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Molecular Characterization of AKAP149, a Novel A Kinase Anchor Protein with a KH Domain

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The cytosolic cAMP activates in eukaryotic cells several isoforms of cAMP-dependent protein kinase (PKAs) involved in signal transduction. The effects of individual PKA isoforms are determined by their cellular localisation, specified through binding to distinct A Kinase Anchor Proteins (AKAPs). A new member of the AKAP family, a membrane-anchored 903 amino acid long protein, designated AKAP149, is characterized in the present work. It is a putative splicing variant of S-AKAP84 with the important new feature of a RNA-binding motif (KH domain). This domain together with the known characteristics of AKAPs suggests the involvement of AKAP149 in the phosphorylation-dependent regulation of RNA-processing.

One of the best characterized second messenger systems in eukaryotes is mediated by the cAMP which activates the cAMP-dependent protein kinases (PKAs). PKAs phosphorylate a wide range of substrates (1).

The PKA heterotetramers are composed of a dimeric catalytic subunit (C) and of a dimeric cAMP-binding regulatory subunit type I (RI) or type II (RII). In the absence of cAMP the catalytic activity is inhibited by the R subunit. The binding of cAMP to R abrogates the inhibition and activates C. While substrate specificity and kinetic rates of catalytic subunits are identical in PKA isoforms, the regulatory subunits exhibit different affinities for cyclic nucleotides analogs and different subcellular locations.

For example, the cAMP-dependent protein kinases with the type I regulatory subunits (type I PKAs) are predominantly cytoplasmic, while type II PKAs may be associated with a particulate fraction of the cell, e.g. the plasma membrane, cytoskeletal components, secretory granules, the Golgi apparatus, centrosomes or the nuclei. The association with particular organelles is the result of binding of the regulatory subunits to the A kinase anchoring proteins (AKAPs, for review see 2, 3).

AKAPs are proteins that possess both a high affinity binding site for PKA subunits RIIα and RIIβ and an independent domain that targets RII-AKAP complexes to specific intracellular sites (2, 3). This arrangement can place different PKA isoforms in proximity of organelle-bound substrates. It has been proposed that the cellular location of particular PKA isoforms determines which substrates are phosphorylated and thereby bestows the site-specificity on the diverse cAMP-mediated signals (1, 2, 3).

In the present work the molecular structure of AKAP149, a new member of the AKAP family, which was named according to its size in SDS-PAGE, has been elucidated. A novel feature of AKAP149 is a KH domain with a RNA-binding potential (4) which suggests functions hitherto not ascribed to AKAPs.

The complete sequence of AKAP149 is available under accession number X97335 in *EMBL database.*

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MATERIALS AND METHODS

Screening of a cDNA expression library with a rabbit antiserum. A λgt11-expression cDNA library (Clontech, Palo Alto, CA) made from RNA isolated from human colonic tissue was screened with a rabbit antiserum induced against colon carcinoma-associated mucins purified on CsCl gradient as described previously (5). Of the 19 clones detected with the serum 3 (B4, B5, M2) originated from one gene which was investigated in the present work.

DNA sequencing and sequence analysis. Radioactive sequencing was performed by the dideoxynucleotide chain termination procedure using a T7 Sequencing Kit (USB/Amersham, Cleveland, Ohio) as described elsewhere (6). Alternatively, cycle sequencing with dyedideoxynucleotides (Perkin Elmer, Weiterstadt, Germany) was performed on an automatic sequencer (373A; ABI /Perkin Elmer, Weiterstadt, Germany). Computer sequence analysis was performed using the HUSAR program package provided by the GENIUS server at the German Cancer Research Center (DKFZ), Heidelberg, Germany. Data base searches were performed using FASTA (7) and BLASTN (8). Protein motif recognition was performed using PROSITE.

Probe generation. A 581bp DNA fragment (B5) detected in the λgt11 library, was used as a probe. It was PCR-amplified, purified by ion exchange HPLC and labeled with [^{32}P]dCTP (6000 Ci/mmol, NEN DuPont, Bad Homburg, Germany) by random DNA labeling kit (Boehringer Mannheim, Mannheim, Germany).

Southern blotting. DNA isolated from human colonic tissue according to a standard protocol (6) was separately digested with 6 rare cutting enzymes. The six digests, 17μg DNA each, were separated on a 1% TAE-agarose gel and capillary blotted onto a nytran membrane (Schleicher and Schuell, Dassel, Germany) according to a standard protocol (6) and hybridized with B5 as a probe.

Northern blotting. A multiple tissue Northern blot loaded with 2 μg mRNA isolated from different human tissues (Clontech Lab.Inc, Palo Alto, CA) was hybridized with B5 probe according to the protocol of the manufacturer.

Rescreening of cDNA libraries. About 2×10^5 colony forming units of an oligo-dT-primed λ ZAP II cDNA library (Stratagene, La Jolla, USA) custom-made from RNA isolated from human colonic carcinoma cell line 5583-S (9) were screened with ^{32}P-labeled B5 probe or with a 388 bp long [^{32}P]dCTP-labeled 5′-terminal Pst I-restriction fragment in a second round for additional cDNA clones.

Production and purification of antibodies directed against AKAP149 peptide. Strongly immunogenic peptide sequences of AKAP149 were selected with the PREDITOP program (10). A 20-mer peptide (CAITPPLPESTVPFS-NGVLK, AKAP149 residues 531−549) was conjugated to activated BSA (Pierce, Rockford, IL) and used for immunization of a rabbit. IgGs were isolated from the serum by affinity chromatography on protein A-Sepharose column (Pharmacia, Freiburg, Germany). Anti-peptide-IgG was obtained by affinity chromatography on SulfoLink matrix (Pierce, Rockford, IL), modified with the 20-mer peptide. The reactivity of the antiserum with the peptide was tested in ELISA as described previously (11).

Western blotting. Human colonic carcinoma cells LS174T were homogenized with an Ultraturrax (IKA, Staufen, Germany) and separated into soluble and particulate fraction by centrifugation at 40.000 × g for 1 h at 4°C. Aliquots of each fraction were separated on 6% SDS-PAGE and blotted onto Immobilon P membrane (Millipore, Bedford, MA) as described previously (5). The proteins were detected with the antiserum (1: 5000) or with the affinity-purified anti-AKAP149 IgG (1:50), peroxidase-labeled protein A and ECL-detection-system (Amersham Int., Buckinhamshire, UK). Preadsorbtion of antiserum (2μl) with synthesized peptide (40μg) was performed at room temperature for 2.5 h prior to detection.

RESULTS

1. Isolation and characterization of cDNA fragments. After initial screening of a λgt11-cDNA expression library with a rabbit antiserum, 3 different DNA fragments were obtained, which yielded different restriction patterns but when used as probes, gave the same Southern blot pattern. Sequencing confirmed that these clones, termed B4 (509bp), B5 (581bp) and M2 (909bp) originated from the same gene due to their overlapping sequences. Their relative positions are shown in Fig.1.

To obtain further fragments, a λZAPII cDNA library was rescreened with the B5 probe. Four additional clones (T1, T2, T3, T4) were obtained. A second round of screening with a 388 bp long ^{32}P-labeled 5′-terminal Pst I-restriction fragment (a portion of T1) yielded 3 new clones (T5, T6, T7) (Fig.1).

2. Complete sequence of AKAP149 cDNA and the deduced domain structure. The complete sequence of the obtained cDNA fragments yielded a 3758bp long sequence (EMBL database, accession number X97335) with a 2712 bp long open reading frame. At position 100, the identical consensus sequence of the 7 clones (T1−T7) is beginning. Upstream of that position

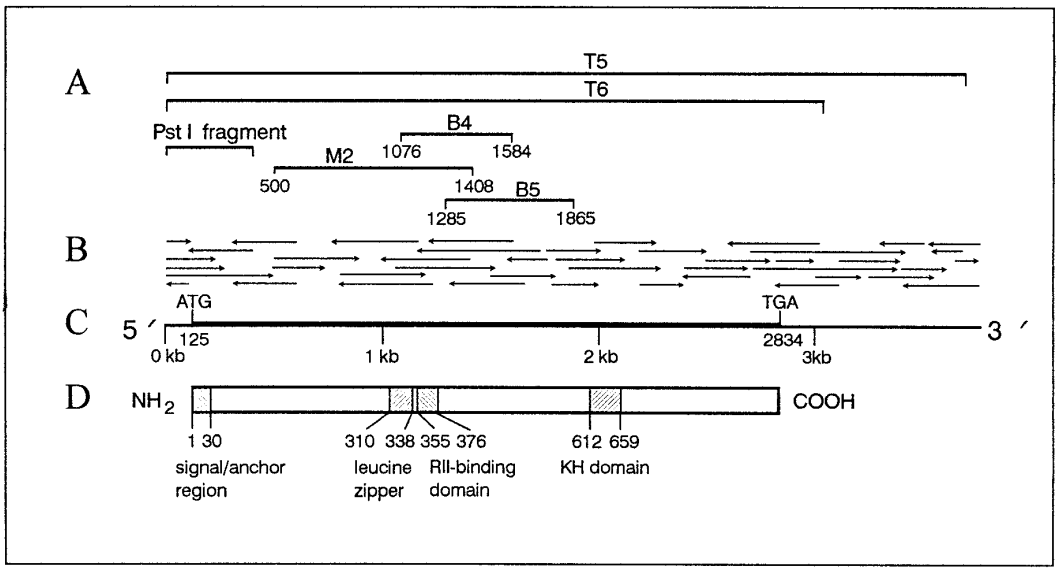

FIG. 1. Relative location of the obtained fragments (**A**) and the direction of sequencing (**B**) of the AKAP149 gene (**C**). Structural domains of AKAP149 (**D**).

in all clones except T4 (which has an unique 65 bp long 5′-sequence upstream of pos.100) there is a common sequence with a G/C content of 83%.

The open reading frame starts at position 125 with a start codon within a sequence fullfilling Kozak criteria for translation initiation (12). An upstream stop codon is located at position 104 and a downstream stop codon is at position 2834, resulting in a protein of 903 amino acids with an expected molecular weight of 97336.2 Da and an isoelectric point of 4.68.

DNA database comparison revealed that a part of the obtained sequence is almost completely identical to the recently described sequence of A kinase anchor protein S-AKAP84 (accession number U34074) (13). According to its size in SDS-PAGE the new protein was named AKAP149. The predicted protein sequence of AKAP149 is identical to S-AKAP84 in the first 571 amino acids except Q and T at amino acids 97–98 instead of H and P. AKAP149 and S-AKAP84 have in common the N-terminal signal/anchor region, which is assumed to anchor S-AKAP84 in the outer mitochondrial membrane thus exposing the catalytic domains in the cytoplasm (13), the leucine zipper motif and the RII-binding domain.

Moreover, there are several sites matching the consensus sequence for phosporylation by casein kinase II (pos. 40, 41, 79, 129, 135, 197, 468, 513, 518, 525) or protein kinase C (pos. 33, 129, 134, 157, 164, 525, 572, 585). At the position 195 there is a potential cAMP- and cGMP-dependent protein kinase phosphorylation site and at the position 247 a ATP/GTP-binding site motif A (P-loop) (14). In contrast to S-AKAP84, the open reading frame of AKAP149 continues to position 2834, resulting in a P/S/T-rich region with 50% of the amino acids being proline, serine and threonine (amino acids 489–540). Most importantly, AKAP149 exhibits a consensus sequence of the KH motif (amino acids 612–659) (Fig.2).

3. Southern and Northern analysis. After digestion of human colonic DNA with Hind III, Bam HI, Eco RI, Pst I and Hinf I and blotting, single bands at 9.2, 23, 15, 1.75 and 1.1 kb respectively, were obtained with the B5 probe. Bgl I digest gave three bands at 12, 8.4 and 7.6 kb (Fig. 3A). These data show the specificity of the B5 probe and indicate that it recognizes a single copy gene.

The same probe detected on Northern blot an abundant mRNA with Mr of about 4.2 kb in

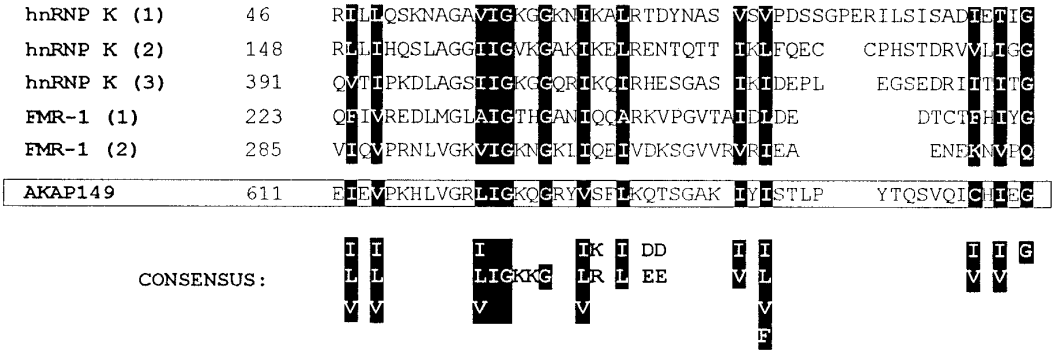

FIG. 2. KH domain alignment. KH domain sequences of human hnRNP K and FMR1 are aligned with the sequence of AKAP149. Numbers on the left indicate positions of the initial amino acid of the KH domain. Highly conserved positions are highlighted, and gaps are introduced for optimal alignment. The consensus sequence of the KH domain was derived from Siomi et al. (18, 19).

RNAs from all investigated epithelial tissues, the strongest signal being in the prostate- and small intestine-mRNA. Additionally, a very strong signal was detected in the testis at 3.2 kb (Fig.3B). After longer exposure, the 4.2 kb signal was visible also in mRNA from spleen and thymus but not from peripheral blood lymphocytes (PBL).

4. Characterisation of AKAP149 protein. The expression of the protein in the colon carcinoma cells LS174T was detected by Western blotting of the particulate cell fraction. After SDS-PAGE under denaturating conditions and blotting, a 149 ± 10 kDa (median ± SD of three determinations) band was detected with the antiserum directed against AKAP149 peptide but not by the preimmune serum (Fig.4; A,B). To further test the specificity of this reaction, the antiserum was preadsorbed with the peptide prior to application to the Western blot. The 149 kDa band was not detected after serum preadsorbtion, whereas the other bands, detected

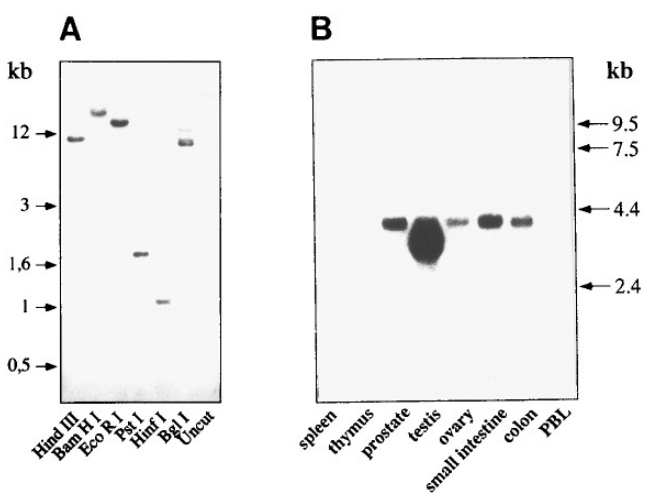

FIG. 3. (**A**) Southern blot of human colonic DNA (17μg/ lane) probed with ^{32}P-labeled B5 fragment of AKAP149. The restriction enzymes are given below each lane. Molecular weights, given in kb, were determined by running in parallel a DNA ladder and staining the gel with ethidium bromide prior to blotting. (**B**) Northern blot of mRNA (2μg per lane) isolated from different human tissues, probed with ^{32}P-labeled B5 probe. The gel was calibrated in kb by running a RNA ladder in parallel and staining with ethidium bromide.

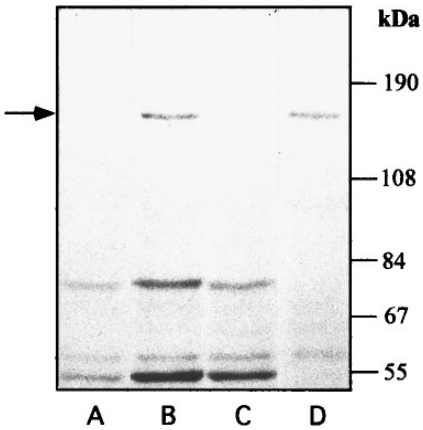

FIG. 4. Detection of AKAP149 in Western blot. Rabbit antiserum directed against a synthetic peptide derived form the B5 fragment was used for detection of the whole AKAP149 protein in the particulate fraction of LS174T cells, obtained as described in Methods, separated on 6 % SDS-PAGE and blotted. (**A**) Binding pattern of the preimmune serum. (**B**) Binding of the anti-peptide-antiserum. The band reactive only with the antiserum is indicated by an arrow. (**C**) Binding of the antiserum preadsorbed with the peptide. (**D**) Binding of the affinity-purified anti-peptide-IgG.

already with the preimmune serum, remained (Fig.4; C). Affinity-purified anti-peptide-IgGs recognized only the 149 kDa band (Fig.4; D). In accord with the expression pattern observed in Northern analysis, no 84 kDa band (S-AKAP84) was detectable in the colonic cell line LS174T.

DISCUSSION

In the present work a new member of A kinase anchor protein (AKAP) family, designated AKAP149, was cloned and characterized in detail.

The cloned cDNA comprised 3758 bp including the complete open reading frame of 903 amino acids. The calculated and experimentally obtained molecular weights (97,336.2 Da versus 149 kDa) were different, probably due to the high content of acidic amino acids and proline, as also observed for other similar proteins (2, 13).

Northern blot analysis revealed a mRNA of about 4.2−4.3 kb in length whereas the cloned cDNA revealed only 3758 bp, indicating that a portion of the untranslated region was not cloned. However, the major part of the 5′-untranslated region seemed to be determined since all but one cDNA clone started within a range of 33 bp. The total 3′-untranslated region was not determined since even in the clone extending over 900 bp beyond the 3′-end of the open reading frame, neither a polyadenylation signal nor a poly-A-tail was found.

The search for homologies in data bases showed, that the obtained sequence is, apart from the two amino acids at positions 97 and 98, identical over the 571 N-terminal amino acids to the sequence of the recently characterized S-AKAP84, a 593 amino acid long, developmentally regulated protein in mammalian spermatozoa (13). Furthermore, a 98.9% identity over 267 bp to a sequence-tagged site (accession number G06061), located on chromosome 17 was found, suggesting also a localization of the gene for AKAP149 on this chromosome.

The Southern blot hybridization with the B5 probe, which is detecting the genes for S-AKAP84 and AKAP149, reveals a pattern of a single copy gene. These data in combination with the finding that seven different clones comprised the unique AKAP149 sequence suggest that AKAP149 and S-AKAP84 are splicing variants of the same gene. This explains also the previously described Northern blot results, demonstrating in addition to the S-AKAP84 mRNA

(3.2 kb) a 4.2–4.3 kb mRNA. Whereas the S-AKAP84 mRNA is exclusively expressed in the testis (and not in colonic tissue), the larger mRNA is widely distributed. This finding is highly suggestive for different functions of either splicing variant.

The previously described structural features of S-AKAP84 are also found in AKAP149: the N-terminal signal/anchor sequence, the leucine zipper motif and the RII-binding domain, shown to bind the regulatory subunits (RIIα and RIIβ) of the cAMP-dependent protein kinases PKAIIα and PKAIIβ (13). The signal/anchor sequence has similarity to the sequence targeting the NADH-cytochrome b_5 reductase (13) to mitochondrial membrane. The S-AKAP84 was indirectly shown to be located in mitochondria. The presence of AKAP149 exclusively in the particulate fraction of LS174T colon carcinoma cell line cells as well as the previous data suggest that AKAP149 is located in a membrane, possibly mitochondrial.

In addition to these common properties of S-AKAP84 and AKAP149, the sequence analysis revealed a number of potential phosphorylation sites and an ATP/GTP binding site motif (P-loop) in the common region of these molecules. Although *in vitro* phosphorylation of AKAPs by PKA or PKC has been observed previously (1, 2), the physiological relevance of these sites in S-AKAP84 and AKAP149 is not clear at present.

The most striking property of AKAP149 gene is the region almost perfectly homologous to the consensus sequence of the KH motif (4), first described 1993 by Siomi et al. (15) and shown recently to be directly involved in RNA binding (16). Indeed, all hitherto described proteins (of which the function is known) with a KH domain are RNA-binding proteins. These proteins are involved in RNA catalysis, mRNA processing and translation (17).

Thus, the protein described in the present work combines the known functions characteristic for AKAPs with a KH RNA binding domain, hitherto not found in AKAPs. It is of interest, how these different domains contribute to the multifunctionality of AKAP149. Indeed, some AKAPs are multifunctional proteins, thought to deliver the cAMP-dependent protein kinases (PKAs) to special compartments in the cell and thus to contribute to the localized action of these kinases or even to merge different signal transduction pathways (20, 21). AKAP149, which is by virtue of its signal/anchor domain most probably located in the outer mitochondrial membrane, has also the potential of binding protein kinase A (via the RII binding domain) and of RNA molecules via the KH domain.

This suggests that the almost ubiquitous AKAP149 is involved in the cAMP-dependent signal transduction pathway and, at the same time, in directing RNA (22) to a special cellular compartment.

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