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Molecular Cloning of a Novel Protein Containing the Association Domain of Calmodulin-Dependent Protein Kinase II[1]

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The cDNA of a novel protein, which contains the association domain of α isoform of calmodulin-dependent protein kinase II (CaM-kinase IIα), was cloned from rat skeletal muscle. This protein, called αKAP, consisted of 200 amino acid residues with a molecular weight of 22,583. αKAP has a highly hydrophobic amino-terminal stretch of 25 amino acids which is absent from CaM-kinase IIα, suggesting that this protein is either a secretory protein or an integral membrane protein. Northern blot analysis with a probe specific for αKAP detected three distinct mRNA species of 4.0, 2.4, and 1.5 kb in rat skeletal muscle. The 4.0- and 2.4-kb RNAs were also detected in heart, and at much lower levels in lung, kidney, and testis. Western blot analysis, using antibody raised against a synthetic peptide corresponding to the carboxyl-terminal 15 amino acids, revealed a single band corresponding in mobility to a molecular weight of 21,000 in crude extracts of both rat skeletal muscle and bacteria transformed with the cDNA, suggesting that no significant post-translational modification, such as excision of the amino-terminal hydrophobic segment, occurred. This, together with the fact that αKAP was recovered in the high-speed pellet in skeletal muscle, indicated that this protein may be an integral membrane protein.

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

Calmodulin-dependent protein kinase II (CaM-kinase II) is abundant 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*). There are at least four genes for CaM-kinase II (*6-11*) and a large number of isoforms are produced by alternative splicing of the transcripts (*8, 12-18*). All isoforms of CaM-kinase II have similar domain structures; the amino-terminal protein kinase domain, a regulatory domain consisting of autoinhibitory and calmodulin-binding regions, and the carboxyl-terminal association domain, although the function of the association domain is not well understood yet.

Among a number of calmodulin-dependent protein kinases, such as phosphorylase kinase, smooth muscle and skeletal muscle myosin light chain kinases, CaM-kinase I, II, III, and IV, and CaM-kinase IV kinase, smooth muscle myosin light chain kinase and CaM-kinase IV genes have been reported to produce other, smaller proteins lacking the amino-terminal protein kinase domains, telokin (*19-21*) and calspermin (*22-25*), respectively. These results suggest the simple possibility that CaM-kinase II gene can also produce a novel protein devoid of the protein kinase domain.

The present study demonstrates that a novel protein which does not contain the amino-terminal protein kinase domain, but does contain the carboxyl-terminal association domain of CaM-kinase IIα is expressed in rat skeletal muscle. Unlike telokin and calspermin, this protein is not only devoid of the protein kinase domain, but possesses a signal peptide-like hydrophobic sequence at the amino terminus which is absent in CaM-kinase IIα.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP, [α-^{32}P]dATP, [α-^{32}P]dCTP, [α-^{32}P]UTP, and [α-^{35}S]dATP were purchased from Amersham. Restriction enzymes and other DNA modifying enzymes were purchased from Takara Shuzo, Toyobo, or New England Biolabs. RNase A and RNase T1 were from Boehringer Mannheim. Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka).

cDNA Cloning and Sequencing—Total RNA was isolated from rat tissues by the guanidinium/cesium chloride method (*26*) and poly(A)$^+$ RNA was isolated by the use of Oligotex-dT30 (Takara Shuzo). An oligo (dT)-primed cDNA library was constructed with λgt10 by the method of Gubler and Hoffman (*27*). The library was screened with an 80-b oligonucleotide corresponding to the complementary sequence of the 3′ non-coding region of CaM-kinase IIα (nucleotides 1438-1517) (*6, 8*). Hybridization was performed in a solution containing 6×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate), 0.25% skim milk, and 50%

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Abbreviations: CaM-kinase. calmodulin-dependent protein kinase; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid].

formamide for 18 h at 42°C, and the membrane filters were washed with a solution consisting of 2.5×SSC and 0.1% SDS at 30°C. The fragments of positive clones were subcloned into pUC119, and the nucleotide sequences were determined by the dideoxy-termination method (*26*). The sequence data were analyzed by the use of the GENETYX program (Software Development, Tokyo).

Northern Blot Analysis—Poly(A)$^+$ RNA was glyoxylated, electrophoresed, and then transferred to a nylon membrane filter. The RNA blots were hybridized with ^{32}P-labeled probes in a solution consisting of 6×SSC, 5× Denhardt's solution (1×Denhardt's solution=0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 0.1 mg/ml heat-denatured salmon sperm DNA, 0.5% SDS, 10% dextran sulfate at 42°C for 16 h, and then washed in 0.1×SSC containing 0.1% SDS at 50°C. The *Pst*I-*Sph*I fragment of CaM-kinase IIα cDNA (nucleotides 6-843) (*6*), prepared from a clone, pKα58, in which a *Pst*I restriction site had been added to an oligo(dG) linked to the 5′ terminus of the cDNA fragment beginning at nucleotide 6 (Tobimatsu, T., unpublished work), was used as a probe for the detection of the amino-terminal side of CaM-kinase II. The *Pvu*II-*Eco*RI fragment (nucleotides 1069-1530) was used as a probe for the carboxyl-terminal side. For analysis of the tissue distribution of αKAP mRNA, the MTN membrane (Clontech), on which 2 μg/lane each of poly(A)$^+$ RNA from a number of rat tissues, was hybridized with a ^{32}P-labeled probe specific for αKAP (nucleotides 1-224) (Fig. 3), and analyzed according to the manufacturer's instructions.

RNase Protection Assay—RNase protection assay was performed by the method of Zinn *et al.* (*28*). Three ^{32}P-labeled RNA probes, probes 225, 758, and 687, were synthesized by T7 RNA polymerase with [α-^{32}P]UTP using the 225-bp *Sph*I-*Pvu*II fragment (nucleotides 844-1068), 758-bp *Sma*I-*Pvu*II fragment (nucleotides 311-1068), and 687-bp *Sph*I-*Eco*RI fragment (nucleotides 844-1530) of CaM-kinase IIα cDNA (*6*), respectively, which had been subcloned into pSP72, as templates, and purified by polyacrylamide/urea gel electrophoresis. RNA samples, rat skeletal muscle poly(A)$^+$ RNA (0.1 μg) or CaM-kinase IIα RNA (1 ng) (as a control) prepared with T7 RNA polymerase from the 1.5-kb *Pst*I-*Eco*RI fragment (nucleotides 6-1530) of CaM-kinase IIα cDNA, were hybridized with 1.24×10^5 cpm each of RNA probes (4.87×10^8 cpm/μg) in 30 μl of a reaction mixture consisting of 40 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES) (pH 6.2), 1 mM EDTA, 0.4 M NaCl, and 80% formamide at 45°C overnight after incubation at 85°C for 5 min. After hybridization, 350 μl of mapping buffer consisting of 10 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 7.5), 300 mM NaCl, 1.9 μl of RNase solution consisting of 0.2 mg/ml RNase A, 0.01 mg/ml RNase T1, 1 mM Tris-HCl (pH 7.5), and 4% glycerol, and 0.5 μl of 10 mg/ml carrier yeast RNA were added and the mixture was incubated at 30°C for 30 min. The samples were subjected to proteinase K treatment, phenol/chloroform extraction, and then ethanol precipitation. The reaction products were electrophoresed on 4% polyacrylamide/8 M urea gel, followed by autoradiography.

Antibody Preparation—A synthetic peptide, CIVHFHRSGAPSVLPH, consisting of the carboxyl-terminal 15 amino acids of CaM-kinase IIα and a cysteinyl residue added to the amino terminus for coupling to a carrier protein, was conjugated to keyhole limpet hemocyanin (Sigma) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce) as the coupling reagent (*29*) under an argon atmosphere after reduction of the peptide by sodium borohydride (*30*), and the conjugate was used to immunize Japanese white rabbits. The rabbit antiserum harvested after immunization was subjected to affinity chromatography on peptide-coupled Cellulofine (Seikagaku). Approximately 21 mg of the antibody was obtained from 20 ml of the antiserum.

Expression of Recombinant Protein in E. coli—For expression of the full-length αKAP, an *Nde*I restriction site was created at the translational initiation site of αKAP cDNA using a mutagenic antisense oligonucleotide, 5′-AAGAGCAGCATATGCAGGCAGCAG-3′, essentially according to the method of Kunkel (*31*), and the mutant *Nde*I-*Eco*RI fragment excised from the pUC119 was inserted into the *Nde*I-*Eco*RI site of pET29a expression vector (Novagen) to generate pET-αKAP1, which encoded the complete αKAP protein from Met^1 to His^{200}. For expression of αKAP lacking the amino-terminal hydrophobic segment, an *Nde*I restriction site was created at the position of Met^{27} substituted for Gly^{27} using an antisense oligonucleotide, 5′-TTTCCTCCACTCTTCATATGTGTGGAGGAGAGAAAG-3′, and the mutant cDNA was inserted into pET29a to generate pET-αKAP2. These constructs were transformed into *Escherichia coli* BL21 (DE3). The transformed bacteria were grown at 37°C to an A_{600} of 0.4, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM. After 1 h at 30°C, the bacteria were harvested by centrifugation, and suspended in the sample buffer consisting of 150 mM Tris-HCl (pH 6.8), 2% SDS, 12.5% glycerol, and 0.02% BPB.

Western Blot Analysis—Rat tissues, which had been frozen in liquid nitrogen immediately after excision and stored at −80°C, were homogenized with a Physcotron in 50 mM Tris-HCl (pH 7.5) containing 10 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of the microbial protease inhibitors, and the residue was removed by centrifugation at 10,000×*g* for 30 min to generate the crude extracts. Crude tissue and bacterial extracts were subjected to SDS-polyacrylamide gel electrophoresis on 15% acrylamide gel, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was incubated with 5% skim milk in phosphate-buffered saline for 0.5 h at 24°C and then with 6.5 μg/ml antibody against the synthetic peptide corresponding to the carboxyl-terminal 15 amino acids of CaM-kinase IIα for 1 h, washed several times in phosphate-buffered saline, and then incubated with 60 μg/ml goat anti-rabbit IgG conjugated with horseradish peroxidase in phosphate-buffered saline containing 5% skim milk. After washing of the membrane in phosphate-buffered saline, the positive bands were detected with the ECL detection system (Amersham).

RESULTS

Analysis of Transcripts of CaM-Kinase IIα Gene—When poly(A)$^+$ RNA from rat skeletal muscle was analyzed by Northern hybridization using either the amino-terminal

or carboxyl-terminal parts of CaM-kinase IIα as a probe, three RNA bands corresponding to sizes of 4.0, 2.4, and 1.5 kb were observed with the carboxyl-terminal probe, but only a much weaker band of 4.2 kb was detected with the amino-terminal probe, as shown in Fig. 1. In contrast to skeletal muscle, brain poly(A)$^+$ RNA gave a much more intense band of 4.8 kb with both the amino-terminal and carboxyl-terminal probes, in agreement with earlier reports that mRNA for CaM-kinase IIα of such a size exists abundantly in the brain (*6, 11*). Thus, poly(A)$^+$ RNAs of 4.0, 2.4, and 1.5 kb hybridizing with only the carboxyl-terminal probe, but not the amino-terminal probe appear to exist in the skeletal muscle. To examine the possibility that the smaller transcripts correspond to the carboxyl-terminal part of CaM-kinase IIα, like calspermin in CaM-kinase IV and telokin in myosin light chain kinase, RNase protection assay was performed using three probes corresponding to the amino-terminal side, the middle, and the carboxyl-terminal side of CaM-kinase IIα, each containing the calmodulin-binding site, as shown in Fig. 2. The two probes, the 225-bp *Sph*I-*Pvu*II fragment and 758-bp *Sma*I-*Pvu*II fragment (see Fig. 2B), both protected a sequence of about 130 bases, and the 687-bp *Sph*I-*Eco*RI fragment (Fig. 2B) protected a sequence of about 600 bases. These results

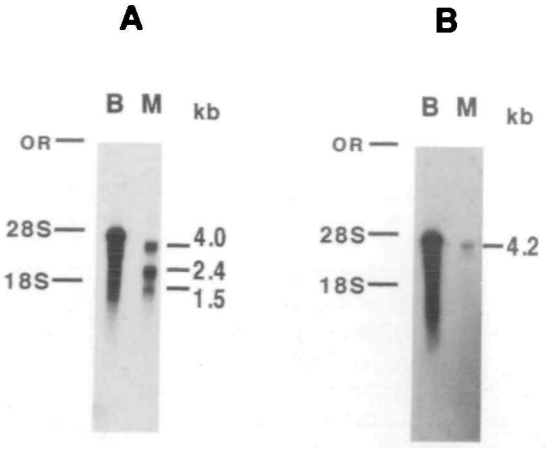

Fig. 1. **Northern blot analysis.** Aliquots of approximately 0.7 μg of poly(A)$^+$ RNAs from rat brain (lane B) and skeletal muscle (lane M) were subjected to Northern blot analysis with ^{32}P-labeled probes for the carboxyl-terminal side (nucleotides 1069-1530) (A) and the amino-terminal side (nucleotides 6-843) (B) of CaM-kinase IIα, as described under "MATERIALS AND METHODS." The results were visualized by autoradiography. The size markers used were 28 S and 18 S rat rRNAs. RNA sizes in kilobases are indicated on the right.

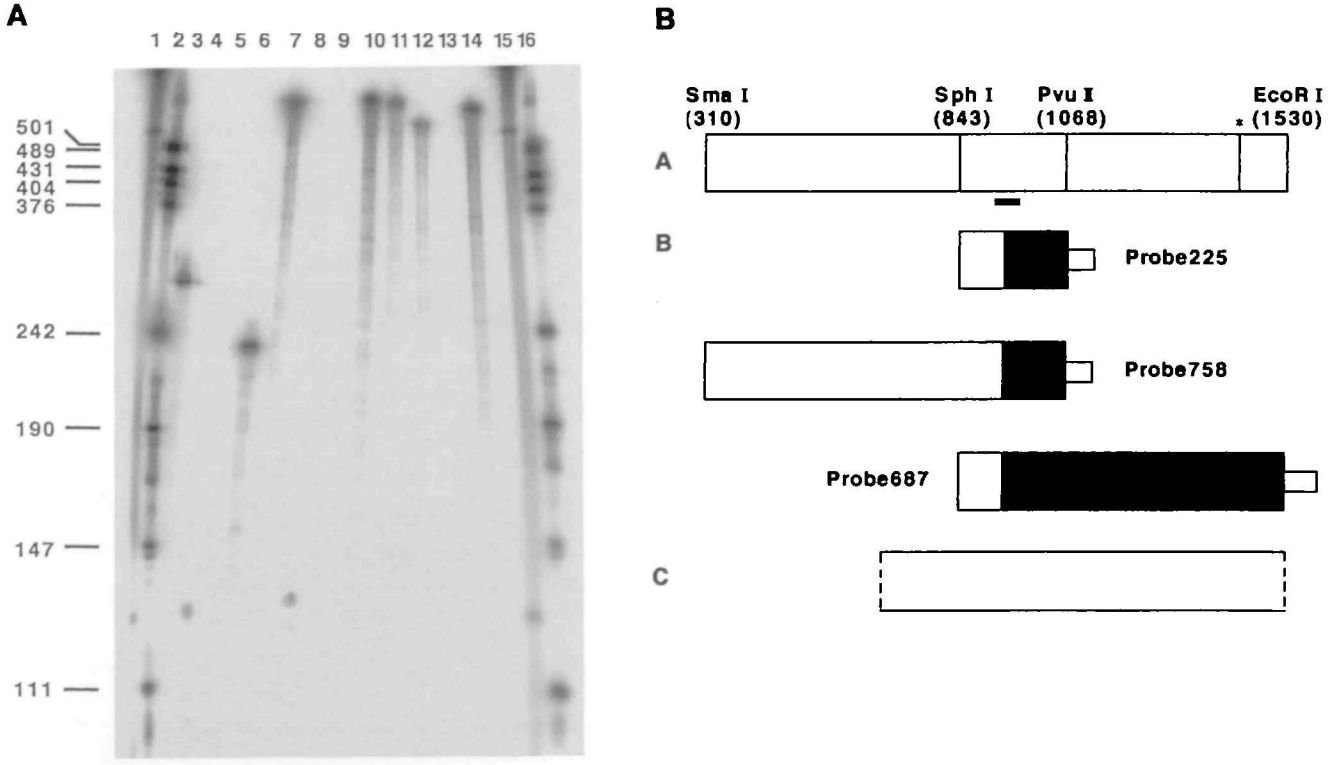

Fig. 2. **RNase protection assay of rat muscle poly(A)$^+$ RNA.** A: λ DNA digested with *Hin*dIII (lanes 1 and 15), pUC119 DNA digested with *Hap*II, as size markers (lanes 2 and 16), undigested probe 225 (lane 3), rat skeletal muscle poly(A)$^+$ RNA hybridized with probe 225, then digested with RNase (lane 4), probe 225 digested with RNase (lane 5), CaM-kinase IIα RNA hybridized with probe 225, then digested with RNase (lane 6), undigested probe 758 (lane 7), the muscle RNA hybridized with probe 758, then digested with RNase (lane 8), probe 758 digested with RNase (lane 9), CaM-kinase IIα RNA hybridized with probe 758, then digested with RNase (lane 10), undigested probe 687 (lane 11), the muscle RNA hybridized with probe 687, then digested with RNase (lane 12), probe 687 digested with RNase (lane 13), and CaM-kinase IIα RNA hybridized with probe 687, then digested with RNase (lane 14) were analyzed by electrophoresis, followed by autoradiography, as described under "MATERIALS AND METHODS." B: Comparison of nucleotides sequences of CaM-kinase IIα (A), probes used for RNase protection assay (B), and skeletal muscle poly(A)$^+$ RNA (C). Black areas represent the regions protected from RNase, and thin boxes represent the sequences derived from the vector pSP72. Restriction sites relevant to the present study, numbered beginning with the first nucleotide of the translational initiation codon of CaM-kinase IIα (*6*), are given in parentheses. The small solid bar and asterisk indicated the positions of the calmodulin-binding site and translational stop codon, respectively.

indicate that the skeletal muscle poly(A)$^+$ RNA hybridizing with the carboxyl-terminal probe is a transcript of CaM-kinase IIα gene and contains the entire amino-terminal part just after the calmodulin-binding site.

Nucleotide Sequence of cDNA Encoding αKAP—A λgt10 cDNA library constructed from rat skeletal muscle was screened by plaque hybridization with a synthetic 80-base oligonucleotide corresponding to the complementary sequence of the 3′ non-coding region of CaM-kinase IIα, and six positive clones were isolated from about 2×10^6 plaques. One of them was subjected to nucleotide sequencing, and the sequence of the coding region is shown in Fig. 3, along with the deduced amino acid sequence. The sequences of nucleotides 223-264 and nucleotides 298-848 were found in a continuous sequence of CaM-kinase IIα (*6*), and the sequence (nucleotides 265-297) lying between the two was not found in CaM-kinase IIα but in α-33 isoform of CaM-kinase IIα isolated from monkey brain (*32*). The sequence of nucleotides 1-222, in which the amino-terminal 25 amino acids of αKAP were encoded, was absent from CaM-kinase IIα cDNA. This amino-terminal segment is highly hydrophobic, as shown in Fig. 4, suggesting that αKAP is either an integral membrane protein or a secretory protein. To determine whether mature αKAP protein present in skeletal muscle has the amino-terminal hydrophobic segment, crude extracts of rat skeletal muscle and bacteria transformed with αKAP cDNA containing the entire coding region or the coding region devoid of the amino-terminal segment were subjected to Western blot analysis using antibody raised against the carboxyl-terminal region of CaM-kinase IIα, as shown in Fig. 5. The crude extract gave a single band corresponding in mobility to a molecular weight of 21,000, the position of which coincided with that in the extract of bacteria transformed with αKAP cDNA containing the entire coding region. The extract derived from bacteria transformed with the cDNA devoid of the amino-terminal segment showed a single faster-migrating band, corresponding to a molecular weight of 20,000. These results of Western blot analysis indicate that αKAP present in skeletal muscle still has the amino-terminal hydrophobic segment, suggesting that αKAP may be an integral membrane protein. The crude brain extract gave a single band corresponding to the position of CaM-kinase IIα with a molecular weight of 50,000, and no band was detectable at the position of αKAP.

Tissue and Intracellular Location of αKAP—The tissue distribution of αKAP mRNA was examined by Northern blot analysis using a probe specific for αKAP, as shown in Fig. 6. Three different mRNA species of 4.0, 2.4, and 1.5 kb were found in rat skeletal muscle. The 4.0- and 2.4-kb species were weakly detected in the heart, and also in the lung, kidney, and testis after long exposure. Thus, αKAP appears to be particularly abundantly expressed in skeletal muscle. Since αKAP possesses a signal peptide-like hydro-

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1     GGCCCACCCTGTGGGTTGTTGACGAGGGAGTAGACGGTGGAAGAAGACACGTGGAAGGAG
61    GAGGAAGTCTCTCACTGGCCGCCAAGCCGACTGTGTTCCAACAGTCCACCTTCCACTACT
121   CTCAGCCGGCTGCTCTGCTGCCTGCAAATGCTGCTCTTCCTCACGCTGTGGGCCCTAGTG
                                  M  L  L  F  L  T  L  W  A  L  V
181   CCCTGCCTGGTGTTGCTAACTCTCTACTTTCTCTCCTCCACAGGAGGGAAGAGTGGAGGA
      P  C  L  V  L  L  T  L  Y  F  L  S  S  T  G  G  K  S  G  G
241   AACAAGAAGAATGATGGCGTGAAGAAAAGAAAGTCCAGTTCCAGCGTTCAGTTAATGGAA
      N  K  K  N  D  G  V  K  K  R  K  S  S  S  S  V  Q  L  M  E
301   TCCTCTGAGAGCACCAACACCACCATCGAGGATGAAGACACCAAAGTGCGCAAACAGGAA
      S  S  E  S  T  N  T  T  I  E  D  E  D  T  K  V  R  K  Q  E
361   ATTATCAAAGTGACAGAGCAGCTGATCGAAGCCATAAGCAATGGAGACTTTGAATCCTAC
      I  I  K  V  T  E  Q  L  I  E  A  I  S  N  G  D  F  E  S  Y
421   ACGAAGATGTGCGACCCTGGAATGACAGCCTTTGAACCGGAGGCCCTGGGGAACCTGGTC
      T  K  M  C  D  P  G  M  T  A  F  E  P  E  A  L  G  N  L  V
481   GAGGGCCTGGACTTTCATCGATTCTATTTTGAAAACCTGTGGTCCCGGAACAGCAAGCCC
      E  G  L  D  F  H  R  F  Y  F  E  N  L  W  S  R  N  S  K  P
541   GTGCACACCACCATCCTGAACCCTCACATCCACCTGATGGGTGACGAGTCAGCCTGCATC
      V  H  T  T  I  L  N  P  H  I  H  L  M  G  D  E  S  A  C  I
601   GCCTACATCCGCATCACTCAGTACCTGGATGCGGGTGGCATCCCCCGCACGGCCCAGTCA
      A  Y  I  R  I  T  Q  Y  L  D  A  G  G  I  P  R  T  A  Q  S
661   GAGGAGACCCGTGTCTGGCACCGCAGGGATGGAAAATGGCAGATCGTCCACTTCCACAGA
      E  E  T  R  V  W  H  R  R  D  G  K  W  Q  I  V  H  F  H  R
721   TCTGGGGCGCCCTCCGTCCTGCCCCATTGAAGGACCAGGCCAGGGTCCCTGCGCTCTTGC
      S  G  A  P  S  V  L  P  H  *
781   TTCGCAGAGATCCATTCTTTGTCCATGGAATGTGGCTGCTGGCTCTCCCTTGGATGTTGC
841   TGGAATTC
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Fig. 3. **Nucleotide and deduced amino acid sequences of αKAP.** The amino acid sequence underlined with a solid line is absent in any form of CaM-kinase II, and the sequence underlined with a broken line is present in the α-33 isoform of CaM-kinase II (*32*).

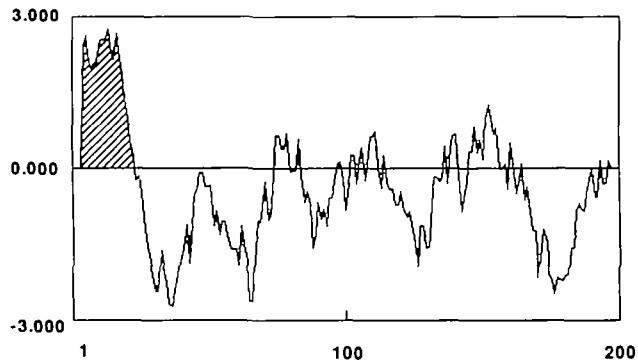

Fig. 4. **Hydropathy profile of αKAP.** Hydrophobicity was calculated by the procedure of Kyte and Doolittle (*33*) with a window of 10 amino acids. Values above the horizontal axis indicate hydrophobic character and those below the axis indicate hydrophilic character. The hatched area is the amino-terminal segment absent from CaM-kinase II.

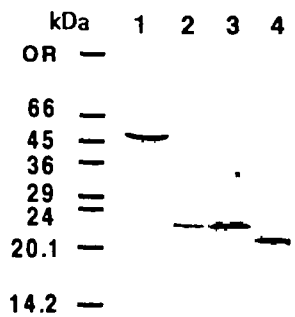

Fig. 5. **Western blot analyses of αKAP from skeletal muscle.** Approximately 50 μg of the crude extract of rat brain (lane 1) or skeletal muscle (lane 2), and 0.47 μg of the extract from bacteria transfected with pET-αKAP1 (lane 3) or pET-αKAP2 (lane 4) were subjected to Western blot analysis using antibody raised against the synthetic peptide corresponding to the carboxyl-terminal 15 amino acids of CaM-kinase IIα and αKAP, as described under "MATERIALS AND METHODS." Molecular masses in kilodaltons are given on the left.

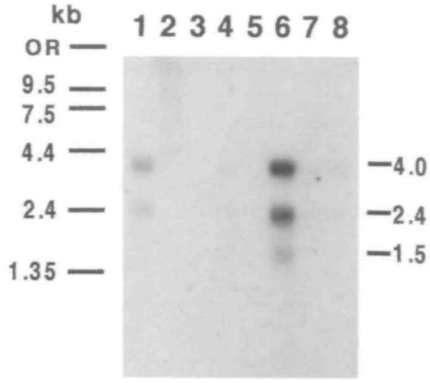

Fig. 6. **Tissue distribution of αKAP mRNA.** MTN membrane (Clontech), on which 2 μg of poly(A)$^+$ RNA isolated from rat heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and testis (lane 8) had been blotted, was hybridized with a ^{32}P-labeled probe specific for αKAP (nucleotides 1-224) and analyzed as described under "MATERIALS AND METHODS." RNA sizes in kilobases are indicated on the left.

phobic segment at the amino terminus (amino acids 1-25) and a nuclear localization signal-like segment (amino acids 39-42) (see "DISCUSSION"), subcellular location of αKAP in skeletal muscle was examined as shown in Fig. 7. The αKAP protein was not detected in the high-speed supernatant, regardless of whether the subcellular fractionation was carried out in an isotonic or hypotonic medium, suggesting that αKAP might be tightly associated with membranes. Even when the fractionation was done in a medium containing 0.1% Triton X-100, αKAP was not detected to any significant degree in the high-speed supernatant (data not shown). Upon fractionation in an isotonic medium, αKAP was recovered not only in the high-speed pellet (P100) but also in the low-speed pellet (P1), but this does not necessarily mean that αKAP is present in the cell nucleus, because our P1 fraction appeared to contain a considerable amount of cell debris.

DISCUSSION

Among a number of calmodulin-dependent protein kinases, smooth muscle myosin light chain kinase and CaM-kinase IV genes produce not only the respective protein kinases, but also other smaller proteins, named telokin (*19-21*) and calspermin (*22-25*), respectively, which are devoid of the amino-terminal protein kinase domains. These findings, together with the previous report that a 2.7-kb RNA from skeletal muscle hybridizes with only the carboxyl-terminal portion of CaM-kinase IIα cDNA, but not the amino-terminal portion (*8*), suggested the possibility that CaM-kinase II gene might also produce a similar smaller protein devoid of the amino-terminal protein kinase domain in skeletal muscle. The results described in the present study show that CaM-kinase IIα gene produced a novel smaller protein, called αKAP, in skeletal muscle, as expected, and that αKAP was devoid of the regulatory domain including the calmodulin-binding region, as well as the amino-terminal kinase domain, *i.e.*, only the carboxyl-terminal association domain of CaM-kinase IIα was included in αKAP. On the other hand, unlike telokin and calspermin, αKAP possesses a highly hydrophobic segment consisting of 25 amino acids at its amino-terminal end, which is absent from CaM-kinase IIα. Western blot analysis showed that αKAP from the crude skeletal muscle extract showed a single band, the mobility of which coincided with that of the recombinant αKAP possessing the amino-terminal hydrophobic segment (Fig. 5), indicating that this signal peptide-like hydrophobic segment remained unexcised in the mature αKAP protein. The sequence, consisting of 33 nucleotides coding for 11 amino acids, KRKSSSSVQLM, indicated by a broken underline in Fig. 3, is not found in CaM-kinase IIα isolated from rat brain (*6*), but is present in α-33 isoform of CaM-kinase IIα isolated from monkey brain (*32*). Similar inserts (KRKSSSSVHLM for CaM-kinase IIγ and KRKSSSSVQMM for CaM-kinase IIδ) are found in CaM-kinase IIγ isolated from rat brain (*10*), and in isoforms of CaM-kinase IIδ isolated from rat aorta (*14*) and heart (*16*). The first three amino acid residues of the inserts, together with the last residue (Lys) of the preceding exon, form the nuclear localization signal fitting the consensus sequence, K-K/R-X-K/R (*34, 35*), the introduction of which into a cytosolic CaM-kinase IIδ isoform has been reported to result in transport to the nucleus (*36*). We have reported that a synthetic peptide, named peptide-γ, corresponding to Lys^{345} to Ser^{358} of the amino acid sequence of CaM-kinase IIγ, is a specific substrate for CaM-kinase IV (*37*). Since the sequence of peptide-γ, KSDGGVKKRKSSSS, contains the putative nuclear localization signal of the insert, we speculate that the location of αKAP may be regulated through phosphorylation by CaM-kinase IV. When rat skeletal muscle was roughly fractionated into nuclear and cell debris (P1), mitochondrial (P10), microsomal (P100), and cytosolic (S100) fractions, and an aliquot of each fraction was examined by Western blot analysis (Fig. 7), the αKAP protein was found to be recovered in P1 and P100 fractions, and no significant reaction was detected in S100 fraction. When the fractionation was carried out in hypotonic medium, even in the presence of 0.1% Triton, no significant amount of αKAP was detected in the high-speed supernatant. These result, taken together, indicate that αKAP is an integral membrane protein and its amino-terminal hydrophobic segment is a signal peptide. Although appreciable amounts of αKAP were recovered in the P1 fraction, the interesting question of whether it is present in the nucleus still remains to be solved, because the P1 fraction contained much cell debris as well as cell nuclei. Thus, further biochemical and immunohistochemical studies are needed for clarification of precise intracellular localization of αKAP.

The αKAP cDNA sequence from the 5′ untranslated region to the coding region for the amino-terminal hydrophobic segment (nucleotides 1-222 in Fig. 3) is not found in CaM-kinase IIα cDNA (*6*), and the following sequence of nucleotides 223 to 264 (Fig. 3) is identical to the CaM-kinase IIα cDNA sequence corresponding to the exon VIII studied with CaM-kinase IIβ (*38*), suggesting that the promoter for αKAP is located within an intron between exons IX and VIII of CaM-kinase IIα gene. Transcriptional activation of this promoter is skeletal muscle-specific as revealed by Northern blot analysis (Fig. 6), in contrast to the brain-specific transcription of CaM-kinase IIα gene. On the other hand, the 33-bp inserts have been found in not only CaM-kinase IIα (*32*), but also CaM-kinase IIγ (*10*) and CaM-kinase IIδ (*14, 16*), as described above. In any

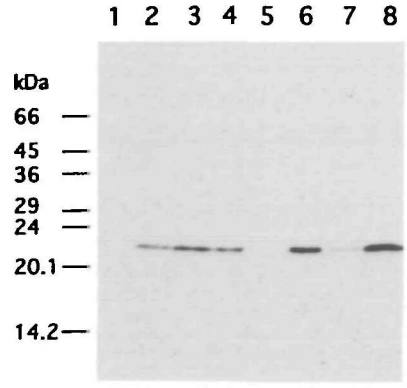

Fig. 7. **Subcellular location of αKAP protein in skeletal muscle.** Fresh rat skeletal muscle was homogenized with a Potter-Elvehjem homogenizer in 9 volumes of an isotonic buffered solution consisting of 50 mM Tris-HCl (pH 7.6), 0.25 M sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of the microbial protease inhibitors, and centrifuged for 10 min at 1,000×g. The pellet was suspended in a hypotonic buffered solution consisting of 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of the microbial protease inhibitors (P1) and the supernatant was then centrifuged for 30 min at 10,000×g. The resultant pellet was suspended in the hypotonic buffered solution (P10) and the supernatant was centrifuged for 60 min at 100,000×g to separate the pellet, which was then suspended in the hypotonic buffered solution (P100) from the supernatant (S100). For the fractionation in the hypotonic buffered medium, fresh rat skeletal muscle was homogenized with a Physcotron in 9 volumes of the hypotonic buffered solution, and centrifuged for 30 min at 10,000×g. The resultant pellet was suspended in the hypotonic buffered solution (P10) and the supernatant was centrifuged for 60 min at 100,000×g to separate the pellet, which was then suspended in the hypotonic buffered solution (P100) from the supernatant (S100). Approximately 5 μg of S100 (lane 1), P100 (lane 2), and P10 (lane 3) fractions in the hypotonic buffered medium, S100 (lane 5), P100 (lane 6), P10 (lane 7), and P1 (lane 8) fractions in the isotonic buffered medium, and 35 ng of the extract from bacteria transfected with pET-αKAP1 (lane 4) were subjected to Western blot analysis using antibody raised against the synthetic peptide corresponding to the carboxyl-terminal 15 amino acids of CaM-kinase IIα and αKAP, as described under "MATERIALS AND METHODS." Molecular masses in kilodaltons are given on the left.

case, this insert has not been found simultaneously with a sequence corresponding to exon VII of CaM-kinase IIβ, suggesting that this insert may be an alternative to exon VII. No significant amount of αKAP mRNA or protein devoid of this sequence was detected in skeletal muscle by RNase protection assay (Fig. 2) or Western blot analysis (Fig. 5), suggesting that transcriptional initiation at the promoter lying within an intron between exons IX and VIII may be associated with usage of this new alternative exon.

Thus, αKAP consists of the association domain of CaM-kinase II and the highly hydrophobic amino-terminal segment. The function of the association domain of CaM-kinase II is not well established, but it is considered to be involved in assembly of subunits of the holoenzyme (*39, 40*), and possibly in the association of the enzyme with other proteins or cellular organelles (*2, 6*). A search for a protein that binds to αKAP and further study on the intracellular localization of αKAP will contribute a great deal to understanding of the functions of the association domain of CaM-kinase II, as well as αKAP.

During preparation of this manuscript, Bayer *et al.* reported cloning and sequencing of the same protein from mouse skeletal muscle (*41*). Homologies between our rat and their mouse sequences are 100% for protein and 96.5% for the nucleotide sequence of the coding region, although there is a marked difference in subcellular localization of this protein between our and their results: our protein was recovered in the high-speed pellet, but their protein was present in the high-speed supernatant. However, we could not reproduce their result on subcellular localization even under the same conditions as they used. When mouse skeletal muscle was homogenized and fractionated into the low-speed pellet (P1), high-speed pellet (P100), and high-speed supernatant (S100) as described in their paper (*41*), the protein was recovered in both the low-speed and high-speed pellets but not significantly detected in the high-speed supernatant. They referred to this protein as αKAP, and accordingly we have used the same name in the present paper.

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