Type II Ca^{2+}/calmodulin-dependent protein kinase binds to actin filaments in a calmodulin-sensitive manner

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Multifunctional type II Ca^{2+}/calmodulin-dependent protein kinase purified from rat brain cytosol was found to bind to actin filaments in vitro. The binding was saturable, and the dissociation constant for the binding was determined to be about 4×10^{-8} M. Electron microscopic observation indicated that the kinase binds to the side of actin filaments. Calmodulin inhibited the binding of the kinase to actin filaments in a Ca^{2+}-dependent manner. The Ca^{2+}/calmodulin-regulated binding of the kinase to actin filaments revealed here may be important for the substrate recognition of the kinase.

Ca^{2+} *Calmodulin dependence* *Protein kinase* *Actin* *Cytoskeleton* *Signal transduction*

1. INTRODUCTION

Brain type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II) has been purified from rat brain cytosol [1–6]. This kinase is also present in membrane cytoskeletal fractions [7]. However, molecular interactions which account for the localization of CaM kinase II in the membrane cytoskeleton remain unclear. As one of the candidates for the molecular interactions between cytoskeleton and this kinase, we focused our attention on the actin-microfilament system because of the following considerations. (i) Actin filaments are preferentially localized in the periphery of cells and constitute the major membrane cytoskeleton. (ii) Various cell functions with which the microfilament system is concerned are thought to be regulated by Ca^{2+}-dependent phosphorylation possibly mediated by CaM kinase II. (iii) Actin filaments bind various kinds of enzymes such as aldolase and other glycolytic enzymes in vitro. (iv) Myosin light chain kinase, one of the Ca^{2+}/calmodulin-dependent protein kinases, has been reported to be associated with stress fibers in cultured cells.

Here, we show that CaM kinase II purified from rat brain binds to actin filaments under physiological ionic conditions. We further present evidence that calmodulin in the presence of Ca^{2+} inhibits the binding of this kinase to actin filaments.

2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

CaM kinase II was purified from rat brain cytosol by the method of Yamauchi and Fujisawa [4] with an additional column chromatography on DEAE-cellulose. Rabbit skeletal muscle actin was prepared as in [8]. Calmodulin was prepared from porcine brain as described [9]. Tropomyosin was purified from bovine stomach smooth muscle according to [10].

2.2. *Preparation of ATP-free F-actin*

Actin was polymerized in 100 mM KCl containing 2 mM $MgCl_2$ and centrifuged at $100000 \times g$ for 2 h at 0°C. The F-actin pellet was suspended and homogenized in a buffer solution containing 10 mM Pipes, 2 mM $MgCl_2$, 0.1 M KCl and 0.1 mM DTT (pH 7.3). The ATP-free F-actin thus obtained was used within 2 h.

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2.3. *Sedimentation assay*

The binding of CaM kinase II to actin filaments was assayed by centrifugation followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [11]. F-Actin (120 μg/ml) was mixed with varied amounts of CaM kinase II in 0.5 mM EGTA or 0.5 mM $CaCl_2$ in both the presence or absence of calmodulin using a standard assay medium (50 mM Pipes, 1 mM $MgCl_2$, 60 mM KCl, 0.1 mM DTT, 5% glycerol; pH 7.3). An aliquot (0.15 ml) of this mixture was centrifuged at 100000 × g for 40 min at 0°C and the supernatant and pellet fractions were electrophoresed on SDS-polyacrylamide gel. Gels were stained with Coomassie brilliant blue, and the intensity of the kinase band (50-kDa subunit) was determined by scanning the gels with a densitometer. In some cases, we used ATP-free F-actin in order to inhibit the kinase reaction. Otherwise, autophosphorylation of CaM kinase II caused apparent shifts of the mobility of its subunits. This made the protein bands diffuse on SDS-polyacrylamide gels and disturbed quantification by densitometry.

2.4. *Electron microscopy*

F-Actin (124 μg/ml) with or without CaM kinase II (60 μg/ml) was incubated for 20 min at 0°C in the absence of calmodulin and Ca^{2+}. Then the sample was diluted 5-fold in a solution containing 0.1% glutaraldehyde, 10 mM Hepes, 2 mM $MgCl_2$, 100 mM KCl and 0.1 mM DTT (pH 7.2). The samples were mounted on glow-discharged Formvar-coated grids and negatively stained with 1.0% uranyl acetate. They were observed with a Hitachi H 7000 electron microscope at an accelerating voltage of 75 kV.

3. RESULTS

Using purified CaM kinase II (fig.1, lane a) and purified muscle actin (fig.1, lane b), we performed the sedimentation assay in order to determine whether CaM kinase II has an affinity for actin filaments in vitro. When mixtures of CaM kinase II and polymerized actin were incubated at 0°C for 10 min and centrifuged, 70–80% of the CaM kinase II sedimented with actin filaments (fig.1B). Only ~5% of the kinase sedimented in the absence of actin filaments. This clearly indicates that CaM

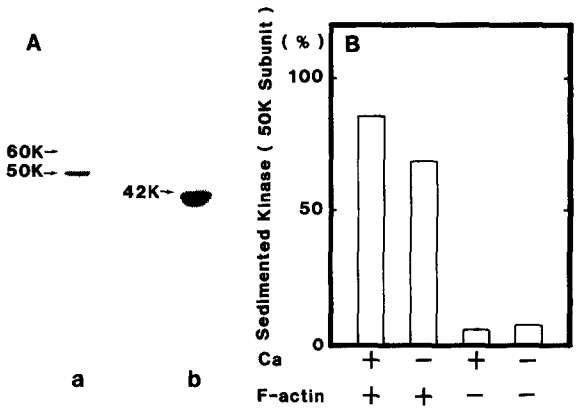

Fig.1. (A) SDS-polyacrylamide gel of the purified preparations of the CaM kinase II and actin. The proteins were subjected to SDS-PAGE (10% acrylamide) and stained with Coomassie blue. (a) Brain type II Ca^{2+}/calmodulin-dependent protein kinase, (b) rabbit skeletal muscle actin. (B) Co-sedimentation of the CaM kinase II with actin filaments in the presence and absence of Ca^{2+}. The CaM kinase II (40 μg/ml) was mixed with ATP-free F-actin (120 μg/ml) in the presence or absence of $CaCl_2$ (0.5 mM), incubated for 10 min at 0°C, and centrifuged. Then the amount of sedimented kinase was determined and is shown as percent of the total amount of the kinase. As a control, the kinase alone was centrifuged.

kinase II binds to actin filaments. The binding was insensitive to Ca^{2+} (fig.1B). Although the CaM kinase II also co-sedimented with actin filaments at physiological temperature (30°C), CaM kinase II alone substantially sedimented at 30°C possibly due to its self aggregation (not shown). Therefore, the sedimentation assays were carried out at 0–4°C. It should be noted that the CaM kinase II did not affect the extent of actin polymerization.

Fig.2A shows the saturation curve for the binding of CaM kinase II to actin filaments. Saturation occurred at about 10 nM CaM kinase II per 2.9 μM actin. The saturable binding suggests the specificity of interaction between CaM kinase II and actin filaments. The dissociation constant of the binding was determined to be about 4 × 10^{-8} M (fig.2B). This implies that the binding is rather strong under the physiologically relevant ionic conditions (1 mM $MgCl_2$+ 100 mM KCl).

Fig.3 shows electron micrographs of actin filaments in the absence or presence of CaM kinase II. Compared to the control actin filaments

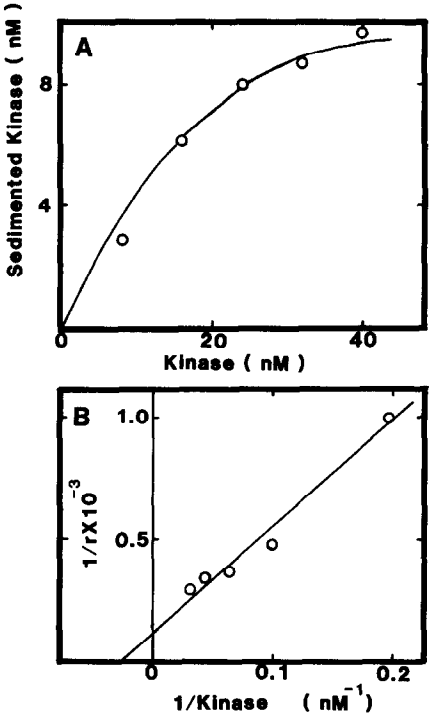

Fig.2. Quantitative analysis of the binding between CaM kinase II and actin filaments. (A) Increasing concentrations of CaM kinase II were mixed with F-actin (120 μg/ml) in the absence of Ca^{2+}, incubated for 10 min at 0°C and centrifuged. Then, the sedimented kinase (50-kDa subunit) was determined. (B) Plot of $1/[\text{kinase}]_{\text{bound}}/[\text{actin}]$ vs $1/[\text{kinase}]$.

(fig.3A), small particles (arrowheads) were attached to the side of actin filaments in the presence of CaM kinase II (fig.3B). The particles seem to be CaM kinase II because the enzyme is thought to be an oligomeric large particle and the same particles were seen when the purified CaM kinase II fraction alone was negatively stained (not shown). No bundles of actin filaments were observed even in the presence of CaM kinase II, indicating that CaM kinase II shows no actin-bundling activity.

A sedimentation assay revealed that tropomyosin, which is known to bind to the side of actin filaments, did not compete with the CaM kinase II for binding to actin filaments (not shown).

As the activity of CaM kinase II is totally dependent on Ca^{2+} and calmodulin, we studied the effect of calmodulin on the binding of CaM kinase II to actin filaments. In the absence of Ca^{2+}, calmodulin scarcely affected the binding (fig.4,

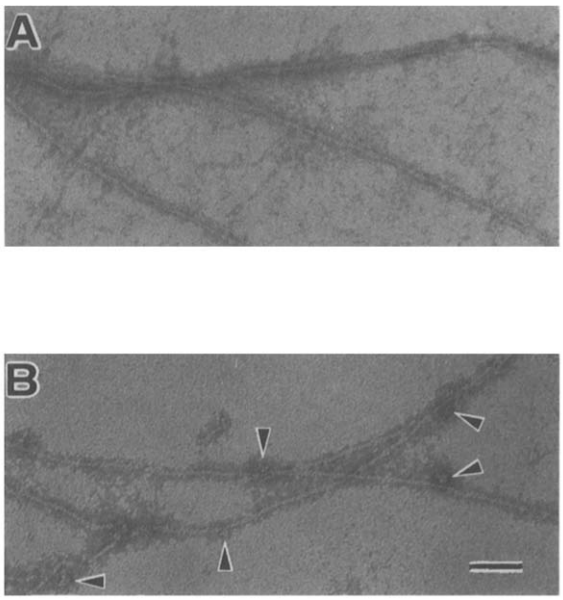

Fig.3. Electron micrographs of negatively stained preparations of actin filaments (A) and actin filaments with CaM kinase II (B). Note that the CaM kinase II, the globular particles (arrowheads), are attached to the actin filaments in (B). Bar, 100 nm.

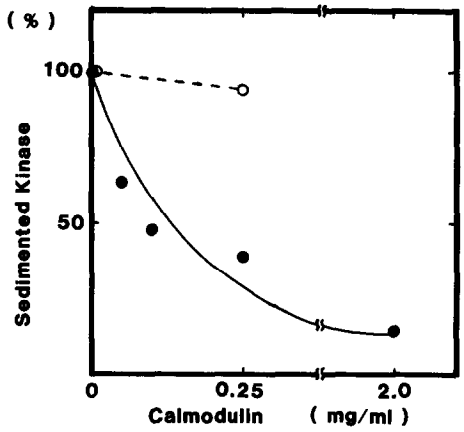

Fig.4. Effect of calmodulin on the binding of CaM kinase II to actin filaments. Increasing concentrations of calmodulin were added to a mixture of ATP-free F-actin (120 μg/ml) and CaM kinase II (15 μg/ml) in the presence (●) or absence (○) of Ca^{2+} and incubated for 10 min at 0°C. Then the sedimented kinase was determined.

○). In contrast, when Ca^{2+} was present, calmodulin inhibited the binding of CaM kinase II to actin filaments in a concentration-dependent manner (fig.4, ●). For half-maximal inhibition an approx. 30-fold molar excess of calmodulin over the 50-kDa CaM kinase II polypeptide was required.

4. DISCUSSION

In this paper we have shown that type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II) from brain binds to actin filaments in vitro. A large number of actin-binding proteins have been purified from various sources, and classified into several functional classes [12–14]. The CaM kinase II might fall under the category of actin filament side binding protein. The sedimentation assay has revealed that the binding of CaM kinase II to actin filaments is saturable and the dissociation constant is about 4×10^{-8} M under physiological ionic conditions. The result suggests that the co-sedimentability of CaM kinase II with actin filaments is not due to artifactual entanglement of the kinase in the actin filaments. In fact, electron microscopic observations clearly indicate that CaM kinase II is attached to the side of actin filaments in the form of a globular particle. Electron microscopy also revealed that the actin filaments are not bundled by the action of CaM kinase II.

A remarkable feature of the binding of the CaM kinase to actin filaments is its sensitivity to calmodulin. In the presence of Ca^{2+}, calmodulin dissociates the kinase from actin filaments. CaM kinase II is known to be distributed in particulate fraction as well as in the soluble cytoplasmic fraction and has been postulated to be in a dynamic subcellular equilibrium [2,15]. Saitoh and Schwartz [15] demonstrated that in *Aplysia* neurons, Ca^{2+}/calmodulin-dependent phosphorylation as well as cAMP-dependent phosphorylation causes release of CaM kinase II from the membrane cytoskeletal complex. Our present findings raise the possibility that Ca^{2+}/calmodulin directly regulates the translocation of the kinase from the membrane cytoskeletal complex which includes actin filaments.

Actin may play important roles in cell secretion and intracellular movement of macromolecules and some of these events are thought to be dependent on protein phosphorylation. Many of the good substrates for the kinase are cytoskeletal proteins [3,5,16,17]. The actin-binding nature of CaM kinase II and its sensitivity to calmodulin may be important for the substrate recognition of the kinase.

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