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In Vivo and In Vitro Association of 14–3-3 Epsilon Isoform With Calmodulin: Implication for Signal Transduction and Cell Proliferation

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Abstract Using a yeast two-hybrid screen, human 14–3-3 epsilon protein was found to interact with human calmodulin. In vitro binding assay between human 14–3-3 epsilon protein/peptide and calmodulin was demonstrated by native gel electrophoresis, and the interaction was shown to be calcium dependent. Our results, along with the association of the 14–3-3 epsilon protein with other signaling proteins, suggest that the 14–3-3 protein could provide a link between signal transduction and cell proliferation. J. Cell. Biochem. 73:31–35, 1999. © 1999 Wiley-Liss, Inc.

Key words: signal transduction; yeast two-hybrid screen; epsilon isoform

The 14–3-3 protein family consists of highly conserved acidic proteins that are present in a wide range of organisms and tissues [Aitken et al., 1992]. Its name was derived by its migration position on two-dimensional gel electrophoresis and DEAE chromatography [Moore et al., 1967]. Seven major mammalian isoforms have been isolated, designated α–η after their respective elution positions on high-performance liquid chromatography (HPLC)[Robinson et al., 1994]. The α and δ isoforms are found to be the phosphorylated form of β and ζ, respectively [Aitken et al., 1995].

One common feature among the 14–3-3 binding proteins is the presence of both cysteine- and serine-rich region with a 14–3-3 binding motif of RSXphosphoSXP [Muslin et al., 1996]. Deletion studies of the 1433ϵ protein revealed that the N-terminal of the 14–3-3 protein is involved in dimerization and binding to the plasma membrane [Jones et al., 1995].

Although there is a high level of sequence similarity among the different 14–3-3 isoforms, each isoform has its own pattern of protein expression and tissue distribution. Some of the functions that have been proposed for the epsilon isoform include calcium-dependent exocytosis in chromaffin cells [Chamberlain et al., 1995], regulation of PKC [Robinson et al., 1994], association with various proteins such as cdc25 [Conklin et al., 1995], phospholipids/actin cytoskeleton [Roth et al., 1995], centrosome [Pietromonaco et al., 1996], synaptic junction [Martin et al., 1994], insulin-like growth factor I receptor (IGFIR), insulin receptor substrate I (IRS-1) [Craparo et al., 1997], a zinc finger protein, A20 [Vincenz et al., 1996], and K8/18 keratin intermediate filament [Liao et al., 1996]. To further study the functions of the 1433ϵ protein in mitogenic signal and cell cycle, we have used a yeast two-hybrid screen to identify the interacting proteins of 1433ϵ, using a HeLa cell two-hybrid cDNA library. A number of putative positive clones were isolated from the yeast two-hybrid screen; calmodulin was identified in four of the six positive clones.

Calmodulin belongs to the troponin C (TnC) superfamily, which are highly conserved among many different cell types and species [Collins et

Abbreviations: CaM, calmodulin; DEAE, diethylaminoethyl; 1433ϵ, 14–3-3 epsilon isoform; Fmoc, 9-fluorenylmethyloxycarbonyl; PKC, protein kinase C; PCR, polymerase chain reaction; RP-HPLC, reversed-phase high-performance liquid chromatography.

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al., 1973], containing a high content of glutamic and aspartic acids. Calmodulin has two major folded N- and C-domains, each containing two Ca^{2+}-binding sites separated by a central helix [Herzberg et al., 1985]. Calmodulin has no N helix of the N-domain, and the four Ca^{2+}-binding sites are thought to be equivalent and compete for both Ca^{2+} and Mg^{2+} [Iida et al., 1986]. Calmodulin can exist in a number of conformations, depending on the number of Ca^{2+} bound per calmodulin molecule. As a predominant intracellular "Ca^{2+}-receptor," calmodulin can bind to and mediate the control of a large number of enzymes through complex formation with calcium. The interactions between 1433ϵ and CaM are described in this paper.

MATERIALS AND METHODS

Cloning and Protein Expression

Polymerase chain reaction (PCR) of 1433e was done using a pair of 1433ϵ cloning primers (Forward: 5′-TAGGGC*GAATTC*ATGGATGATCAGAGGATCTGG-3′; Reverse: 5′-TAGGGC-*GTCGAC* TGGTTTCTCTTGTTGGCTTATGTC-3′) and a human fetal heart cDNA library (Clontech, Palo Alto, CA) as the template. The procedures were performed as described previously [Hwang et al., 1995]. An *Eco*R1 site and a *Sal*I site, shown as the underlined bases, were designed in the 1433ϵ forward and reverse cloning primers, respectively. An end-clamp, TAGGGC was added to the 5′ end of the primer to facilitate cleavage by the restriction enzymes. After cutting with restriction enzymes, the PCR product was subcloned into a two-hybrid expression vector, pGBT9. The recombinant plasmid pGBT9–1433ϵ was transformed into the *Escherichia coli* strain JM109 and plated out onto LBA plates.

Expression of the 1433ϵ Protein Using pQE30 Expression Vector

The PCR product of 1433ϵ was subcloned into the T5 expression vector, pQE-30. The recombinant plasmid pQE-30–1433ϵ was transformed into the *Escherichia. coli* strain M15 (pREP4), and the 1433ϵ protein was expressed as a 6× His-tagged recombinant protein. The cloning procedures were performed as described previously [Luk et al., 1998]. CaM (human brain) was purchased from Sapphire Bioscience (Alexandria, NSW).

Yeast Two-Hybrid Screen

Saccharomyces cerevisiae strains HF7c and SFY526, two-hybrid expression vectors, pGAD10 and pGBT9 were obtained as the Matchmaker two-hybrid system of Clontech and were used for the yeast two-hybrid screen. A HeLa cell two-hybrid cDNA library purchased from Clontech was used in the screen. The 1433ϵ cDNA sequence was cloned into the GAL4 DNA-binding domain fusion plasmids-pGBT9 and the HeLa cDNA library was cloned into GAL4 transcriptional activation domain fusion plasmids-pGAD10. The *Escherichia coli* strain of JM109 was used in the cloning of the plasmids. pGBT9–1433ϵ was cotransformed along with the HeLa Matchmaker cDNA library into HF7c and SFY526, using the lithium acetate procedure. Double-transformed cells were plated on Leu^-, Trp^-, and His^- triple minus plates for yeast strain HF7c and Leu^-, Trp^- plates for yeast strain SFY526. The plates were incubated for 5 days at 30°C before colonies were picked, restreaked, and assayed for the lacZ phenotype, using 5-bromo-4-chloro-3-indolyl-β-D-galactoside as the chromogenic substrate.

In situ colony assay for β-galactosidase activity was performed by lifting the colonies onto a Whatman No.1 filter paper. The colonies were permeabilized by submerging into liquid nitrogen and placed onto another Whatman No.1 filter paper that had been soaked with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM $MgCl_2$, 50 mM β-mercaptoethanol) and incubated at 30°C for 5 min to 1 h. Library clones that activated the lacZ and His3 reporter genes only in the presence of both pGBT9–1433ϵ and the library plasmid were chosen for DNA sequencing using the Pharmacia ALF automatic sequencer.

Peptide Synthesis

Helix α4 of 1433ϵ, residues 75–96 (NH_2-GEDKLKMIREYRQMVETELKLI-COOH) was synthesized using the standard Fmoc chemistry procedures for solid-phase peptide synthesis [Erickson et al., 1976] on an Applied Biosystems 432A solid phase peptide synthesizer (Foster City, CA). The purity of the peptide was confirmed by reversed-phase (RP)-HPLC analysis.

In Vitro Binding Assay

Mixtures of 1 mg/ml of 1433ϵ protein/helix α4 peptide (residues 75–96) of 1433ϵ and 1 mg/ml of calmodulin (Alexis Corp., San Diego, CA) were dissolved in a native alkaline buffer (124 mM glycine, 20 mM Tris-HCl, pH 8.6) complemented with either 3 mM $CaCl_2$ (in the presence of calcium), or 1 mM EDTA (in the absence of calcium). These protein mixtures were allowed to equilibrate with occasional mixing at 4°C for 1 h before running on a 10% native polyacrylamide gel electrophoresis (PAGE). Protein bands were detected by Coomassie Blue staining.

RESULTS

In Vivo Interaction Studies

In a yeast two-hybrid screen using 1433ϵ as the "bait" plasmid, a number of putative positive clones were isolated from the two-hybrid HeLa cell two-hybrid library. They were screened with the appropriate reporter gene, either lacZ for yeast strain SFY526 or lacZ and HIS3 for yeast strain HF7c to eliminate the false-positive clones. After series of screening, six putative positive clones were obtained. Of the six clones that were further characterized, four appear to be identical to the size of the PCR products. These clones were then sequenced and they were identified as calmodulin. Specificity of the interaction between 1433ϵ and calmodulin was further tested by co-transforming the library plasmid with the control vectors provided by Clontech's matchmaker hybrid system, and by in vitro binding assay (Figs. 1–3).

In Vitro Interaction Studies

From the in vitro binding assay, the 1433ϵ-CaM protein complex was stable within the pH range of 7.0–8.6 (Fig. 1, lane 3). From previous investigation of CaM, it was demonstrated that CaM was capable of interacting with basic amphiphilic helices [O'Neil et al., 1990]. From primary sequence analysis of the 1433ϵ protein, we postulated that helix α4 of 1433ϵ may be a possible candidate for binding to calmodulin, since of the nine helices of 1433ϵ protein, only helix α4 satisfies the binding criteria of calmodulin.

Alkaline polyacrylamide gel electrophoresis on the study of the interaction between the 1433ϵ protein/helix α4 of 1433ϵ and calmodulin

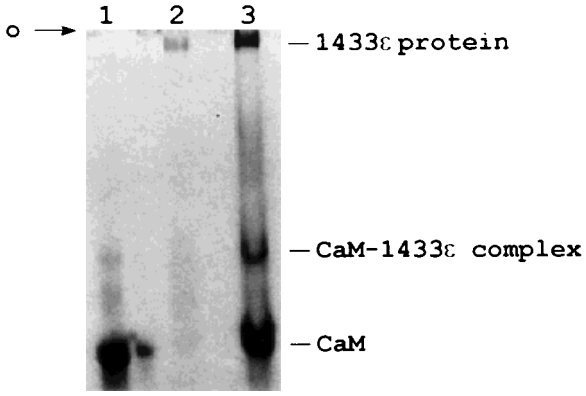

Fig. 1. In vitro binding assay of calmodulin and 1433ϵ protein in the presence of 3mM of $CaCl_2$. **Lane 1,** calmodulin only; **lane 2,** 1433ϵ protein only; **Lane 3:** calmodulin and 1433ϵ protein. 0 denotes the origin.

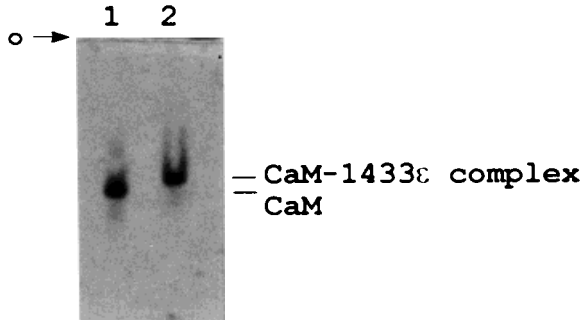

Fig. 2. In vitro binding assay of calmodulin and 1433ϵ helix α4 peptide in the presence of 3mM of $CaCl_2$. **Lane 1,** calmodulin only; **lane 2,** calmodulin and 1433ϵ helix α4 peptide. 0 denotes the origin.

was carried out by the modified method of Head and Perry [Head et al., 1974]. Unlike the negatively charged CaM protein (Fig. 1, lane 1), the native recombinant 1433ϵ protein (Fig. 1, lane 2) does not enter the gel matrix. This is probably due to its overall positive surface charge similar to that of the 1433ϵ protein, especially for the basic groove residues, Lys 49, Arg 56, Arg 60, and Arg 127 [Petosa et al., 1998, Liu et al., 1995]. Complex forming between the 1433ϵ protein and CaM was observed on lane 3 of Figure 1.

Our result demonstrated that both 1433ϵ protein and 1433ϵ helix α4 peptide (residues 75–96) were capable of forming a weak (Fig. 1, lane 3) and a tight complex (Fig. 2, lane 2) with calmodulin, respectively. This protein complex was not observed in the absence of calcium (Fig. 3, lane 2). In conclusion, the binding between 1433ϵ and calmodulin is calcium dependent.

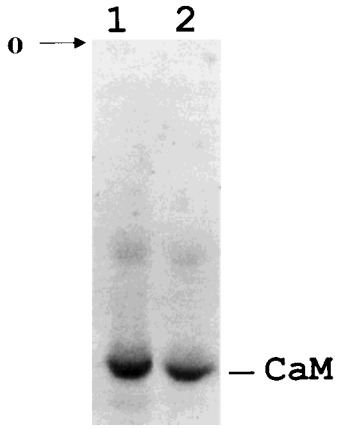

Fig. 3. In vitro binding assay of calmodulin and 1433ϵ helix α4 peptide in the presence of 1mM of EDTA. **Lane 1,** calmodulin only; **lane 2,** calmodulin and 1433ϵ helix α4 peptide. 0 denotes the origin.

DISCUSSION

14–3-3 protein and calmodulin are found to participate in signal transduction and cell proliferation but this is the first study demonstrating that 1433ϵ interacts directly with calmodulin. Based on the similar pI values of CaM and 1433ϵ, 3.9–4.3 [Klee et al., 1980], and 4.48 respectively, the interaction between these two proteins was not strong as expected (Fig. 1, lane 3); however, a tighter interaction was found between CaM and the 1433ϵ helix α4 peptide (Fig. 2, lane 2). From the X-ray crystallographic structure of the 1433 protein indicated that the amphathic helix, helix α4 (residues 75–96), is exposed in the dimeric form of 14–3-3 protein. Upon calcium binding to CaM, CaM will adopt a more open structure that binds to amphiphilic helices [O'Neil et al., 1990]. We speculate that other regions of the 1433ϵ protein may be involved in modulating the interaction between the helix α4 of 1433ϵ with CaM at different calcium concentration level based on our results of the in vitro binding assay. Thus, it is possible that the 1433ϵ protein is involved in governing the signal transduction process similar to previous investigation on the TnC amphiphilic helix interaction [Swenson et al., 1992].

The direct interaction observed between 1433ϵ and CaM in the yeast two-hybrid screen added to the accumulating evidences in their role in signal transduction and cell proliferation. It has been found that in serum-stimulated cells, 1433ϵ and calmodulin are found to be localized at the centrosome and spindle apparatus along with Ca^{2+}/CaM-dependent protein kinase II [Pietromonaco et al., 1996]. The binding specificity of 14–3-3 protein with other protein seems to be cell cycle specific and that would only bind to the interphase, but not to the M-phase form, of the Cdc25 [Kumagai et al., 1998]. Therefore, the interaction between 1433ϵ and CaM might also be cell cycle dependent, since their binding is sensitive to changes in calcium concentration and intracellular Ca^{2+} level is known to fluctuate during G2 to M transition [Lu et al., 1993]. Overall, the experimental reports suggest that these two proteins might play a vital role in the G2-M transition, particular in the DNA damage checkpoint.

Centrosome replication and separation during the cell cycle requires EGF, calcium, calmodulin, and Ca^{2+}/CaM-dependent protein kinase II (CaM-kinase II). CaM-kinase II are crucial for the proper G2-M transition, its role is to regulate the ubiquitination and degradation of cyclins [Lorca et al., 1993]. CaM-kinase II can autophosphorylate, and this phosphorylation state can remain active in the absence of calcium; thus, it can prolong the duration of the kinase activity after the initial activation by the calcium ion [Alberts et al., 1994]. The CaM-kinase II kinase activity is maintained until it is overcome by the activity of the protein phosphatase. It is possible that interaction between 1433ϵ and CaM could regulate the activities of CaM-kinase II in response to calcium concentration. CaM-kinase II can phosphorylate and activate tyrosine hydroxylase, which is the rate-limiting enzyme in catecholamine synthesis. Recently, 14–3-3 proteins were also found to be able to enhance the activity of the tryptophan hydroxylase [Furukawa et al., 1993]; therefore, the effect of the 14–3-3 protein on tryptophan hydroxylase might be through the action of CaM-kinase II. Further investigation is still needed to determine the role of 14–3-3 protein in the regulation of the CaM-kinase II and of tryptophan hydroxylase in conjunction with CaM.

14–3-3 interaction among Raf-1, cdc25 phosphatase, calmodulin, and CaM-kinase II implies that 14–3-3 may play an important role in mediating between mitogenic signaling and cell cycle at the centrosome/spindle apparatus. The identification of the interaction between 1433ϵ and CaM may provide a better understanding of the role of the 14–3-3 protein in governing these processes. Further investigation will be needed to identify the specific binding domain

between 1433ϵ and CaM and the effects of their interaction in signal transduction and cell proliferation.

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