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An Alternative, Nonkinase Product of the Brain-Specifically Expressed Ca^{2+}/Calmodulin-Dependent Kinase II α Isoform Gene in Skeletal Muscle

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The gene for the α isoform of Ca^{2+}/calmodulin-dependent kinase II (αCaMKII) codes for a multifunctional protein kinase that is found exclusively in the brain. Here we show that in skeletal muscle, an alternative nonkinase product, hereafter referred to as αKAP (αCaMKII association protein), is expressed from the same gene. αKAP consists of a C-terminal region that is identical to the association domain of αCaMKII, with the exception of 11 amino acids inserted in the variable region. The N-terminal sequence of αKAP is highly hydrophobic and not present in any known CaMKII protein. The catalytic and regulatory domains of αCaMKII are missing in αKAP. Analysis of the exon-intron structure revealed that the αKAP transcript is derived from the αCaMKII gene by alternative promoter usage and RNA splicing. The transcriptional start site of αKAP mRNA is located within an intron of the αCaMKII gene. Therefore, the relationship between αKAP and αCaMKII is that of a gene within a gene. Immunostaining using anti-αKAP antibodies suggests that αKAP is associated with sarcomeres of skeletal muscle fibers. On the basis of its primary structure and specific location, the possible function of αKAP as an anchoring protein for CaMKII is discussed.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) constitutes a family of closely related multifunctional serine/threonine kinases that mediate the effect of Ca^{2+} as second messenger in various tissues (see references 8, 15, and 36 for reviews). A hallmark of CaMKII that distinguishes it from other multifunctional protein kinases is its conversion into a Ca^{2+} independent form by autophosphorylation, a process that may potentiate Ca^{2+} action and could play an important role in synaptic plasticity (27). The enzyme is most abundant in the brain, where it seems to be involved in diverse functions such as the synthesis and secretion of neurotransmitters, ion channel regulation, structural modification of the cytoskeleton, axonal transport, and long-term potentiation. Important functions for CaMKII have also been demonstrated outside the neural system (see, for example, references 23 and 43). Of particular interest for the present study is the observation that in skeletal muscle, CaMKII is involved in the regulation of a calcium ion channel, which allows movement of Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm during muscle activation (44).

At least four distinct genes that give rise to multiple CaMKII isoforms have been defined. The genes for the α and β isoforms of CaMKII (α- and βCaMKII) are expressed more or less exclusively in the nervous system (2, 6, 20, 22). By contrast, transcripts of the γ- and δCaMKII genes are present in many nonneural tissues as well as in the brain (41, 42). All four isoforms have a high degree of sequence homology and contain three structural domains referred to as the catalytic, regulatory, and association domains (22). The catalytic domain comprises the N-terminal half of each isoform and is responsible for the kinase activity. This is followed by a region of about 40 amino acids termed the regulatory domain since it contains an autoinhibitory segment and the calmodulin-binding segment. The C-terminal region of about 150 amino acids has been termed the association domain and is thought to be responsible for the assembly of subunits into a larger multimeric structure and possibly also for association with intracellular targets. Electron microscopic analyses indicate that multimers of 8 to 10 subunits are assembled with the association domains into a single globular hub, surrounded like flower petals by smaller particles containing the catalytic and regulatory domains of the individual subunits (19). However, recent data suggest that monomeric CaMKII molecules are also present in the cell (33, 45).

The N-terminal region of the association domain is the least conserved sequence of the different CaMKII isoforms and has therefore been referred to as the variable region (2). In the case of βCaMKII, this variable region is encoded by five exons, and deletion of one exon by alternative splicing results in the β′CaMKII variant (20). By analogy, new variants that have recently been described for γ- and δCaMKII are most likely also the result of alternative splicing in this region (12, 24, 30, 37). The functional significance of these different splice variants is not clear. In one case, it has been shown that alternative exon usage in the variable region creates a nuclear localization signal which targets the protein to the nucleus (39).

In this report, we show that alternative promoter usage and RNA splicing of the αCaMKII gene in skeletal muscle results in a transcript encoding a truncated protein that lacks the catalytic and regulatory domains and therefore has biological properties completely different from those of the brain-specific αCaMKII protein. As this new protein consists mainly of the association domain, it will be referred to in this report as αKAP (αCaMKII association protein).

(This article is based on a doctoral study by K.-U. Bayer at the Faculty of Biology, University of Hamburg.)

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MATERIALS AND METHODS

RNA analysis, reverse transcriptase-dependent PCR (RT-PCR), rapid amplification of 5′ DNA ends (5′-RACE), primer extension, and sequencing. The

isolation and analysis of total cellular RNA by Northern (RNA) blotting and the preparation of radioactive probes have been described previously (20). In all cases, adult BALB/c mice were used as a source for RNA. A 1.6-kb *Eco*RI fragment from the 3′ untranslated region of the mouse αCaMKII cDNA was used as a probe. This probe is specific for the αCaMKII transcript and does not hybridize with RNAs from other CaMKII genes (1).

Amplification of transcripts by RT-PCR was performed essentially as described previously (20). The PCR was carried out in a thermocycler (Perkin-Elmer) for 25 cycles. PCR products were separated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized to a specific oligonucleotide radioactively labeled at the 5′ end.

The 5′ end of the αKAP transcript in skeletal muscle was amplified by using a kit for 5′-RACE as described in the instruction manual (Life Technologies Inc.). The oligonucleotide 5′-CTGTCATTCCAGGGTCGCAC-3′ (positions 1247 to 1266 [14]) was used as a gene-specific reverse transcription primer, and oligonucleotide 5′-CGATCAGCTGCTCTGTCACT-3′ (positions 1187 to 1206 [14]) was used as a nested gene-specific amplification primer. The 5′-RACE products were directly cloned into the vector pCRII by using a TA cloning kit (Invitrogen).

Primer extension analysis for mapping the transcriptional start site of the αKAP gene was performed by using a kit as instructed by the supplier (Promega). The DNA products were fractionated on an 8% polyacrylamide gel in the presence of 6 M urea.

The nucleotide sequence was determined by the dideoxy method, using fluorescence-labeled dideoxy terminators (35). Subsequent separation and analysis were performed on a model 373A DNA sequencer (Applied Biosystems, Inc.).

Genomic cloning. A 129 SV mouse genomic library in λ FIXII (Stratagene) was screened with a radiolabeled probe derived from a subfragment of the αKAP cDNA clone (nucleotide residues 1 to 534; see Fig. 2). Positive plaques were purified by further rounds of screening. All recombinant DNA manipulations were done according to standard procedures (34).

Expression of αKAP in *Escherichia coli* and purification of the protein. A DNA fragment encoding an αKAP protein that lacked the 18 N-terminal amino acids was obtained by PCR and inserted into the *Bam*HI site of the vector pH6EX3 (4). The resulting fusion gene has an open reading frame coding for a histidine hexapeptide and a thrombin cleavage site at its N terminus and the recombinant αKAP (amino acid residues 19 to 200; see Fig. 2) at its C terminus. Expression of this fusion protein was induced in a growing bacterial culture by adding isopropyl-β-D-thiogalactoside (IPTG) to 1 mM and incubating the culture for 3 h at 37°C. The fusion protein was purified by a Ni-nitrilotriacetic acid agarose column (Qiagen Inc.) as described by the manufacturer. Fractions were collected and dialyzed against 0.5× phosphate-buffered saline. Protein concentrations were determined by the method of Bradford (5).

Antiserum preparation. Chinchilla bastard rabbits were injected intramuscularly with 300 μg of recombinant protein expressed from the vector pH6EX3 as described above. The antigen was emulsified with complete Freund's adjuvant. The rabbits were subsequently given two boosters in 4-week intervals. Blood was collected from the dorsal ear vein 2 weeks after every booster.

Immunoblot analysis. Protein extracts were prepared by homogenization of different tissues from adult mice in lysis buffer (80 mM Tris-HCl [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol); 40 μg of protein per lane was fractionated on an SDS–17.5% polyacrylamide gel (21) and transferred to nitrocellulose by the semidry electroblotting procedure. Blots were blocked in TNT buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk.

The antiserum was diluted 1:500 in TNT buffer containing 1% nonfat dry milk; the blot was incubated for 1 h at room temperature and then washed for 1 h with four changes of TNT buffer. Proteins were detected by peroxidase-conjugated goat antibodies directed against rabbit immunoglobulins (Bio-Rad) and chemiluminescence staining (ECL kit; Amersham).

Subcellular fractionation. Skeletal muscle tissue was prepared from hindlegs of BALB/c mice and homogenized in a hypotonic lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] buffer [pH 7.4], 5 mM sodium PP_i, 5 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*,*N*′-tetraacetic acid (EGTA), 1 mM $MgCl_2$, 1 mM Na_2MoO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin and pepstatin per ml) in a Polytron homogenizer. Cell fractionation was performed as described previously (29). Nuclei and cell debris were removed by low-speed centrifugation (1,000 × *g* for 5 min). A crude nuclear fraction (P1) was prepared by resuspending and centrifuging the pellet in lysis buffer. The remaining supernatant was fractionated into a cytosolic fraction (S100) and a particulate fraction (P100) by ultracentrifugation at 100,000 × *g* for 45 min. Aliquots from each fraction (20 μg of protein) were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with an antiserum against αKAP.

Immunohistochemistry. Rat muscle tissue specimens dissected from musculus quadriceps femoris and musculus biceps femoris were mounted by use of 7% gum tragacanth (Sigma) in distilled H_2O on pieces of thick blotting paper and flash frozen in precooled (−130°C) isopentane (Riedel-de Haen, Hannover, Germany) as described for the preparation of diagnostic muscle biopsies (13). Four-micrometer sections were cut on a cryomicrotome (Frigocut 2800 E; Leica, Heidelberg, Germany) at −20°C and fixed in acetone at room temperature for 15 min. For immunostaining, the avidin-biotin-peroxidase complex method was used (17). After blocking of endogenous avidin-binding activity by treatment with avidin and biotin solutions from a commercial kit (Zymed Laboratories, San Francisco, Calif.), sections were incubated with 5% bovine albumin in Tris-buffered (pH 7.2)–0.9% NaCl solution to reduce nonspecific background staining. Incubation for 90 min with diluted (1:400) rabbit anti-αKAP antiserum or normal rabbit serum as a control was followed by reaction with biotinylated $F(ab')_2$ fragments of sheep anti-rabbit immunoglobulin G (Sigma). Subsequently sections were overlaid with either Cy2- or Cy3-conjugated streptavidin (Rockland, Gilbertsville, Pa., and Jackson-Dianova, Hamburg, Germany, respectively) or streptavidin-biotinylated peroxidase complex (DAKO Diagnostika, Hamburg, Germany). The peroxidase was developed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and H_2O_2. Immunofluorescence was visualized and photographed under epi-illumination in a Zeiss photomicroscope III (Zeiss, Oberkochem, Germany).

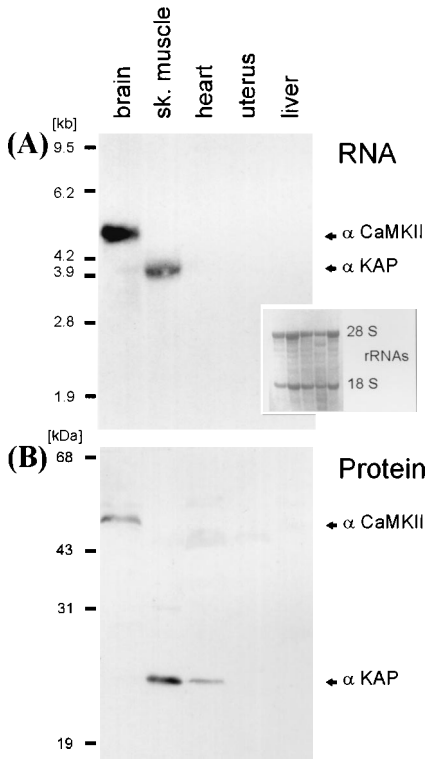

FIG. 1. Expression of the αCaMKII and αKAP genes. (A) Northern blot analysis of total RNAs from different tissues of adult mice. A fragment from the 3′ untranslated region of the αCaMKII gene was used as a probe. The amount of RNA on each lane was checked by staining the rRNAs with methylene blue (inset). (B) Immunoblot analysis of protein extracts from different tissues of adult mice with an antiserum against recombinant αKAP protein.

Nucleotide sequence accession number. The GenBank accession number of the sequence reported in this paper is X87142.

RESULTS

Characterization of the αKAP transcript in skeletal muscle. When RNAs from several tissues of adult mice were hybridized with a probe from the 3′ untranslated region of the αCaMKII gene, transcripts were detected in the brain and skeletal muscle (Fig. 1A). The 4.8-kb brain-specific RNA corresponds to the transcript of the αCaMKII gene, whose partial cDNA sequence has been described previously (14). The skeletal muscle-specific RNA is about 4.1 kb long and will be referred to in this report as αKAP RNA. After long exposure times, minor amounts of the αKAP RNA were also detected in the heart, uterus, and brain. The expression level in these tissues is about 1% or less of that in skeletal muscle (data not shown). No αKAP transcripts, even after long exposures, were found in RNA from the liver, lung, thymus, kidney, spleen, and

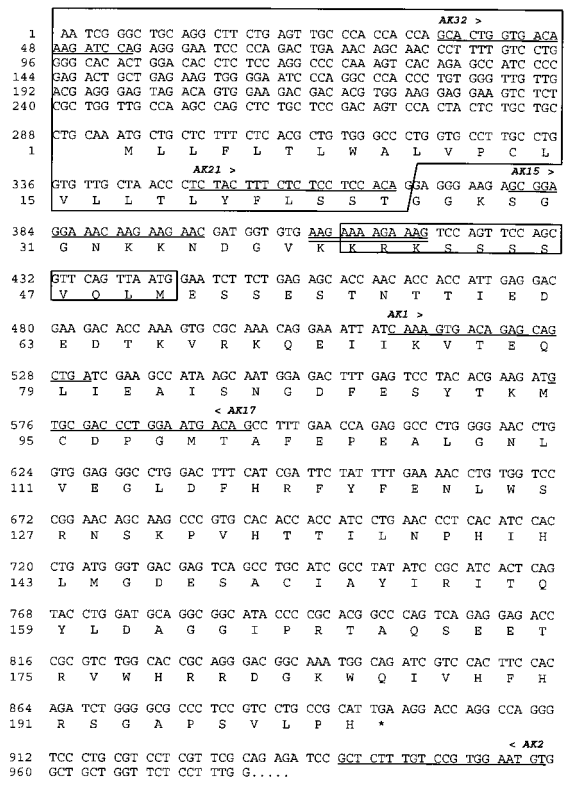

FIG. 2. Nucleotide sequence of αKAP cDNA and deduced amino acid sequence of the protein. Sequences specific for the αKAP transcript and not present in the previously described αCaMKII cDNA are boxed (14). The nucleotide sequence encoding the amino acid sequence KKRK, a potential nuclear localization signal, is doubly underlined. Sequences of oligonucleotides used for the RT-PCR analysis shown in Fig. 4 are underlined, and the designations are given above the sequences.

testes. For practical purposes, we will therefore refer to the αKAP transcript as skeletal muscle specific. To compare the sequences of the αCaMKII and αKAP transcripts, RT-PCR was performed with different oligonucleotides derived from the published cDNA sequence of the mouse αCaMKII gene (14). The results indicated that the muscle-specific αKAP RNA did not contain sequences corresponding to the kinase domain and regulatory domain of the αCaMKII gene. However, sequences corresponding to the association domain and 3′ untranslated region were present in RNAs from both tissues (data not shown). Further analysis of PCR-derived cDNA clones confirmed that the αKAP transcript contained a region that was identical in sequence to the association domain of the brain-specific αCaMKII transcript.

To characterize the 5′ end of the αKAP transcript, this region was amplified by the 5′-RACE, using an oligonucleotide located in the association domain as a gene-specific primer. The resulting cDNAs were cloned in a plasmid vector and sequenced. By combining sequence data from these 5′-RACE-derived cDNA clones with those obtained from RT-PCR products of the association domain, the partial sequence of the αKAP cDNA shown in Fig. 2 was derived. The sequence 3′ to the association domain (3′ to nucleotide residue 978 in Fig. 2) is not known. However, PCR analysis and restriction mapping suggest that this region is identical to the 3′ untranslated region of the brain-specific αCaMKII transcript. This possibility is confirmed by the fact that both transcripts are coded for by the same gene (see below). In nucleotide position 663 of the region encoding the association domain, a C was found instead of a T as in the published sequence of αCaMKII (position 1335 [14]). This C was also found by sequencing an αCaMKII cDNA clone isolated from a brain cDNA library. The difference from the published sequence does not result in an amino acid change and is most likely due to the fact that different mouse strains were used for the isolation of RNA.

The sequence shown in Fig. 2 contains a major open reading frame of 600 nucleotides beginning with a potential ATG initiator codon at nucleotide 294 and ending with a TGA terminator codon at position 894. The encoded protein contains 200 amino acids and has a calculated molecular mass of about 25 kDa. As shown below, a protein of that size was found in skeletal muscle extracts by immunoblotting, indicating that the postulated open reading frame is indeed translated.

The schematic drawing in Fig. 3A shows the structural relationship at the cDNA level between the skeletal muscle-specific αKAP transcript identified in this study and the previously described brain-specific transcript of the αCaMKII gene. The region coding for the association domain of αCaMKII and the 3′ untranslated region are shared by the two cDNAs. However, in the case of αKAP, 33 nucleotides (residues 411 to 443 in Fig. 2) have been inserted at the 5′ end of this region. These results are supported by the RT-PCR analysis shown in Fig. 4 (lanes AK1 and AK15). The cDNA fragment obtained by amplification with oligonucleotide AK15 as the 5′ primer is about 33 bp longer in skeletal muscle than in the brain. At the protein level, insertion of the corresponding 11 amino acids (residues 40 to 50 in Fig. 2) has introduced a potential nuclear localization signal (^{39}KKRK) which is closely related to that of simian virus 40 T antigen (18).

As expected from our initial experiments, sequences corresponding to the catalytic and regulatory domains of αCaMKII are missing in αKAP, and therefore RT-PCR analysis with oligonucleotides from this region detects a transcript in the

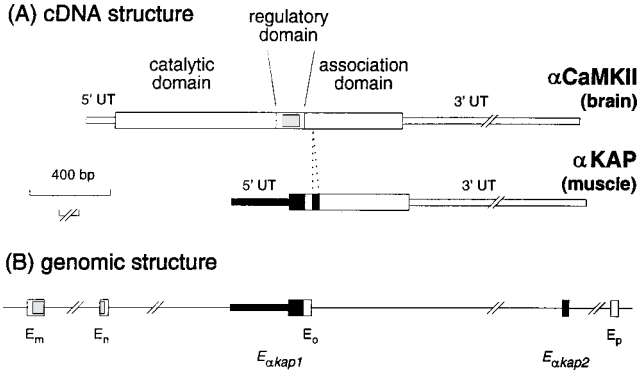

FIG. 3. Schematic representation of cDNAs and genomic structures of αCaMKII and αKAP. (A) Comparison of cDNA structures of αCaMKII and αKAP. Boxed areas indicate translated regions. The protein domains were assigned as specified by Lin et al. (22). The calmodulin binding site within the regulatory domain is shaded. The untranslated regions at the 5′ and 3′ ends are labeled 5′ UT and 3′ UT, respectively. Black areas represent regions present only in the muscle-specific αKAP transcript. For details, see Results. (B) Partial genomic structures of the αCaMKII and αKAP genes. As the complete exon-intron structure of the αCaMKII gene is not known, exons were arbitrarily designated E_m, E_n, E_o, and E_p. The exons specific for αKAP are in black and designated $E_{\alpha kap1}$ and $E_{\alpha kap2}$. The part of exon $E_{\alpha kap1}$ that is not drawn in black represents exon E_o of the αCaMKII gene (see Results for details). Exons correspond to the following nucleotide residues of the published αCaMKII cDNA sequence (14): E_m, 948 to 1031, E_n, 1032 to 1074, E_o, 1075 to 1115; and E_p, 1116 to 1164. Exons correspond to the following nucleotide residues of the αKAP cDNA sequence shown in Fig. 2: $E_{\alpha kap1}$, 1 to 410, $E_{\alpha kap2}$, 411 to 443; and E_p, 444 to 492.

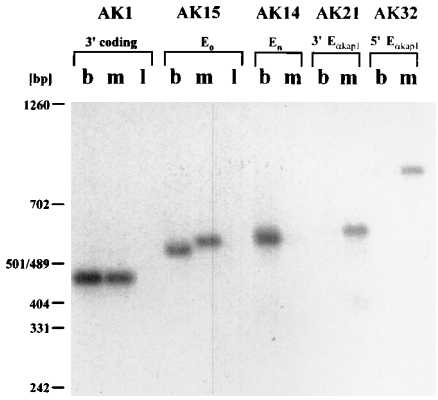

FIG. 4. Structure and expression of the αKAP cDNA as revealed by RT-PCR analysis. RNA from brain (b), skeletal muscle (m), and liver (l) was amplified by RT-PCR. The positions and sequences of oligonucleotides used in this analysis are underlined in Fig. 2. The only exception is oligonucleotide AK14, which is not present in αKAP and whose sequence corresponds to positions 1043 to 1062 of the region coding for the regulatory domain of αCaMKII (14). In all reactions, an oligonucleotide complementary to AK2 was used as the 3′ primer. The 5′ primers (AK1, AK14, AK15, AK21, and AK32) used for the different reactions are indicated above the lanes. The positions of these primers with respect to the different exons of the αKAP gene shown schematically in Fig. 3B are also indicated. After electrophoresis and blotting, filters were hybridized to radioactively labeled oligonucleotide AK17.

brain but not in skeletal muscle (Fig. 4, lane AK14). The 5′ untranslated region as well as the first nucleotides of the translated region (nucleotides 294 to 369; Fig. 2) of the αKAP transcript show no homology to any of the published CaMKII sequences. RT-PCR amplification of RNA by using oligonucleotides from this region confirmed that transcripts containing these sequences are present more or less exclusively in skeletal muscle (lanes AK21 and AK32; Fig. 4). After higher rounds of PCR amplification and/or longer exposure times of the blot, these sequences could also be detected in RNA from the brain, heart, and uterus (data not shown). The peptide (amino acid residues 1 to 25; Fig. 2) encoded by the αKAP-specific sequence consists of a cluster of hydrophobic amino acids typical of membrane-spanning domains. Taken together, these structural features clearly suggest that αKAP is not a protein kinase, and therefore its physiological role in the cell must be quite different from that of αCaMKII.

The αKAP transcript is derived from the αCaMKII gene by alternative promoter usage and RNA splicing. The above-described observation that sequences corresponding to the association domain are shared by the αCaMKII and αKAP transcripts suggests that the two transcripts arise from the same structural gene by alternative promoter usage and/or RNA splicing. We therefore screened a mouse genomic λ library with a probe derived from a subfragment of the αKAP cDNA and isolated several overlapping λ clones. To analyze in more detail the structural relationship between the two transcripts, part of the exon-intron structure of the gene was determined. For this purpose, genomic λ clones were characterized by restriction mapping and partial sequence analysis using oligonucleotide primers deduced from the cDNA sequences of the two transcripts. Exon-intron junctions were identified by comparison of genomic and cDNA sequences. In all cases, canonical splice donor-acceptor sequences were present at exon-intron junctions. Figure 3B schematically shows an exon-intron map of the region relevant for this study. Exons E_m and E_n code for the regulatory domain and are not present in the αKAP transcript. Exons E_o and E_p are found in both transcripts and encode the variable region and the N-terminal end of the association domain. Assignment of these exons to different protein domains is based on sequences of the mouse and rat αCaMKII cDNAs (2, 14, 22) and on comparison with the exon-intron structure of the closely related mouse βCaMKII gene (20).

There are two exons, $E_{\alpha kap1}$ and $E_{\alpha kap2}$, in Fig. 3B, which are specific for the αKAP transcript. Exon $E_{\alpha kap1}$ represents the most 5′ exon of the αKAP transcript and contains all of exon E_o plus 369 bp of sequence 5′ to E_o which are not present in the αCaMKII transcript. Addition of these new sequences, which are intronic with respect to the αCaMKII transcript, is obviously due to skeletal muscle-specific transcription initiation in this intron and to the fact that the splice acceptor site ^{368}AG/G is not used. The new 369-bp sequence codes for the 5′ untranslated region, for the start ATG, and for the N-terminal strongly hydrophobic region of αKAP. Primer extension analysis confirmed that transcription of the truncated RNA in skeletal muscle initiates at the 5′ end of the above-described αKAP cDNA (Fig. 5). These results were also supported by nuclease S1 mapping experiments (data not shown). The putative promoter region 5′ to the transcriptional start site does not contain a TATA box or CAAT box motif, both of which are common to a variety of regulated eukaryotic promoters. However, sequence motifs characteristic for muscle-specific promoters such as MyoD binding sites are present (data not shown). Further structural and functional studies are necessary to define this region. The other exon, specific for αKAP, designated $E_{\alpha kap2}$ in Fig. 3B, is derived by alternative splicing and lies in between two exons shared by the two transcripts. As mentioned above, insertion of $E_{\alpha kap2}$ creates a

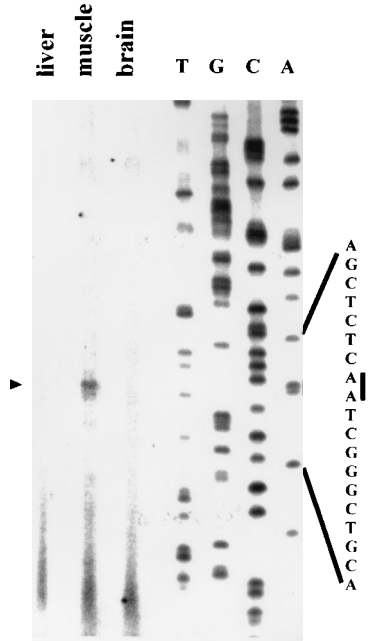

FIG. 5. Mapping of the transcriptional start site of the αKAP gene. An oligonucleotide complementary to the coding sequence AGACTGAAAC AG CAACCCTT (positions 68 to 87; Fig. 2) was end labeled, hybridized to RNA from the liver, skeletal muscle, and brain, and extended with reverse transcriptase as described in Materials and Methods. The extension products were separated on a gel along with a sequencing ladder (lanes T, G, C, and A) in which the same oligonucleotide was used to prime sequencing reactions from the cloned promoter region. The vertical bars from the A residues indicate the transcriptional start sites.

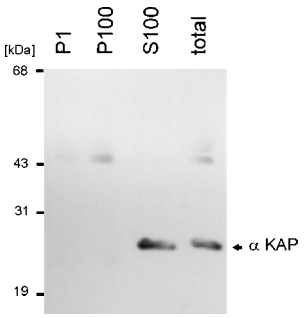

FIG. 6. Subcellular location of the αKAP protein. Mouse skeletal muscle tissue was fractionated into crude nuclear (P1), membrane (P100), and cytoplasmic (S100) fractions as described in Materials and Methods. Aliquots of each fraction were analyzed by immunoblotting with an antiserum against the recombinant αKAP protein.

putative nuclear localization signal. In summary, the αKAP transcript present in skeletal muscle is derived from the αCaMKII gene by alternative promoter usage and RNA splicing.

The αKAP cDNA encodes a 25-kDa protein that is associated with sarcomeres of skeletal muscle fibers. To identify the protein encoded by the αKAP transcript, antibodies were raised against the recombinant protein deduced from the cDNA sequence shown in Fig. 2. For this purpose, a partial cDNA clone containing the postulated open reading frame with the exception of the 5′ region that encodes the N-terminal 18 hydrophobic amino acids was fused to a histidine tag and overexpressed in *E. coli*. The purified recombinant protein was used to raise antibodies in rabbits. When Western blots (immunoblots) containing protein extracts from different mouse tissues were probed with an anti-αKAP antiserum, a protein of about 25 kDa was detected in skeletal muscle extracts (Fig. 1B). This size is in good agreement with the size of the protein inferred from the open reading frame of the cDNA sequence (Fig. 2) and makes it unlikely that the ATG codon in nucleotide position 441 (Fig. 2) is used for initiation of translation, as this would result in protein with a molecular mass of about 18 kDa. In agreement with the RNA data, minor amounts of the 25-kDa protein were also found in heart, uterus, and brain extracts but not in extracts from the liver. A protein corresponding in molecular weight to the mouse αKAP was detected in extracts from rat skeletal muscle, indicating that there was a rat homolog to the mouse protein identified in this study (data not shown). Because of the common association domain, the anti-αKAP antiserum recognized in brain extract a protein of 54 kDa, corresponding to the molecular mass of brain-specific αCaMKII (22). As expected, this protein was not present in skeletal muscle extracts. The antiserum seems to be specific for the association domain of the α isoform, as bands corresponding to the β, γ, and δ isoforms, which are expressed in skeletal muscle (20, 42), were not detected by immunoblot analysis (Fig. 1B).

Of particular interest was the subcellular localization of αKAP, as its sequence contains a potential nuclear localization signal and a stretch of hydrophobic amino acids which might serve as a membrane anchor. Therefore, skeletal muscle tissue was fractionated into nuclear, cytoplasmic, and membrane-containing fractions, and aliquots of each fraction were analyzed by immunoblotting. The results in Fig. 6 show that the protein was present only in the S100 supernatant. A nuclear location of αKAP or a tight association with membranes is therefore unlikely.

Evidence for a specific localization of αKAP came from immunostaining of sections of rat hindleg skeletal muscle with the anti-αKAP antiserum. As shown in Fig. 7, a relatively weak yet distinct labeling signal was found associated with the typical cross-striation pattern of skeletal muscle tissue. We tentatively assigned the antibody decoration to the I band. This conclusion was based on phase-contrast examination of immunoperoxidase-labeled frozen sections, which allowed us to identify the Z line in the middle of the stained band in areas where an optimal longitudinal section plane was achieved (data not shown). However, further experiments are needed to confirm our interpretation.

DISCUSSION

The results presented here show that two functionally different proteins are encoded by the αCaMKII locus: a previously described brain-specific Ca^{2+}/calmodulin-dependent protein kinase and a non-protein kinase, αKAP, that is predominantly found in skeletal muscle. An RNA corresponding in length to the αKAP transcript has previously been detected in the rat skeletal muscle and diaphragm by using a probe containing part of the region coding for the association domain of αCaMKII (42). The relationship between αKAP and αCaMKII is clearly one of a gene within a gene. The transcription initiation site, the 5′ noncoding region, and part of the 5′ coding region including the AUG start codon of the muscle-specific αKAP mRNA correspond to intron sequences in the brain-specifically expressed αCaMKII gene. These intron sequences are directly adjacent to an exon of the αCaMKII gene and together form the 5′-most exon of the αKAP gene (Fig. 3). The αKAP transcriptional start site and the skeletal muscle-specific promoter are located within the intron separating the regulatory domain and the variable region of the association domain. The complete exon-intron structure of the αCaMKII gene has not been determined, and therefore the location of this intron relative to that of the brain-specific αCaMKII promoter is unknown. However, preliminary genomic mapping studies indicate that the skeletal muscle-specific and the brain-specific promoters are at least 10 kb apart. This spacing should allow independent regulation by tissue-specific transcription factors. The fact that minute amounts of the αKAP mRNA are also present in the brain might suggest that the αKAP promoter is not strictly muscle specific and that opening up of the chromatin structure during transcription in the brain might activate a basal activity of the promoter that does not require muscle-specific factors. Experiments aimed at defining the *cis*- and *trans*-regulatory elements of the αKAP promoter are in progress.

The αCaMKII and αKAP genes share the region corresponding to the association domain and the 3′ untranslated region. The only difference is a 33-bp insertion that is specific for αKAP. As these 33 bp correspond to an exon (E_{kap2} in Fig. 3), insertion is most likely the result of alternative splicing. The fact that this insert was not found in brain-specific transcripts of the αCaMKII and αKAP genes suggests that at least in the mouse, this splicing event is muscle specific (1). Insertion of this exon has occurred in the variable region of the association domain. This region shows considerable length variation in different CaMKII isoforms and splice variants (see, for example, reference 12). Sequences identical or related to the αKAP-specific exon have also been found in variants of the CaMKII α, γ, and δ isoforms of monkeys and rats (3, 12, 37, 42).

In most previously described cases in which more than one protein is expressed from a single genetic locus, alternative RNA initiation and splicing produced functionally related pro-

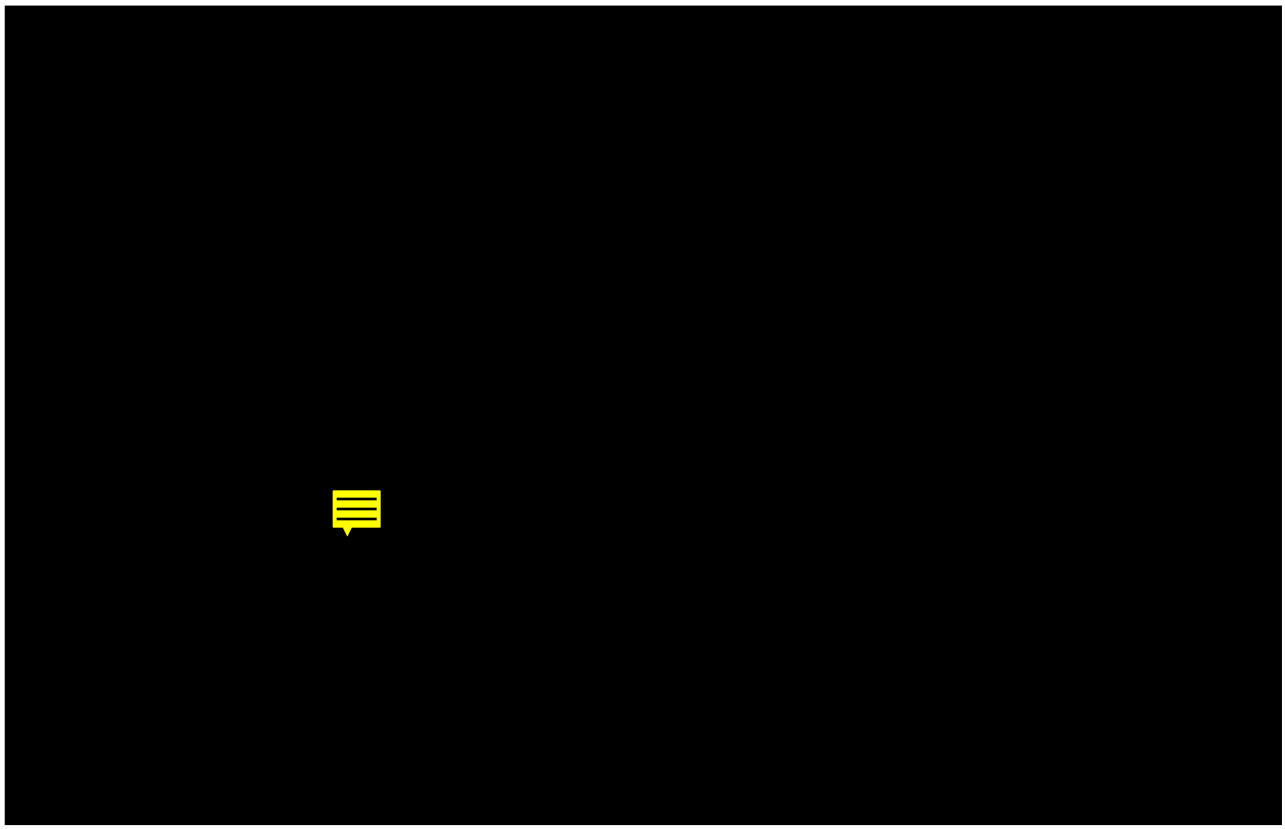

FIG. 7. Immunofluorescence localization of αKAP in skeletal muscle tissue by use of a triple-layer avidin-biotin method (magnification, ×700). Frozen sections of rat hindleg skeletal muscle were incubated with a rabbit anti-αKAP antiserum (A) or, as a control, with normal rabbit serum (B) as described in Materials and Methods.

teins, usually in different tissues and/or at different developmental stages (see reference 25 for a review). However, there are also a few precedents wherein two functionally different proteins are derived from the same genetic locus, similar to what has been shown here for αCaMKII and αKAP (9, 11, 16, 26, 31). Remarkably, two of these cases involve Ca^{2+}/calmodulin-dependent protein kinases: the locus for CaMKIV in rat encodes the protein kinase and a male germ-specific calmodulin-binding protein, calspermin (26, 31, 40); the locus for myosin light-chain kinase in the chicken encodes a Ca^{2+}/calmodulin-regulated protein kinase and a calmodulin-binding protein, KRP, that has no kinase activity (9). In contrast to αKAP, both calspermin and KRP have retained the calmodulin binding sites present in their respective protein kinases. Although most likely not a frequent event, the functional diversity of a gene as described here for the αCaMKII gene has to be taken into account when one analyzes the phenotypes of knockout mice. However, in the case of the αCaMKII knockout mouse, it is unlikely that transcription of the αKAP gene has been affected, as the mutation was introduced into the genomic region representing the catalytic domain (38).

The amino acid sequence of αKAP suggests a two-domain structure of the protein: an N-terminal hydrophobic domain specific for αKAP and a large C-terminal domain identical to the association domain of αCaMKII. These two domains are separated by a variable region (amino acid residues 26 to 50) which might serve as a linker as has been discussed for different CaMKII isoforms (19). Part of this variable region (amino acid residues 26 to 39) is a conserved peptide present in all CaMKII isoforms and variants. In the case of the α isoform, it is encoded by exon E_o (Fig. 3). The other half of the variable region is specific for αKAP and corresponds to exon $E_{\alpha kap2}$ (Fig. 3). Insertion of this αKAP-specific peptide creates at its border a potential nuclear localization site closely related to that of simian virus 40 T antigen (18). Subcellular fractionation of skeletal muscle tissue, however, gave no evidence for a nuclear location of αKAP. Recently another example in which alternative splicing in the variable region introduces an identical nuclear localization signal in a CaMKII isoform has been described (39). In this case, evidence that this sequence motif is required for targeting the protein to the nucleus was presented. The two results are not contradictory, as it is clear from other studies that the molecular context of the nuclear localization signal very much determines its targeting function (10).

The physiological function of αKAP is unknown, and therefore the following discussion is based on indirect evidence obtained from its primary structure and subcellular location. The two-domain structure in combination with the specific subcellular location leads us to propose that αKAP may serve as an anchoring protein for CaMKII molecules. Specific anchoring proteins located at various sites within the cell are thought to compartmentalize protein kinases to their sites of action and thereby provide specificity of the responses mediated by each kinase. Anchoring proteins have been described for protein kinases A and C but not for Ca^{2+}/calmodulin-dependent kinases (see reference 28 for a review). Recently, however, proteins binding to CaMKII were identified by the use of a gel overlay assay (25a). In its simplest version, our model predicts that αKAP functions as an anchoring protein by binding CaMKII molecules via the association domain and

by being attached via its N-terminal hydrophobic domain to a specific subcellular structure or molecule, which might serve directly as a substrate or might be close to a substrate for the kinase. Interaction of CaMKII association domains has been shown by different experimental approaches (see reference 33 for a review).

The role of the N-terminal domain of αKAP as an anchor peptide is largely speculative. As this domain is highly hydrophobic, we tested the possibility that it attaches αKAP to membranes of the skeletal muscle sarcoplasmic reticulum and thereby targets CaMKII molecules to a subcellular structure that has been shown to be associated with this type of protein kinase (7, 44). However, the subcellular fractionation experiments shown in Fig. 6 do not support this hypothesis and make it unlikely that αKAP is inserted into membranes via its N-terminal domain.

The best evidence for a specific location of αKAP in the cell came from immunostaining of skeletal muscle sections with anti-αKAP antibodies (Fig. 7). The results suggest that αKAP is associated with sarcomeres of skeletal muscle fibers, most likely with the actin-containing I band. The nature of this association is unclear at present. The observation that in the cell fractionation experiments αKAP is found in the high-speed supernatant and not in the pellet, where one would expect myofibrils and associated proteins, suggests that αKAP is not an integral part of the myofibril and that its association can be disrupted in low-ionic-strength buffer. There is some evidence that low-ionic-strength buffers favor CaMKII solubilization (20a). However, further experiments are needed to support this hypothesis and to show which domain of the αKAP molecule interacts with which protein of the sarcomeres. Earlier in vitro studies have suggested an association between purified skeletal muscle actin and brain CaMKII (32). Therefore, actin may be a candidate for the interaction with αKAP. The identification and characterization of anchoring proteins for CaMKII will be a major contribution to understanding the diverse functions of this family of multifunctional protein kinases.

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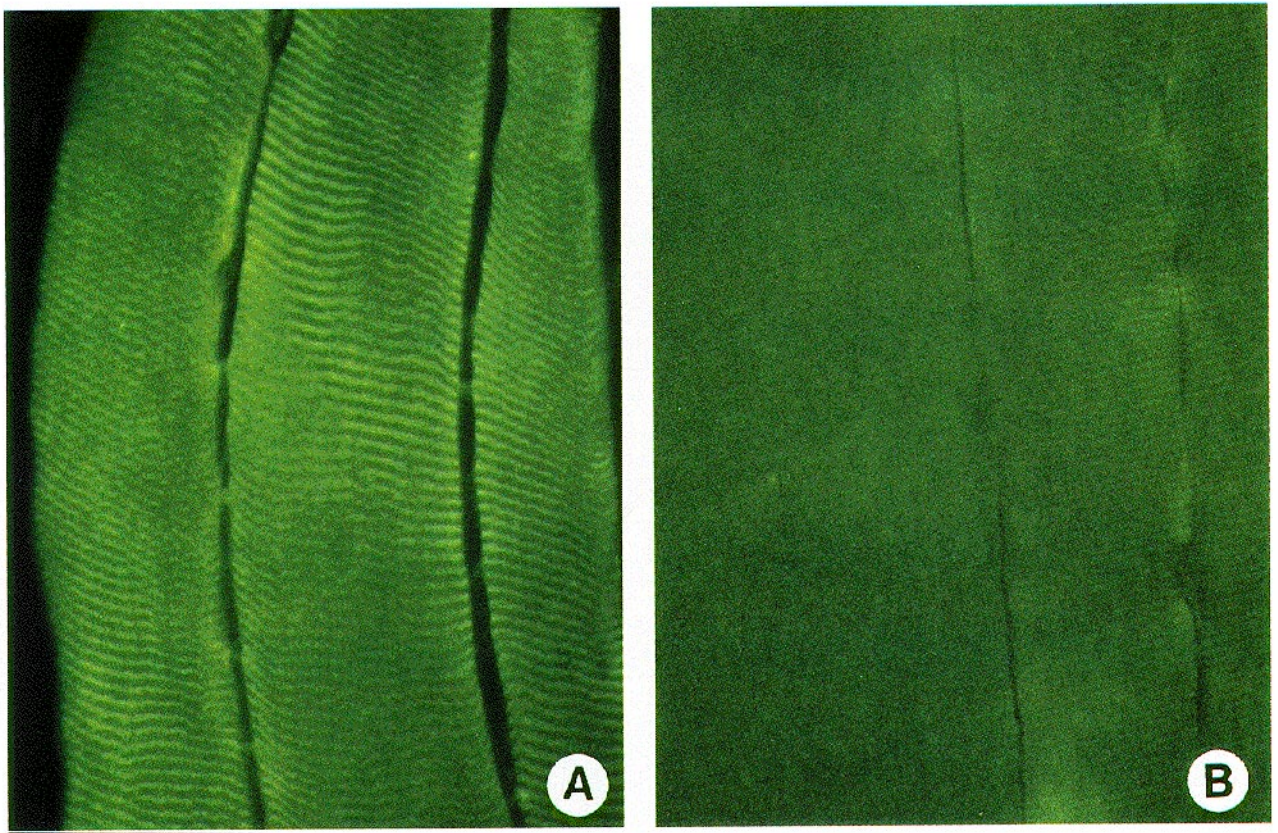
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