Rapid Communication J. Biochem. 122, 494-497 (1997)

Perinuclear Membrane Localization of αKAP, a Protein Produced from a Gene within the Gene of Calmodulin-Dependent Protein Kinase IIα[1]

Masayuki Takeuchi and Hitoshi Fujisawa[2]

Department of Biochemistry, Asahikawa Medical College, Asahikawa, Hokkaido 078

Received for publication, June 25, 1997

αKAP is a protein produced from a gene within the gene of the α isoform of calmodulin-dependent protein kinase II (CaM-kinase IIα). It consists of the association domain of CaM-kinase IIα and a highly hydrophobic amino-terminal stretch consisting of 25 amino acids which is absent from CaM-kinase IIα. We previously demonstrated that αKAP is an integral membrane protein by subcellular fractionation analysis [Sugai, R., Takeuchi, M., Okuno, S., and Fujisawa, H. (1996) *J. Biochem.* 120, 773-779], but the exact subcellular localization of αKAP was not well understood. Here we demonstrate that αKAP is localized on the nuclear membrane of COS-7 cells transiently expressing αKAP. The nuclear membrane and perinuclear small vesicles were immunostained with an antibody against a synthetic peptide corresponding to the carboxyl-terminal 15 amino acids of αKAP. In contrast to the intact αKAP, the mutant αKAP, from which the hydrophobic amino-terminal segment had been deleted, accumulated within nuclei. Thus, αKAP may function as an anchoring protein for CaM-kinase II and/or other proteins in the perinuclear membrane.

Key words: CaM-kinase II, αKAP, nuclear membrane, signal sequence, skeletal muscle.

Calmodulin-dependent protein kinase II (CaM-kinase II) occurs abundantly in the central nervous system and is thought to be involved in a variety of cellular functions mediated by Ca^{2+} in the brain (*1-5*). A novel protein, designated as αKAP, was recently reported to be produced from a gene within the gene of the α isoform of CaM-kinase II (CaM-kinase IIα) in skeletal muscle (*6, 7*). This protein does not contain the protein kinase and regulatory domains of CaM-kinase IIα, but consists of the association domain of CaM-kinase IIα and a highly hydrophobic amino-terminal stretch consisting of 25 amino acids which is absent from CaM-kinase IIα (Fig. 1). Our previous cell-fractionation analysis revealed that αKAP is recovered in the particulate fraction (*7*), suggesting that the hydrophobic amino-terminal segment might be a signal sequence. Although the exact function of the association domain of CaM-kinase II is not well understood, its possible involvement in assembly of the subunits into a holoenzyme (*8, 9*) and association of the enzyme with other proteins or cellular organelles (*3*) has been suggested. These facts, taken together, led to the postulation that αKAP might be an anchoring protein for CaM-kinase II or other proteins. In the present study, the subcellular localization of αKAP was examined to elucidate the function of this protein.

When COS-7 cells transiently expressing αKAP were stained with an anti-αKAP antibody raised against the carboxyl-terminal 15 amino acids of αKAP and CaM-kinase IIα, which reacts specifically with αKAP and CaM-kinase IIα (*7*), and the stained cells were analyzed by fluorescence microscopy, strong fluorescence signals were found on the nuclear membrane and perinuclear small vesicles, as shown in Fig. 2B. The perinuclear small vesicles were also observed under a phase-contrast light microscope

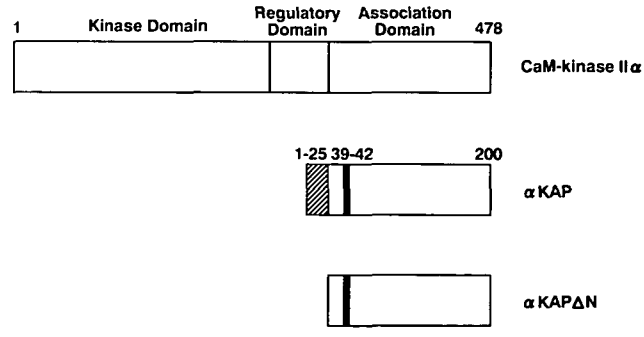

Fig. 1. **Schematic representation of the structures of CaM-kinase IIα, αKAP, and αKAPΔN.** CaM-kinase IIα comprises three domains, the protein kinase domain, regulatory domain, and association domain. αKAP consists of the association domain and a highly hydrophobic amino-terminal stretch consisting of 25 amino acids (represented by the shaded bar) which is absent from CaM-kinase IIα. αKAPΔN consists of only the association domain, being constructed through deletion of the amino-terminal hydrophobic segment from αKAP. A putative nuclear localization signal, KKRK, produced on the insertion of an 11-amino acid segment through alternative splicing is represented by the solid bars.

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, the Byotai Taisha Research Foundation, the Uehara Memorial Foundation, and the Mitsubishi Foundation.

[2] To whom correspondence should be addressed. E-mail: fujisawa@asahikawa-med.ac.jp

Abbreviations: CaM-kinase, calmodulin-dependent protein kinase; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; NLS, nuclear localization signal.

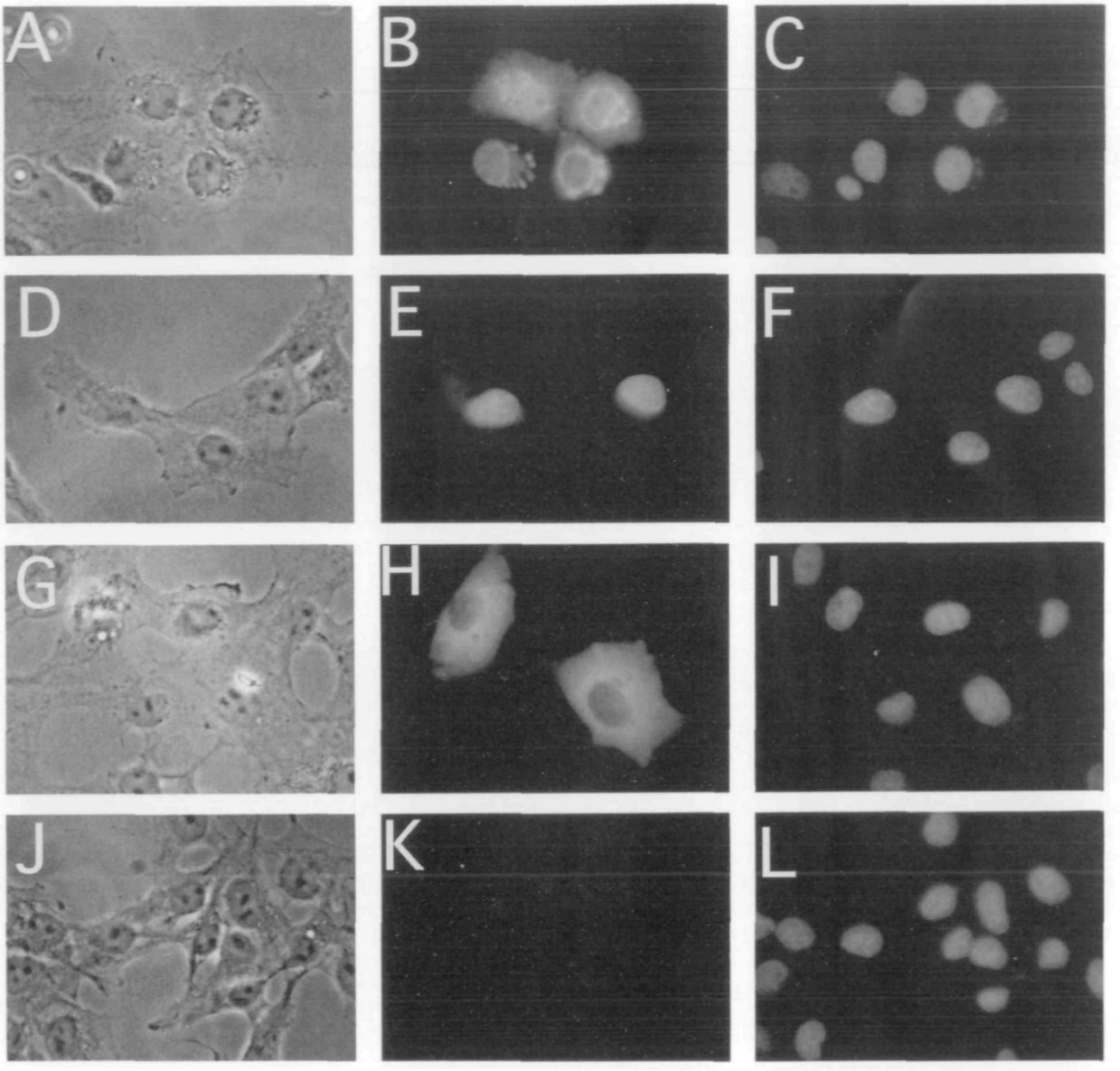

Fig. 2. **Immunocytochemical analysis of αKAP, αKAPΔN, and CaM-kinase IIα.** For expression of the full-length αKAP, the 0.7-kb *Nde*I-*Eco*RI fragment containing the entire αKAP cDNA of pET-αKAP (7) was blunt-ended with the Klenow fragment, ligated with *Eco*RI linkers, and then subcloned into a mammalian expression vector, pGFP-N1 (CLONTECH), to generate pαKAP. For expression of αKAP lacking the amino-terminal hydrophobic segment, the 0.6-kb *Nde*I-*Eco*RI fragment of pET-αKAP2 (7) was blunt-ended with the Klenow fragment, ligated with *Eco*RI linkers, and then subcloned into pGFP-N1 to generate pαKAPΔN. For expression of CaM-kinase IIα, the 2-kb *Kpn*I-*Kpn*I fragment of pKα58 (7) was blunt-ended with T4 DNA polymerase, and then subcloned into the filled-in *Bam*HI site of pET11a (Novagen). The resultant plasmid, pET-KIIα, encodes CaM-kinase IIα possessing a T7 tag, consisting of 11 amino acids, on the amino-terminal side of Ile[4]. The T7 tag was introduced into CaM-kinase IIα to distinguish it from other isoforms of CaM-kinase II for future use, but it was not utilized in the present study. The restriction enzyme site of the 1.6 kb *Nde*I-*Eco*RI fragment of pET-KIIα was filled in with the Klenow fragment, ligated with *Eco*RI linkers, and then subcloned into pGFP-N1 to generate pKIIα. African green monkey kidney COS-7 cells (*14*) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a humidified incubator at 37°C under an atmosphere of 5% CO_2. Transfection was carried out using the calcium phosphate-DNA precipitation method (*15*), followed by glycerol shock (*16*). COS-7 cells were plated at 1×10^5 cells/ml on a 60-mm dish in 3 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After 20-24 h of culture, the cells were incubated for 4-6 h in 3 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2.5 mM HEPES-NaOH (pH 7.1), 14 mM NaCl, 75 μM Na_2HPO_4, 12.5 mM $CaCl_2$, and 8 μg of plasmid DNAs for transfection. The cells were rinsed with phosphate-buffered saline (PBS), treated with 10% glycerol in PBS for 1 min, rinsed with PBS, and then cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Analysis was performed 1-2 days after transfection. Transfected COS-7 cells on coverslips were rinsed with PBS, fixed with 4% formaldehyde for 30 min, washed three times in PBS, and then permeabilized with methanol for 5 min. After washing in PBS, the cells were incubated with 0.5% skim milk in PBS for 30 min, rinsed with PBS, incubated with 0.5 μg/ml of anti-αKAP antibody (7) for 16 h, washed three times in PBS, and then incubated with 6 μg/ml of fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (DAKO) in PBS containing 0.5% skim milk for 1 h. After washing three times in PBS, cell nuclei were visualized by staining with 1 μg/ml of Hoechst 33258 (Polysciences) for 1 h, followed by washing in three changes of PBS. The cells were mounted in Vectashield (Vector Laboratories), and then examined by fluorescence microscopy under a Zeiss Axioskop microscope (Carl Zeiss) equipped with a standard FITC filter set, #487909 (Carl Zeiss), and a Hoechst 33258 filter set, #487902 (Carl Zeiss), using a Zeiss 40× Plan-NEOFLUOR lens (Carl Zeiss). COS-7 cells transfected with pαKAP (A, B, and C), pαKAPΔN (D, E, and F) pKIIα (G, H, and I), or the pGFP-N1 vector (J, K, and L) were stained by means of indirect immunofluorescence with the anti-αKAP antibody (B, E, H, and K), and the cell nuclei were stained with Hoechst 33258 (C, F, I, and L). Phase-contrast images of the cells are shown in panels A, D, G, and J.

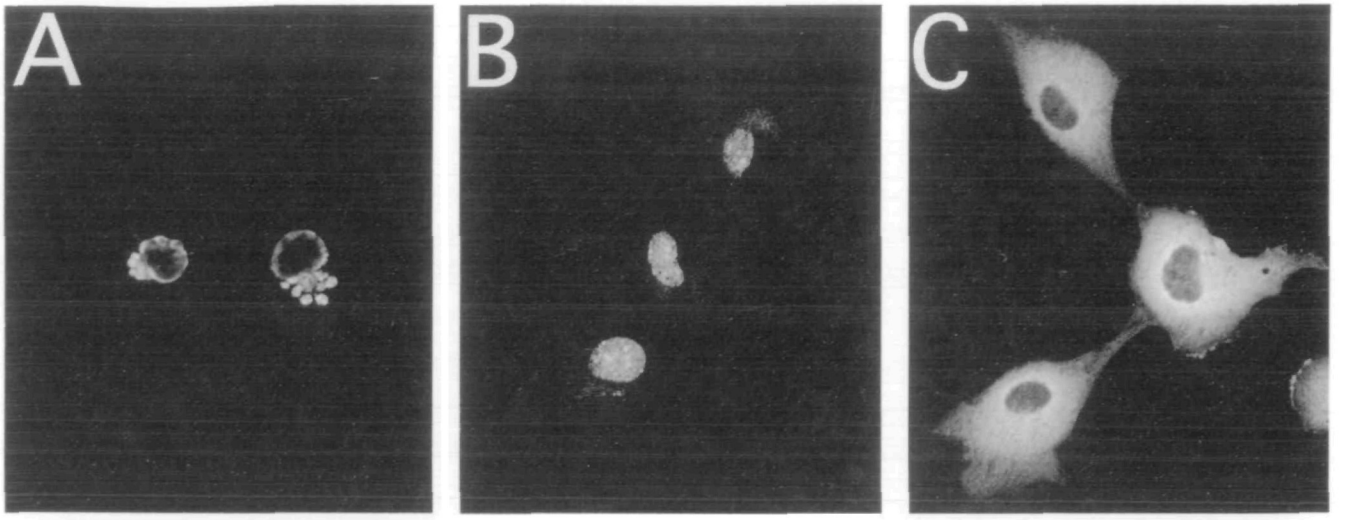

Fig. 3. **Confocal laser scanning images of αKAP, αKAPΔN, and CaM-kinase IIα.** COS-7 cells transfected with pαKAP (A), pαKAP ΔN (B), or pKIIα (C) were stained by means of indirect immunofluorescence with the anti-αKAP antibody as described in Fig. 2. Confocal images were obtained with a Bio-Rad MRC-600 Confocal System (Bio-Rad) and an Olympus BH-2 microscope (Olympus) equipped with a 40× objective lens. The FITC label was excited at 488 nm with an argon laser and collected through a 515-nm long-pass filter. Data were collected and analyzed using Comos software (Bio-Rad).

(Fig. 2A), while such vesicles were not observed in cells transfected with the empty expression vector (Fig. 2J), suggesting that these perinuclear vesicles were produced owing to overexpression of αKAP. Analysis by laser scanning confocal microscopy was carried out to confirm the fluorescence microscopy results and to determine the precise subcellular location of αKAP, as shown in Fig. 3A. Immunofluorescence signals were found only on the perinuclear membrane, *i.e.* they were not seen in any other regions. While our earlier finding that αKAP is recovered in the high-speed pellet (*7*) suggested that it is an integral membrane protein and the amino-terminal hydrophobic segment is a signal sequence (the transmembrane region), it should be noted that αKAP was found only in the perinuclear membrane, possibly because αKAP may serve as an anchoring protein for the association of CaM-kinase II or some other proteins with the cell nuclear membrane. To confirm that the hydrophobic amino-terminal segment of αKAP functions as a transmembrane segment rendering αKAP a membrane protein, αKAPΔN, from which the hydrophobic amino-terminal segment had been deleted, was constructed and expressed in COS-7 cells, and its subcellular location was examined by fluorescence microscopy (Fig. 2E) and confocal laser scanning microscopy (Fig. 3B). Both analyses showed that the mutant αKAP protein lacking the hydrophobic amino-terminal segment was broadly distributed within the nucleus, no significant immunofluorescence being seen in any other subcellular regions. Thus, the removal of the hydrophobic amino-terminal segment from αKAP altered its subcellular location from the perinuclear membrane to the nucleoplasm, confirming that the hydrophobic segment is a transmembrane region. In cells expressing CaM-kinase IIα, immunofluorescence signals exhibited a broad distribution outside the nucleus, no significant immunofluorescence being detected in the nucleus (Figs. 2H and 3C). Srinivasan *et al.* (*10*) reported that a sequence, KKRK, produced on the insertion of an 11-amino acid segment (KRKSSSSVQMM) through alternative splicing into CaM-kinase IIδ (*11, 12*), serves as a nuclear localization signal (NLS) for translocation of the enzyme from the outside to the inside of the nucleus. The sequence, KKRK, was originally discovered as an NLS of the SV40 T antigen (*13*). They also demonstrated that on the insertion of a segment containing this sequence into CaM-kinase IIα, which is excluded from the nucleus and is broadly distributed within the cell in the cytosol/cytoskeleton, the enzyme exhibits distinct nuclear localization and is not significantly detected in the cytoplasm (*10*). These results, taken together, suggest that the sequence, KKRK, which is not found in CaM-kinase IIα but is produced in the αKAP protein on the insertion of an 11-amino acid segment (KRKSSSSVQLM) through alternative splicing (*7*), plays an important role as an NLS determining the subcellular location of αKAP.

REFERENCES

1. Fujisawa, H. (1990) Calmodulin-dependent protein kinase II. *BioEssays* **12**, 27-29
2. Kelly, P.T. (1991) Calmodulin-dependent protein kinase II. Multifunctional roles in neuronal differentiation and synaptic plasticity. *Mol. Neurobiol.* **5**, 153-177
3. Soderling, T.R. (1993) Calcium/calmodulin-dependent protein kinase II: role in learning and memory. *Mol. Cell. Biochem.* **127-128**, 93-101
4. Nairn, A.C. and Picciotto, M.R. (1994) Calcium/calmodulin-dependent protein kinases. *Semin. Cancer Biol.* **5**, 295-303
5. Braun, A.P. and Schulman, H. (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu. Rev. Physiol.* **57**, 417-445
6. Bayer, K.U., Löhler, J., and Harbers, K. (1996) An alternative, nonkinase product of the brain-specifically expressed Ca^{2+}/calmodulin-dependent kinase II α isoform gene in skeletal muscle. *Mol. Cell. Biol.* **16**, 29-36
7. Sugai, R., Takeuchi, M., Okuno, S., and Fujisawa, H. (1996) Molecular cloning of a novel protein containing the association domain of calmodulin-dependent protein kinase II. *J. Biochem.* **120**, 773-779