 100000 *g* supernatant was submitted to CaM-affinity chromatography, and the Ca^{2+}-dependent CaM-binding proteins were eluted with buffer C.

Fig. 1 shows the result of RBA of the eluate from the CaM–Sepharose column. The eluate contained five phosphorylated bands, of 90 kDa, 80 kDa, 60 kDa, 49 kDa and 42 kDa, and the phosphorylation of the 80 kDa, 60 kDa, 49 kDa and 42 kDa bands was increased in the presence of Ca^{2+}/CaM, which suggests that these phosphorylations are Ca^{2+}/CaM-dependent. The 60 kDa and the 49 kDa bands should be CaM kinase II, and the 42 kDa band was presumed to be CaM kinase I. The 80 kDa

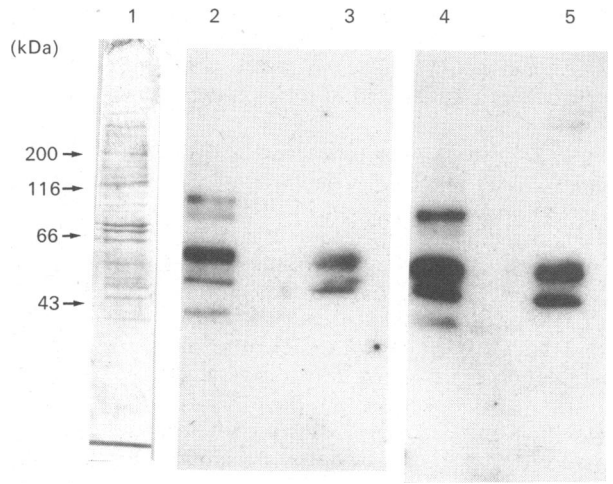

Fig. 1. RBA of the CaM-binding protein in rabbit brain

Rabbit brain (5 g) was homogenized with 20 ml of Buffer B in a Teflon/glass homogenizer at 1000 rev./min with 10 up-and-down strokes. The homogenate was centrifuged at 100000 *g* for 30 min, and the supernatant was applied to the CaM–Sepharose column (1 cm × 5 cm). Ca^{2+}-dependent CaM-binding proteins were eluted with Buffer C. Then the eluate was subjected to SDS/PAGE (10%-acrylamide), transferred to a PVDF membrane and analysed by RBA. Lane 1: SDS/PAGE of the eluate, stained with Coomassie Blue. Lanes 2 and 3 were overlaid with [γ-^{32}P]ATP in the presence of 10 mM-EGTA. Lanes 4 and 5 were overlaid with [γ-^{32}P]ATP in the presence of 1 mM-$CaCl_2$ and 0.1 μM-CaM. Lanes 2 and 4, eluate; lanes 3 and 5, CaM kinase II from rat brain for the control.

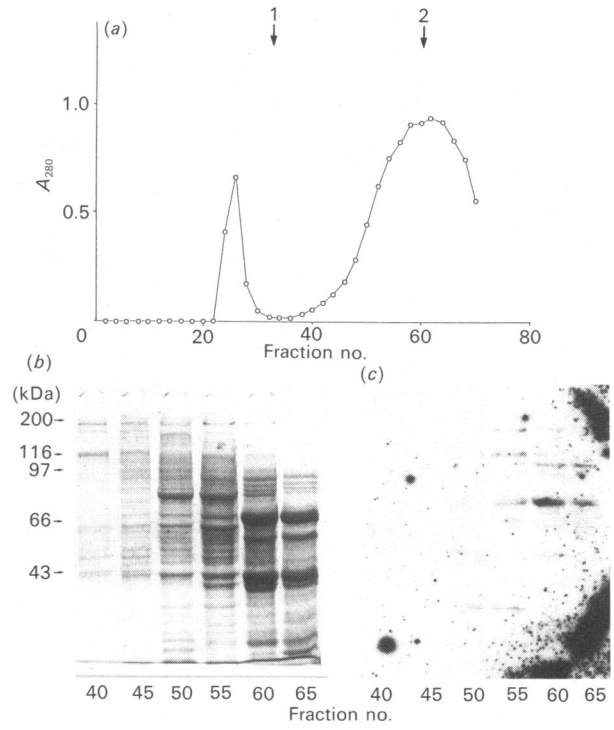

Fig. 2. Sepharose CL-4B gel-filtration chromatography of the 80 kDa kinase

Flow rate was 25 ml/h and each fraction was collected into a 10 ml tube. (*a*) The elution positions of standard proteins were determined in separate runs by their A_{280} and are indicated by arrows. Standard proteins were: (1) thyroglobulin (660 kDa); (2) aldolase (160 kDa). (*b*) SDS/PAGE stained with Coomassie Blue. (*c*) RBA of each fraction. From fractions 55–65, 80 kDa phosphorylated bands appeared, and these fractions were applied to CaM-affinity chromatography.

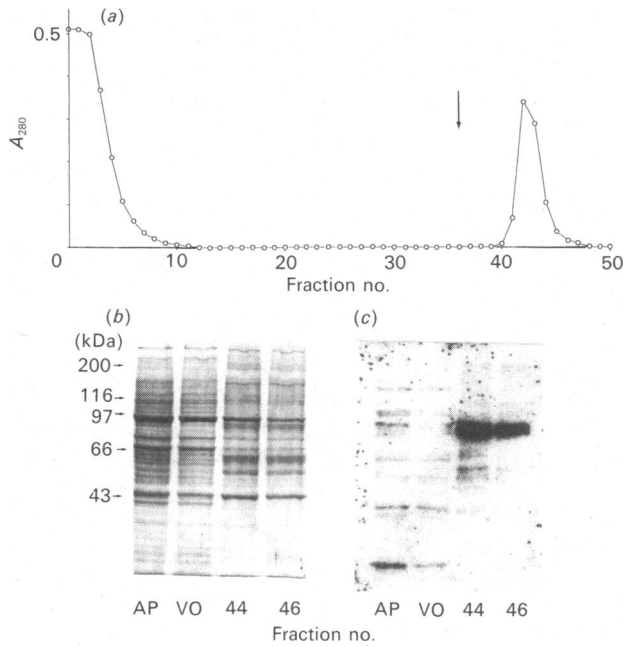

Fig. 3. CaM-coupled Sepharose 4B chromatography of the 80 kDa kinase

In (*a*), from fraction 38, the buffer was changed to Buffer C, and bound protein was eluted from fraction no. 42. (*b*) SDS/PAGE stained with Coomassie Blue. (*c*) RBA of each fraction. Abbreviations: AP, applied sample; VO, void-volume fraction.

Table 1. Partial purification of new Ca^{2+}/CaM-dependent kinase

Fraction	Volume (ml)	Protein (mg)	Activity (pmol/min)	Specific activity (pmol/min per mg)
100000 *g* supernatant	200	1660	1943	1.17
DEAE-cellulose	100	310	1982	6.39
Gel filtration	100	59	482	8.17
CaM-affinity	4	2.1	59.1	28.1

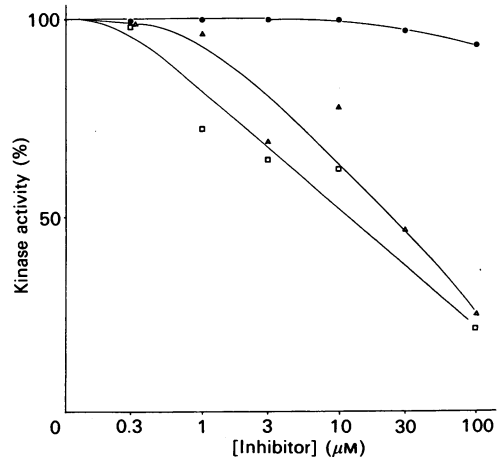

Fig. 4. Effects of W-7 (□), ML-9 (△) and KN-62 (●) on 80 kDa kinase activity

Each compound was dissolved in dimethyl sulphoxide and added to the assay mixture.

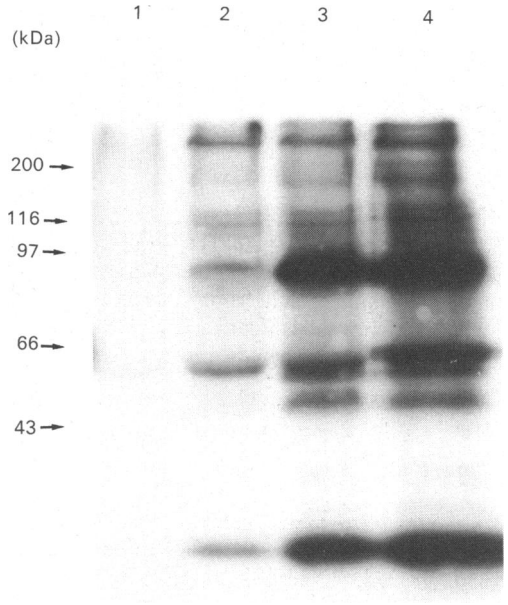

Fig. 5. Intrinsic substrates of the 80 kDa kinase in the rabbit brain cytosol

Each lane contains 20 μl of boiled rabbit brain homogenate as substrate, and 20 μl of enzyme fraction was added to lanes 2, 3 and 4. Lane 1: absence of enzyme. Lane 2: instead of $CaCl_2$, 10 μM-EGTA was added to the assay mixture. Lane 3: presence of 1 mM-$CaCl_2$ but absence of CaM. Lane 4: presence of 1 mM-$CaCl_2$ and 0.1 μM-CaM. The incubations were carried out for 5 min at 30 °C and terminated by adding SDS sample buffer containing 8 M-urea. Then they were applied to the 10%-polyacrylamide slab gel and SDS/PAGE was carried out. Phosphorylated proteins were detected by autoradiography.

band could not be attributed to any reported Ca^{2+}/CaM-dependent protein kinase. Thus we tried to purify the 80 kDa protein kinase and characterize it.

Isolation of 80 kDa Ca^{2+}/CaM-dependent protein kinase

After three-step column chromatography, the 80 kDa phosphorylated band was separated from four other phosphorylated bands on RBA.

RBA after gel-filtration chromatography (Fig. 2*c*) shows 80 kDa autophosphorylated bands from fraction nos. 55–65, and the molecular mass was estimated to be 160 kDa. These fractions were applied to the CaM–Sepharose column, and the Ca^{2+}-dependent CaM-binding proteins were eluted with 2 mM-EGTA. RBA revealed the concentrated 80 kDa autophosphorylated band (Fig. 3*b*). The purification procedures are summarized in Table 1, with histone IIIs as an exogenous substrate.

Substrate specificity

We examined whether this enzyme fraction can phosphorylate several exogenous substrates. Histone IIIs (305 pmol of P/min per mg of substrate) and myosin light chain (368 pmol of P/min per mg of substrate) were phosphorylated in a Ca^{2+}/CaM-dependent manner. But there was no incorporation of ^{32}P into casein, phosvitin nor myelin basic protein. From the view of substrate specificity, this result supported our finding that this enzyme is different from CaM kinase II or MLCK.

Effects of various inhibitors on kinase activity

Fig. 4 shows the effects of three specific inhibitors on this kinase activity, with histone IIIs as a substrate. KN-62, a specific inhibitor for the CaM kinase II, had no effect on this kinase

activity. ML-9, a specific inhibitor for MLCK, weakly inhibited the kinase activity, with 50% inhibition estimated at 20 μM. W-7, a specific inhibitor for calmodulin, also inhibited the activity, with 50% inhibition at approx. 8 μM. This value is almost the same as that for CaM kinase II [14].

Intrinsic substrates

Fig. 5 shows that three bands (80 kDa, 60 kDa, 43 kDa) were phosphorylated in a Ca^{2+}/CaM-dependent manner. The 80 kDa phosphorylated band may result from autophosphorylation of this kinase itself, which was also detected by RBA. The 60 kDa and 43 kDa proteins should be the intrinsic substrate of this kinase.

DISCUSSION

Ferrell & Martin [8] demonstrated that the SDS-denatured protein kinases could re-acquire kinase activity on the blot after treatment with guanidine, and, if overlaid with [γ-^{32}P]ATP, radiolabel is incorporated into various regions of the blot. They decided that the radiolabel represents phosphate residues transferred from the γ-position of ATP to serine and threonine residues, and the proteins responsible for this transfer are protein kinases or catalytic subunits of oligomeric protein kinase holoenzymes. They did not determine whether these phosphorylated residues are phosphorylated albumin, used as a blocking protein, or autophosphorylated protein kinases.

We changed several kinds of proteins in the renaturation buffer and the blocking buffer to determine the best conditions for renaturation of Ca^{2+}/CaM-dependent protein kinases. Casein renatured them most efficiently, but albumin, histone IIIs and protamine did also; the number and the estimated molecular masses of the phosphorylated residues appearing on autoradiography were unchanged when the blocking protein was changed (results not shown). The 80 kDa kinase could not phosphorylate casein in the tube, but the phosphorylated residue appeared in RBA using casein as the blocking protein. Moreover, MLCK, which has little autophosphorylation, could not be detected by RBA at any time. These findings suggest that the phosphorylated residues appearing on RBA represent autophosphorylation of the protein kinases.

Since RBA could reveal the Ca^{2+}/CaM-dependent autophosphorylation of each protein kinase, we overlaid the renatured blots with ^{125}I-labelled CaM and found that the CaM-binding sites were also renatured and bound with ^{125}I-CaM in a Ca^{2+}-dependent manner (results not shown). These data showed us that the 80 kDa protein has a CaM-binding site as well as an ATP-binding site. Among the Ca^{2+}/CaM-dependent protein kinases, the reported molecular mass of the holoenzyme is as follows: CaM kinase I, 42400 Da [2]; CaM kinase II, 56000 Da [3]; CaM kinase III, 140000 Da [4]; phosphorylase kinase, 1340000 Da [6]; MLCK, 130000 Da [5]. And molecular masses of the catalytic subunits on SDS/PAGE are as follows: CaM kinase I, 37 kDa, 39 kDa, 42 kDa [2]; CaM kinase II, 60 kDa, 49 kDa [3]; CaM kinase III, unknown [4]; phosphorylase kinase, 145 kDa, 128 kDa [6]; MLCK, 130 kDa [5]. The molecular mass of the 80 kDa kinase that we found was estimated to be 160000 Da on CL-4B gel-filtration chromatography, and the catalytic subunit appeared as an 80 kDa band on RBA.

Our 80 kDa protein kinase phosphorylated smooth-muscle myosin light chain and histone IIIs and did not phosphorylate casein or myelin basic protein, both of which are good substrates for CaM kinase II. On the other hand, phosphorylase kinase and MLCK have narrow substrate specificities and cannot phosphorylate histone IIIs. CaM kinase I also cannot phosphorylate smooth-muscle myosin light chain. CaM kinase III is reported as a specific protein kinase for elongation factor 2. Moreover, KN-62, a specific inhibitor for CaM kinase II, did not affect the kinase activity. These findings suggest that this 80 kDa kinase is different from already known Ca^{2+}/CaM-dependent protein kinases.

One of the great benefits of RBA is that the molecular mass of the kinase or the catalytic subunit of the kinase can be decided before the purification, and one can detect the target enzyme from a crude sample. Besides, since many samples can be examined at once by using slab gels, RBA can replace the usual fraction assay. Especially when a good substrate is unknown or not available, there are even more advantages from the use of RBA.

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