Subcellular distribution of α and β subunit proteins of Ca^{2+}/calmodulin-dependent protein kinase II expressed in Chinese hamster ovary cells

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When two cDNAs respectively encoding the entire coding regions of α and β subunits of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) were introduced into Chinese hamster ovary cells, the expressed α and β subunits were differently associated with subcellular structure. Although α subunit was loosely associated with subcellular structure, about 80% of CaM kinase II acitivity of α subunit was found in soluble fraction. More than 50% of the β subunit bound to the membrane, and the remainder was soluble but was loosely associated with subcellular structure. The relative rate of phosphorylation for substrate proteins of the β subunit bound to membrane was significantly different from that of the soluble form.

Calcium ion; Calmodulin; Protein kinase; Protein phosphorylation; Subcellular distribution; Expression of cDNAs

1. INTRODUCTION

Since Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) was first demonstrated by gel filtration of brain calmodulin-dependent protein kinases [1], there have been many reports concerning the properties of CaM kinase II, and the physiological importance of CaM kinase II has been increasingly recognized (for a review, see [2,3]). CaM kinase II is expressed in great quantities in brain neurons and is the most abundant protein kinase in mammalian brain [2–6]. CaM kinase II is composed of two distinct but related protein subunits of 50 kDa (α) and 60/58 kDa (β/β). They are the products of highly homologous transcription units, with amino acid insertions at the carboxyl-terminal side of calmodulin binding domain accounting for the larger size of β subunit [7,8]. There is a variation in the α and β subunit compositions of the kinase in different regions of adult rat brain [9–12]. CaM kinase II exists in both soluble and particulate fractions [3,13–15]. However, it is not clear that the association of the two subunits in a variety of ratios is correlated to the association of CaM kinase II with subcellular structure in neuronal cells. We have recently reported the ligation of the α and β cDNAs encoding the entire coding regions of α and β subunits of CaM kinase II into an expression vector and their introduction into Chinese hamster ovary (CHO) cells, demonstrating that the α and β subunits exhibit enzyme activity individually and that basic properties of expressed enzyme are comparable to brain CaM kinase II [16].

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In the present study, we demonstrated that the α and β subunits associated differently with subcellular structure in CHO cells transfected with α and β cDNAs separately or simultaneously, suggesting that the α and β subunit composition of CaM kinase II is important to the association of the kinase with subcellular structure in vivo.

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP was purchased from Du Pont-New England Nuclear. ^{125}I-Labeled antimouse IgG (Fab') was from Amersham International. Stable cell lines transfected with α and/or β cDNAs and specific antibody against brain CaM kinase II were prepared as described previously [16]. The CaM kinase II substrate peptide syntide 2 (Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys) [17] was synthesized by the solid-phase method using a model 430A Applied Biosystems peptide synthesizer.

2.2. *Assay of CaM kinase II*

CaM kinase II activity was assayed by phosphorylation of syntide 2 or protein substrate [16,17]. Reaction mixture contained 50 μM [γ-^{32}P]ATP, 8 mM $Mg(CH_3COO)_2$, 20 μM syntide 2 or indicated amounts of protein substrate, 2 μM calmodulin, 0.2 mM $CaCl_2$, 0.1 mM EGTA, 50 mM Hepes buffer, pH 8.0, and suitable amounts of enzyme. Control reaction was carried out in the presence of 1 mM EGTA.

2.3. *Preparation of soluble and particulate fractions*

The transfected cells were cultured to confluency in F-12 medium supplemented with 10% fetal bovine serum in 10 cm dish. The cells were washed with 10 ml of phosphate-buffered saline, scraped by a rubber policeman, and collected by centrifugation at 170 × *g* for 5 min. The cell pellet was suspended in 0.2 ml/dish of 40 mM Tris buffer, pH 7.6, containing 1 mM dithiothreitol (DTT), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml each of antibiotic protease inhibitors antipain, leupeptin, and pepstatin A, and was sonicated for 15 s. After centrifugation at 18500 × *g* for 30 min, the supernatant was designated as the soluble

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fraction. The resulting precipitate was suspended in the same volume of extraction buffer containing 0.1% Triton X-100, and stirred for 20 min at 0°C. After centrifugation at 18500 × g for 30 min, the supernatant was designated as the first washings. The resulting precipitate was treated by the same method. The supernatant was designated as the second washings and the resulting precipitate as the particulate fraction.

2.4. *Subcellular fractionation*

Subcellular fractionation of the transfected cells was carried out by differential centrifugation in isotonic sucrose medium. The transfected cells in 4 dishes were homogenized in 0.8 ml of a solution containing 0.28 M sucrose, 10 mM Tris buffer, pH 7.6, 1 mM DTT, 1 mM EGTA, 1 mM PMSF, and 10 μg/ml each of antipain, leupeptin and pepstatin A with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 900 × g for 10 min. The precipitate was washed by brief homogenization in the sucrose medium and centrifuged to obtain the nuclear fraction. The supernatant fluid was pooled and centrifuged at 11500 × g for 20 min. The precipitate was donated the mitochondrial fraction. The supernatant fluid was centrifuged at 100000 × g for 60 min. The precipitate was designated as the microsomal fraction and the supernatant fluid as the supernatant fraction. Lactate dehydrogenase activity was assayed by the method of Pesce et al. [18].

Table I

CaM kinase II activity of soluble and particulate fractions in transfected cells

Clone	Fraction	Protein	CaM kinase II activity		
		(%)	Total activity (nmol/min)	%	Specific activity (nmol/min/mg)
α	soluble	54.0	97.9	79.9	12.0
	1st washings	13.5	14.5	11.8	7.1
	2nd washings	5.8	2.7	2.2	3.0
	particulate	26.5	7.3	6.0	1.8
β	soluble	53.6	30.5	30.2	4.82
	1st washings	14.7	10.9	10.8	6.26
	2nd washings	6.3	4.5	4.4	6.14
	particulate	25.4	55.0	54.5	18.3
αβ	soluble	54.3	233.0	76.1	40.5
	1st washings	13.1	27.0	8.8	19.4
	2nd washings	8.4	9.0	2.9	10.2
	particulate	24.2	37.2	12.1	14.4

CaM kinase II activity was measured as Ca^{2+}-dependent phosphorylation of syntide 2.

3. RESULTS AND DISCUSSION

Table I shows the CaM kinase II activity of α and β subunits and αβ complex expressed in transfected cells, when transfected cells were homogenized in hypotonic medium. About 80% of CaM kinase II activity of α subunit was found in soluble fraction and the activity in particulate fraction was 6.0%, indicating that the α subunit might exist in cytosol. On the other hand, about 30% of the activity of β subunit was found in soluble fraction and more than 50% of the activity was in particulate fraction. The activity of the first and second washings of β subunit was about 15% and more than 50% of the activity was not solubilized from particulate fraction by the treatment of 0.1% Triton X-100. The treatment of 0.1% Triton X-100 was thought to extract the enzyme associated loosely with membrane and the enzyme existed in occluded vesicle, but not to extract the enzyme bound to membrane tightly. Therefore, about half of the β subunit which was obtained in particulate fraction must have been bound to membrane tightly. α and β subunits in the αβ clone polymerized to form an αβ complex [16]. About 76% and 12% of the activity of αβ complex were found in soluble and particulate fractions, respectively. The activity of αβ complex in particulate fraction was significantly higher than that of α subunit. This result indicates that the α and β subunits associated differently with subcellular structure in transfected cells and that the β subunit might play a role in the association of αβ complex with membrane.

The α and β subunits in soluble and particulate fractions were examined by immunoblot analysis using a specific antibody as shown in Fig. 1. The α subunit was found in great quantities in soluble fraction, and the β subunit was found in particulate fraction more than in soluble fraction. The αβ complex was found in soluble fraction more than in particulate fraction. This result was consistent with the distribution of CaM kinase II activity of α and β subunits and αβ complex.

Since the β subunit existed in both soluble and particulate fractions, the enzymatic properties of β subunit in two fractions were examined. The substrate specificity of β subunit in soluble fraction was compared with that in particulate fraction as shown in Table II. The β subunit in soluble and particulate fractions demonstrated a nearly identical relative rate of phosphorylation for MAP 2, τ protein and syntide 2. The relative rate of phosphorylation for myosin light chain in soluble fraction was lower than that in particulate fraction and that for casein was higher than in particulate fraction. This result indicates that the relative rate of phosphorylation for protein substrates of β subunit was significantly different in soluble and particulate fractions.

The distribution of α and β subunit in subcellular fraction was examined by cell fractionation using differential centrifugation in isotonic sucrose medium as shown in Fig. 2. α subunit was concentrated in microsomal fraction and about 27% and 20% of the activity of α subunit were found in supernatant and mitochondrial fraction, respectively. Since about 80% of the activity of α subunit was found in soluble fraction, the α subunit in microsomal and mitochondrial fractions was thought to be associated loosely with these subcellular structures. The activity of β subunit was concentrated in mitochondrial and microsomal fractions and was very low in supernatant fraction. The distribution of αβ complex was similar to that of α subunit. Lactate dehydrogenase, a marker enzyme of

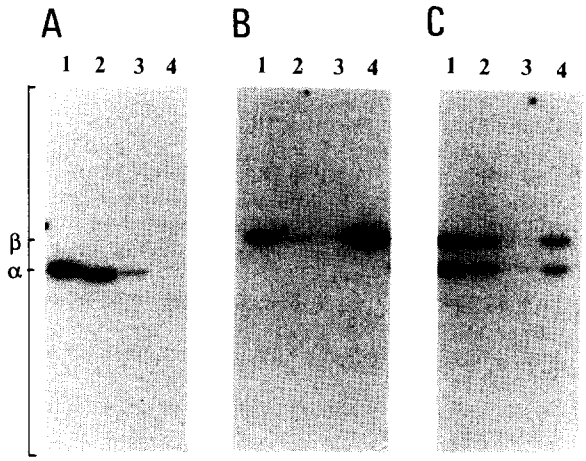

Fig. 1. Immunoblot analysis of α and β subunits in soluble and particulate fractions. Protein in each fraction prepared from cell homogenate (200 μg protein) was separated on SDS polyacrylamide slab gel electrophoresis and immunoblotted as described in [16]. (A) α clone. (B) β clone. (C) $\alpha\beta$ clone. (Lane 1) Soluble fraction; (lanes 2 and 3) the first and second washings of particulate fraction, respectively; (lane 4) particulate fraction.

cytosol, was almost found in supernatant fraction, indicating that the α and β subunits were different from a cytoplasmic enzyme and some parts of the two subunits were associated with subcellular structure cell membrane, endoplasmic reticulum and mitochondria.

In the present study, we demonstrated that the α and β subunits were associated differently with subcellular structure in transfected cells. More of the β subunit than of the α subunit was found in particulate fraction and more than 50% of the β subunit bound to membrane. The amino acid insertions of β subunit may be important to the association of CaM kinase II with membrane. It has been reported that the subcellular distribution of CaM kinase II is under developmental control [19]. The forebrain of 5 day postnatal rats contains about 80% cytoplasmic CaM kinase II with α and β subunit ratio of about 1:4. Between 5 and 20 days, 80% of the kinase is particulate with α and β subunit ratio of 7:1. On the other hand, more of the cerebellar CaM kinase II ($\alpha:\beta$ = 1:4) than of the forebrain isoform ($\alpha:\beta$ = 3:1) is recovered in particulate fraction [9]. These previous reports suggest that the subcellular distribution of CaM kinase II is not correlated to a variation of α and β subunit composition of the kinase. However, the result presented here was consistent with the result reported that more of cerebellar CaM kinase II than of the forebrain isoform is recovered in particulate fraction, suggesting that the association of α and β subunit is correlated to the association of the kinase with subcellular structure. The change of distribution of α and β subunits in the development was not explained from our result. It could be that the distribution of CaM kinase II was regulated by the membrane composition rather than by the structure of the kinase in developing neuronal cells. Further studies are required to explain the observation.

We demonstrated that the substrate specificity of β subunit was significantly changed in vitro by the association of the kinase with membrane. Subcellular distribution of CaM kinase II may be important for the

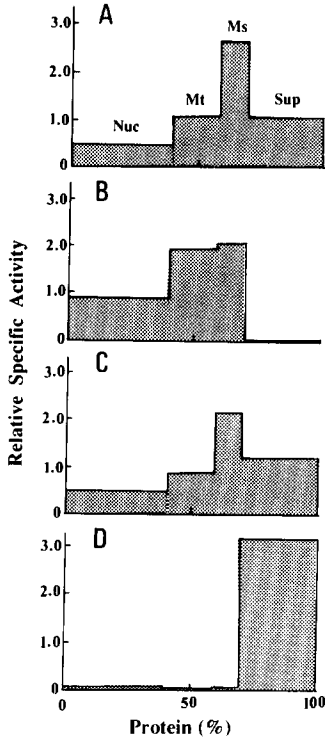

Fig. 2. Subcellular fractionation of CaM kinase II activity and lactate dehydrogenase in transfected cells. Subcellular fractionation of α-, β-, and $\alpha\beta$-clones was carried out as described in section 2. (A, B, C) CaM kinase II activity of α-, β-, and $\alpha\beta$-clones, respectively. (D) Lactate dehydrogenase activity of $\alpha\beta$-clone. Nuc, nuclear fraction; Mt, mitochondrial fraction; Ms, microsomal fraction; Sup, supernatant fraction.

Table II

Substrate specificity of β subunit in soluble and particulate fractions

Substrate	*Relative activity:* CaM kinase II activity	
	Soluble fraction	Particulate fraction
Microtubule associated protein 2	1.00	1.00
τ protein	0.78	0.80
Myosin light chain	1.60	1.35
Casein	0.44	1.02
Syntide 2	8.40	7.43

CaM kinase II activity was measured as Ca^{2+}-dependent phosphorylation of syntide 2 (20 μM) or protein substrate (0.23 mg/ml) under the standard conditions, except that phosphorylation of casein was carried out at a concentration of 1 mg/ml in Mes buffer, pH 6.6. The enzyme activity is shown as relative activity to MAP 2

regulation of enzyme activity in neuronal cells. It has been reported that CaM kinase II is the major protein of postsynaptic density [13,14], in which the α and β subunits may form an $\alpha\beta$ complex. The enzymatic properties of CaM kinase II in postsynaptic density may be different from that in soluble fraction. The present study provides valuable information to investigate the properties of CaM kinase II in soluble fraction and in postsynaptic density. Introduction of the cDNAs into cultured neuronal cells would seem a useful approach to understand the physiological significance of different association of CaM kinase II with subcellular structure.

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