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Cloning and characterization of a cDNA encoding an A-kinase anchoring protein located in the centrosome, AKAP450

Oliwia Witczak, Bjørn S.Skålhegg, Guy Keryer[1], Michel Bornens[1], Kjetil Tasken, Tore Jahnsen and Sigurd Ørstavik[2]

Institute of Medical Biochemistry, Faculty of Medicine, University of Oslo, PO Box 1112, Blindern N-0317 Oslo, Norway and [1]Institut Curie, Biologie du Cycle Cellulaire et de la Motilite, 75248 Paris Cedex 05, France

[2]Corresponding author
e-mail: sigurd.orstavik@basalmed.uio.no

A combination of protein kinase A type II (RII) overlay screening, database searches and PCR was used to identify a centrosomal A-kinase anchoring protein. A cDNA with an 11.7 kb open reading frame was characterized and found to correspond to 50 exons of genomic sequence on human chromosome 7q21-22. This cDNA clone encoded a 3908 amino acid protein of 453 kDa, that was designated AKAP450 (DDBJ/EMBL/GenBank accession No. AJ131693). Sequence comparison demonstrated that the open reading frame contained a previously characterized cDNA encoding Yotiao, as well as the human homologue of AKAP120. Numerous coiled-coil structures were predicted from AKAP450, and weak homology to pericentrin, giantin and other structural proteins was observed. A putative RII-binding site was identified involving amino acid 2556 of AKAP450 by mutation analysis combined with RII overlay and an amphipatic helix was predicted in this region. Immunoprecipitation of RII from RIPA-buffer extracts of HeLa cells demonstrated co-precipitation of AKAP450. By immunofluorecent labeling with specific antibodies it was demonstrated that AKAP450 localized to centrosomes. Furthermore, AKAP450 was shown to co-purify in centrosomal preparations. The observation of two mRNAs and several splice products suggests additional functions for the AKAP450 gene.

Keywords: AKAP/cAMP/protein kinase A/centrosomes/microtubules

Introduction

The physiological effects of a large number of hormones and neurotransmitters are mediated by tightly regulated processes of protein phosphorylation and dephosphorylation by protein kinases and protein phosphatases. Key cellular processes such as metabolism, gene transcription, ion channel conductivity, the release of synaptic vesicle contents, cell growth and cell division utilize cyclic AMP (cAMP) as a second messenger in response to hormonal stimuli (Scott, 1991; Francis and Corbin, 1994). Cyclic AMP mediates most of its effects by activating the cAMP-dependent protein kinase A (PKA). PKA, which is a tetrameric holoenzyme, contains a regulatory (R) subunit dimer and two catalytic (C) subunits (Beebe and Corbin, 1986). To date, four genes encoding human R subunits (RIα, RIβ, RIIα and RIIβ) and three genes encoding human C subunits (Cα, Cβ and Cγ) have been identified (Skålhegg and Tasken, 1997). Binding of four cAMP molecules, two to each R subunit, activates the PKA holoenzyme resulting in the release of free active C subunits.

How the discrete physiological effects observed in response to cAMP can be mediated by the broad specificity protein kinase PKA has been subject to a great deal of speculation. A growing body of evidence demonstrates that anchoring of the regulatory subunit of PKA type II (RII) to A-kinase anchoring proteins (AKAPs) targets PKA in close proximity to relevant substrates conveying specificity in the cAMP/PKA signaling pathway (Scott, 1991; Rubin, 1994). Microtubule-associated protein 2 (MAP2) was the first protein identified as an AKAP, anchoring PKA to the microtubular network in neurons (Lohmann *et al*., 1984; Schwartz and Rubin, 1985). Since then, a large number of different AKAPs have been identified from different tissues and species. Some of these AKAPs are tissue specific, while others have a more ubiquitous distribution (Hausken and Scott, 1996). Although early studies on subcellular distribution of PKA demonstrated that centrosomes both in cultured cells (Nigg *et al*., 1985) and in the central nervous system (De Camilli *et al*., 1986) contain an anchoring site for PKA type II, none of the AKAPs cloned so far have been shown to target to centrosomes. PKA modifies microtubular dynamics or organization (Lane and Kalderon, 1994; Gradin *et al*., 1998) and it is anticipated that the centrosomal localization of PKA is of importance to these functions. To further investigate the function of PKA in centrosomes, we have therefore studied the molecular mechanism for PKA anchoring in centrosomes.

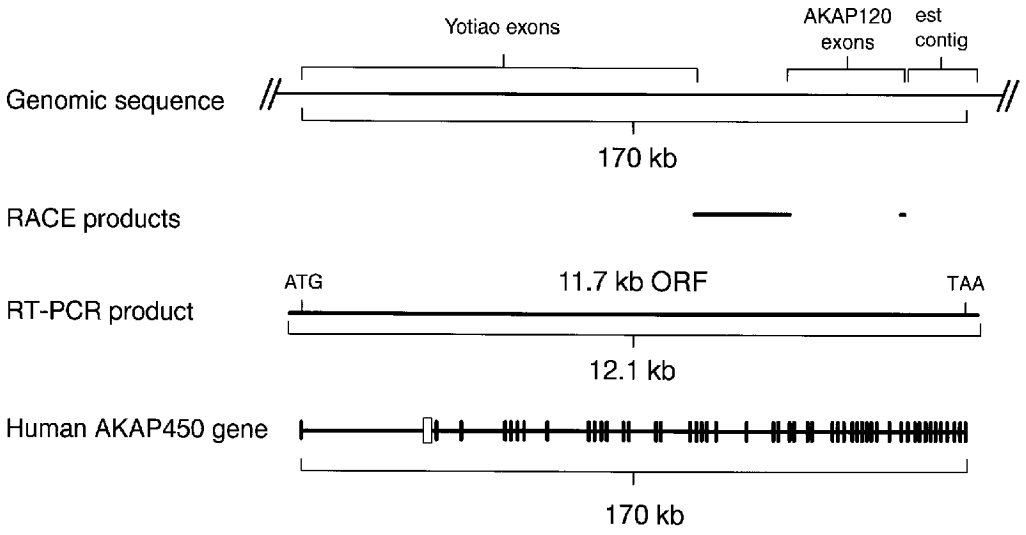
Previously, we characterized the biochemical properties of AKAPs located in human centrosomes (Keryer *et al*., 1993). An AKAP with an apparent mol. wt of 350 kDa co-purifies with centrosomes. In the present study, using a combination of RII overlay screening, database searches and PCR, we have identified and sequenced a 12.1 kb cDNA containing an 11.7 kb open reading frame (ORF) encoding a 453 kDa protein, designated AKAP450 (DDBJ/EMBL/GenBank accession No. AJ131693). AKAP450 was demonstrated to be co-immunoprecipitated with PKA type II, and by immunofluorescent labeling and centrosomal preparations it was shown that AKAP450 is targeted to centrosomes. AKAP450 represents a novel centrosomal protein with numerous coiled-coils as previously shown for several other centrosomal proteins.

Results

Cloning and sequencing of AKAP450 cDNA

The majority of PKA type II (RII) in lymphocytes has been shown to be located in the Golgi–centrosomal area

A



B

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1     MEDEERQKKL EAGKAKLAQF RQRKAQSDGQ SPSKKQKKKR KTSSSKHDVS AHHDLNIDQS QCNEMYINSS QRVESTVIPE STIMRTLHSG EITSHEQGFS
101   VELESEISTT ADDCSSEVNG CSFVMRTGKP TNLLREEEFG VDDSYSEQGA QDSPTHLEMM ESELAGKQHE IEELNRELEE MRVTYGTEGL QQLQEFEAAI
201   KQRDGIITQL TANLQQARRE KDETMREFLE LTEQSQKLQI QFQQLQASET LRNSTHSSTA ADLLQAKQQI LTHQQQLEEQ DHLLEDYQKK KEDFTMQISF
301   LQEKIKVYEM EQDKKVENSN KEEIQEKETI IEELNTKIIE EEKKTLELKD KLTTADKLLG ELQEQIVQKN QEIKNMKLEL TNSKQKERQS SEEIKQLMGT
401   VEELQKRNHK DSQFETDIVQ RMEQETQRKL EQLRAELDEM YGQQIVQMKQ ELIRQHMAQM EEMKTRHKGE MENALRSYSN ITVNEDQIKL MNVAINELNI
501   KLQDTNSQKE KLKEELGLIL EEKCALQRQL EDLVEELSFS REQIQRARQT IAEQESKLNE AHKSLSTVED LKAEIVSASE SRKELELKHE AEVTNYKIKL
601   EMLEKEKNAV LDRMAESQEA ELERLRTQLL FSHEEELSKL KEDLEIEHRI NIEKLKDNLG IHYKQQIDGL QNEMSQKIET MQFEKDNLIT KQNQLILEIS
701   KLKDLQQSLV NSKSEEMTLQ INELQKEIEI LRQEEKEKGT LEQEVQELQL KTELLEKQMK EKENDLQEKF AQLEAENSIL KDEKKTLEDM LKIHTPVSQE
801   ERLIFLDSIK SKSKDSVWEK EIEILIEENE DLKQQCIQLN EEIEKQRNTF SFAEKNFEVN YQELQEEYAC LLKVKDDLED SKNKQELEYK SKLKALNEEL
901   HLQRINPTTV KMKSSVFDED KTFVAETLEM GEVVEKDTTE LMEKLEVTKR EKLELSQRLS DLSEQLKPKP GEISFLNEEV KSLKPEKEPV SLRCRELEII
1001  INHNRAENVQ SCDTQVSSLL DGVVTMTSRG AEGSVSKVNK SFGEESKIMV EDKVSFENMT VGEESKQEQL ILDHLPSVTK ESSLRATQPS ENDKLQKELN
1101  VLKSEQNDLR LQMEAQRICL SLVYSTHVDQ VREYMENEKD KALCSLKEEL IFAQEEKIKE LQKIHQLELQ TMKTQETGDE GKPLHLLIGK LQKAVSEECS
1201  YFLQTLCSVL GEYYTPALKC EVNAEDKENS GDYISENEDP ELQDYRYEVQ DFQENMHTLL NKVTEEYNKL LVLQTRLSKI WGQQTDGMKL EFGEENLPKE
1301  ETEFLSIHSQ MTNLEDIDVN HKSKLSSLQD LEKTKQLEEQ VQELESLISS LQQQLKETEQ NYEAEIHCLQ KRLQAVSEST VPPSLPVDSV VITESDAQRT
1401  MYPGSCVKKN IDGTIEFSGE FGVKEETNIV KLLEKQYQEQ LEEEVAKVIV SMSIAFAQQT ELSRISGGKE NTASSKQAHA VCQQEQHYFN EMKLSQDQIG
1501  FQTFETVDVK FKEEFKPLSK ELGEHGKEIL LSNSDPHDIP ESKDCVLTIS EEMFSKDKTF IVRQSIHDEI SVSSMDASRQ LMLNEEQLED MRQELVRQYQ
1601  EHQQATELLR QAHMPQMERQ REDQEQLQEE IKRLNRQLAQ RSSIDNENLV SERERVLLEE LEALKQLSLA GREKLCCELR NSSTQTQNGN ENQGEVEEQT
1701  FKEKELDRKP EDVPPEILSN ERYALQKANN RLLKILLEVV KTTAAVEETI GRHVLGILDR SSKSQSSASL IWRSEAEASV KSCVHEEHTR VTDESIPSYS
1801  GSDMPRNDIN MWSKVTEEGT ELSQRLVRSG FAGTEIDPEN EELMLNISSR LQAAVEKLLE AISETSSQLE HAKVTQTELM RESFRQKQEA TESLKCQEEL
1901  RERLHEESRA REQLAVELSK AEGVIDGYAD EKTLFERQIQ EKTDIIDRLE QELLCASNRL QELEAEQQQI QEERELLSRQ KEAMKAEAGP VEQQLLQETE
2001  KLMKEKLEVQ CQAEKVRDDL QKQVKALEID VEEQVSRFIE LEQEKNTELM DLRQQNQALE KQLEKMRKFL DEQAIDREHE RDVFQQEIQK LEQQLKVVPR
2101  FQPISEHQTR EVEQLANHLK EKTDKCSELL LSKEQLQRDI QERNEEIEKL EFRVRELEQA LLVSADTFQK VEDRKHFGAV EAKPELSLEV QLQAERDAID
2201  RKEKEITNLE EQLEQFREEL ENKNEEVQQL HMQLEIQKKE STTRLQELEQ ENKLFKDDME KLGLAIKESD AMSTQDQHVL FGKFAQIIQE KEVEIDQLNE
2301  QVTKLQQQLK ITTDNKVIEE KNELIRDLET QIECLMSDQE CVKRNREEEI EQLNEVIEKL QQELANIGQK TSMNAHSLSE EADSLKHQLD VVIAEKLALE
2401  QQVETANEEM TFMKNVLKET NFKMNQLTQE LFSLKRERES VEKIQSIPEN SVNVAIDHLS KDKPELEVVL TEDALKSLEN QTYFKSFEEN GKGSIINLET
2501  RLLQLESTVS AKDLELTQCY KQIKDMQEQG QFETEMLQKK IVNLQKIVEE KVAAALVSQI QLEAVQEYAK FCQDNQTISS EPERTNIQNL NQLREDELGS
2601  DISALTLRIS ELESQVVEMH TSLILEKEQV EIAEKNVLEK EKKLLELQKL LEGNEKKQRE KEKKRSPQDV EVLKTTTELF HSNEESGFFN ELEALRAESV
2701  ATKAELASYK EKAEKLQEEL LVKETNMTSL QKDLSQVRDH LAEAKEKLSI LEKEDETEVQ ESKKACMFEP LPIKLSKSIA SQTDGTLKIS SSNQTPQILV
2801  KNAGIQINLQ SECSSEEVTE IISQFTEKIE KMQELHAAEI LDMESRHISE TETLKREHYV AVQLLKEECG TLKAVIQCLR SKEGSSIPEL AHSDAYQTRE
2901  ICSSDSGSDW GQGIYLTHSQ GFDIASEGRG EESESATDSF PKKIKGLLRA VHNEGMQVLS LTESPYSDGE DHSIQQVSEP WLEERKAYIN TISSLKDLIT
3001  KMQLQREAEV YDSSQSHESF SDWRGELLLA LQQVFLEERS VLLAAFRTEL TALGTTDAVG LLNCLEQRIQ EQGVEYQAAM ECLQKADRRS LLSEIQALHA
3101  QMNGRKITLK REQESEKPSQ ELLEYNIQQK QSQMLEMQVE LSSMKDRATE LQEQLSSEKM VVAELKSELA QTKLELETTL KAQHKHLKEL EAFRLEVKDK
3201  TDEVHLLNDT LASEQKKSRE LQWALEKEKA KLGRSEERDK EELEDLKFSL ESQKQRNLQL NLLLEQQKQL LNESQQKIES QRMLYDAQLS EEQGRNLELQ
3301  VLLESEKVRI REMSSTLDRE RELHAQLQSS DGTGQSRPPL PSEDLLKELQ KQLEEKHSRI VELLNETEKY KLDSLQTRQQ MEKDRQVHRK TLQTEQEANT
3401  EGQKKMHELQ SKVEDLQRQL EEKRQQVYKL DLEGQRLQGI MQEFQKQELE REEKRESRRI LYQNLNEPTT WSLTSDRTRN WVLQQKIEGE TKESNYAKLI
3501  EMNGGGTGCN HELEMIRQKL QCVASKLQVL PQKASERLQF ETADDEDFIW VQENIDEIIL QLQKLTGQQG EEPSLVSPST SCGSLTERLL RQNAELTGHI
3601  SQLTEEKNDL RNMVMKLEEQ IRWYRQTGAG RDNSSRFSLN GGANIEAIIA SEKEVWNREK LTLQKSLKRA EAEVYKLKAE LRNDSLLQTL SPDSEHVTLK
3701  RIYGKYLRAE SFRKALIYQK KYLLLLLGGF QECEDATLAL LARMGGQPAF TDLEVITNRP KGFTRFRSAV RVSIAISRMK FLVRRWHRVT GSVSININRD
3801  GFGLNQGAEK TDSFYHSSGG LELYGEPRHT TYRSRSDLDY IRSPLPFQNR YPGTPADFNP GSLACSQLQN YDPDRALTDY ITRLEALQRR LGTIQSGSTT
3901  QFHAGMRR
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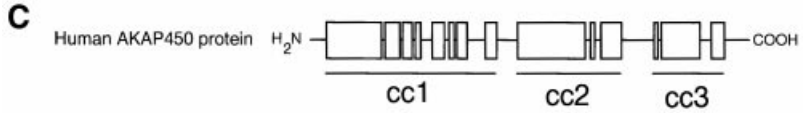
C



(Keryer *et al*., 1993; Skålhegg *et al*., 1994). A Jurkat T lymphocyte expression library was therefore used in an RII overlay-based screening procedure to identify centrosomal AKAPs. Several positive clones were isolated, sequenced and found to be identical to previously cloned human AKAP cDNAs. One clone was found to encode parts of a protein highly similar to a rabbit AKAP with a predicted mol. wt of 120 kDa (Dransfield *et al*., 1997), indicating that this represented the human AKAP120 homologue (results not shown). Interestingly, the rabbit AKAP120 peptide sequence revealed some homology to pericentrin, as determined by the BLASTP search program [basic local alignment and search tool (Altschul *et al*., 1997)], which suggested that AKAP120 could be a centrosomal protein.

Using the BLAST program (Altschul *et al*., 1997) to compare the nucleotide sequence of rabbit AKAP120 with all previously published nucleotide sequences (BLASTN), revealed that a segment of the human chromosome 7q21 encoding a protein highly homologous to rabbit AKAP120 had been sequenced and submitted to the DDBJ/EMBL/GenBank database (accession No. AC004013). However, this sequence did not have the in-frame stop codon found previously in the rabbit AKAP120 sequence. Also, no in-frame stop codon could be identified in the exon that was found upstream of the translation initiation codon. Together with the fact that no evidence of an endogenous AKAP120 protein has been presented, this led us to believe that the AKAP120 cDNA could encode parts of a larger protein.

In an attempt to identify the translation initiation and termination signals of the human AKAP120 cDNA sequence, 5′ and 3′ rapid amplifications of cDNA ends (RACE) were performed using RACE-ready cDNA from two different human sources (prostate and brain) and several primers specific for the human sequence. Sequencing the resulting 5′-RACE PCR products did not reveal any in-frame stop codons upstream of the ATG corresponding to the suggested translation initiation signal in rabbit AKAP120. In fact, the resulting cDNA sequence contained an ORF that was identical in its most 5′ part with the 3′ end of the previously published cDNA sequence encoding the Yotiao protein (Lin *et al*., 1998), indicating that the two cDNAs were derived from one gene. Furthermore, the exons encoding the Yotiao protein were found in close proximity to the exons encoding the human AKAP120 homologue (Figure 1A, top line). The cDNAs generated by 3′-RACE contained an ORF in continuation with the AKAP120 sequence, with no identifiable stop codon. In addition, these PCR products were shown to overlap the 5′ end of an exon (predicted by sequence analysis) in the human genomic sequence. The 3′ end of this exon was shown to overlap a contig of expressed sequence tag (EST) clones previously sequenced, suggesting that an additional 15 exons were present in the gene encoding the human homologue of AKAP120 (Figure 1A, line 2, right part, and line 4). Based on this, and the exons predicted in a contig of four overlapping BAC clones containing human genomic DNA from chromosome 7q21-22 (Figure 1A, line 1), it was possible to assemble a hypothetical cDNA sequence with an ~12 kb ORF. In order to investigate the possible presence of such a gene product, oligonucleotides matching the 5′ (sense orientation) and 3′ (antisense orientation) ends of this hypothetical cDNA were used in an RT–PCR reaction with isolated human T lymphocyte total RNA as template. This resulted in a single 12 kb PCR product identified by agarose gel electrophoresis and ethidium bromide staining. This product was excised from the gel, purified and cloned into the vector pCR-XL-TOPO, and sequenced. The resulting sequence is schematically represented in Figure 1A, line 3. By comparing this sequence to the contig of BAC clones, a total of 50 protein-encoding exons were identified (Figure 1, line 4, vertical lines; Table I). The cDNA sequence was corrected for single nucleotide mismatches based on the genomic sequencing data. The resulting cDNA sequence contained an ORF of 11 724 nucleotides encoding a 3908 amino acid protein (Figure 1B). This protein was given the name AKAP450, based on its calculated mol. wt of 453 kDa. The most N-terminal part of AKAP450 (amino acids 1–1626) was shown to be identical to the Yotiao protein, but with two exceptions. Amino acids 17–28 of the Yotiao protein sequence could not be found in the AKAP450 sequence, but an exon encoding these amino acids could be found

Fig. 1. (**A**) Characterization of DNA fragments for AKAP450. Top line: schematic drawing of a genomic region of human chromosome 7q21-22 obtained from four separate BAC clones sequenced as part of the human genome project [DDBJ/EMBL/GenBank accession Nos: AC003086 (clone RG104F04, BAC1) 7q21; AC000066 (clone RG293F11, BAC2) 7q21-22; AC004013 (clone GS541B18, BAC3) 7q21; AC000120 (RG161K23, BAC4) 7q21]. This contig was shown to contain exons encoding a protein highly homologous to the rabbit AKAP120 protein (Dransfield *et al*., 1997). Downstream of the putative human AKAP120 gene, several exons had been predicted by computer analysis, and these exons were confirmed to be transcribed by the generation of a sequence contig based on overlapping EST clones. Upstream of the putative AKAP120 gene, exons were identified encoding the previously characterized Yotiao protein (Lin *et al*., 1998). Second line: to identify the start and stop of the putative human AKAP120 cDNA, the ends of the corresponding cDNA were amplified in 5′- and 3′-RACE reactions. A contig of 5′- and 3′-RACE products was made and compared with the human genomic sequence. The horizontal lines represent the obtained composite cDNA sequences and are drawn to indicate what parts of the genomic sequence they correspond to, and thus which exons they are derived from. Third line: using a primer corresponding to nucleotide sequences in the first exon encoding the Yotiao protein and a primer complementary to the last exon in the suggested EST contig, a 12.1 kb PCR product was amplified in an RT–PCR using total human T-lymphocyte RNA as a template. The PCR product was cloned into a bacterial vector (pCR-XL-TOPO), sequenced in one direction and the resulting sequences were compared with the genomic DNA sequence. A composite sequence was made from sequencing of the cDNA, and corrected for single nucleotide sequencing errors based on comparison to the human genomic sequences. This revealed an ORF of 11 724 nucleotides in the 12.1 kb PCR product (excluding the translation termination signal). The ORF encoded a protein of 3908 amino acids, which was named AKAP450 based on its calculated mol. wt of 453 kDa. Fourth line: identification of exons in the AKAP450 gene. Exons are represented by vertical lines, and are not drawn to scale. Fifty exons were demonstrated to encode AKAP450. The open box represents the position of an additional exon present in the Yotiao cDNA which was not present in the AKAP450 cDNA. (**B**) Deduced amino acid sequence of the human AKAP450 protein. The peptide sequences used for immunization are boxed (amino acids 19–33 and 2572–2586). A putative RII-binding motif (amino acids 5252–5265) is underlined and the amino acid (L) mutated in Figure 2 is marked by an asterisk above the sequence. (**C**) Predicted coiled-coils in the human AKAP450 protein. The human AKAP450 peptide sequence was analyzed using the logarithm of Lupas *et al*. (1991) and a window of 28 amino acids. Regions with a probability of coiled-coils >0.5 are indicated as boxes and demonstrate three clusters designated cc1, cc2 and cc3 (underlined) of coiled-coil structure in the AKAP450 protein.

Table I. Organization of the human gene encoding AKAP450

Exon no	Nuc. No.[a] in the AKAP450 cDNA	5′ intron sequence	Exon sequence	3′ intron sequence	BAC No.[b]	Nuc. No. in BAC clones
1	–48		...caaagccaag	gtaggagagc...	1	–96643
2	NP	...tttctttcag	attgaagaac.....tgtcagacag	gtatgtgtta...	2	15775–15810
3	49–306	...tttttcttag	cttgcccagt.....ctctgtggaa	gtaagtattc...	2	19013–19270
4	307–351	...tttgttttag	ctggaaagtg.....cagttcagag	gtaagactaa...	2	25591–25635
5	352–405	...ttttatacag	gtaaatggtt.....tttattaagg	gtacagtatt...	2	37460–37513
6	406–576	...attgatttag	gaagaagaat.....actgcagcag	gtatgtttat...	2	38186–38357
7	577–732	...tttctttcag	ttacaagaat.....atttcagcaa	gtaagtatta...	2	39923–40078
8	733–930	...ctattttcag	ttacaggcta.....atatgaaatg	gtatgtttat...	2	40905–41102
9	931–3318	...tttcctaaag	gaacaagata.....atcagaacag	gtatgtttac...	2	46150–48537
10	3319–3532	...ttactttaag	aatgatttaa.....caagaaacag	gtaaaatggt...	2	57731–57944
11	3533–3612	...gttctattag	gtgatgaagg.....ttttttacag	gtaaaatgtt...	2	59551–59630
12	3613–3751	...ttgttaacag	actttatgca.....gaagttcaag	gtaataaaag...	2	61431–61569
13	3752–3837	...taccatccag	actttcaaga.....actaagcaag	gtctgtgaga...	2	62319–62404
14	3838–3952	...ttttcaatag	atctggggac.....gaagacattg	gtaaattttg...	2	67540–67654
15	3953–4151	...tataacatag	atgtcaatca.....ttccgccaag	gtattcatct...	2	68116–68311
16	4152–4248	...ttatattcag	cttacctgtt.....tacaatagag	gtattatatt...	2	75197–75293
17	4249–4341	...ttaaatgtag	ttttctggtg.....agtagctaag	gtaggcttat...	2	76814–76906
18	4342–4695	...atttctttag	gttattgtgt.....tagacagtct	gtaagtatgc...	2	83721–84074
19	4696–4920	...actattaaag	attcatgatg.....attagcccag	gtaagggtct...	2	85976–86200
20	4921–5061	...tcttatttag	agatcctcca.....gcaaacacag	gtagtatgga...	2	87348–87488
21	5062–5165	...acttaaacag	aatggaaatg.....ctaatgaaag	gtatacaaaa...	2	87970–88073
22	5166–5371	...atctatacag	gtatgcactc.....catacaagag	gtactagttt...	2	90310–90515
23	5372–5604	...gttattctag	ttacagatga.....tagcagtcag	gtaacctcct...	3	3268–3500
24	5605–5767	...ctttgtttag	cttgaacatg.....aaggctgagg	gtgagcaatt...	3	11802–11964
25	5768–5980	...tgtattatag	gcgtcattga.....gttgaacaac	gtaagtattt...	3	12816–13028
26	5981–6213	...taataaacag	aattactaca.....atttttagat	gtaagtattc...	3	15773–16005
27	6214–6333	...taaactttag	gagcaagcca.....aactagagag	gtaagaactt...	3	16969–17088
28	6334–6510	...tatatttcag	gttgaacagt.....ttttcaaaag	gtgtggcatt...	3	20572–20748
29	6511–6615	...atatttaaag	gtagaggacc.....ggaaaaagag	gtaaggagtt...	3	21447–21551
30	6616–6768	...gttattaaag	attacaaact.....attatttaag	gtaattagtt...	3	27397–27549
31	6769–6948	...ttgttcctag	gatgacatgg.....agataacaag	gtatactcat...	3	28238–28417
32	6949–8022	...taattattag	gttattgaag.....agttctcaag	gttagttttg...	3	29621–30694
33	8023–8163	...tgtcattcag	acaactactg.....agagcttttg	gtaagataag...	3	33064–33204
34	8164–8649	...tacgttttag	gtaaaagaaa.....aagtaaagag	gtatttggtt...	3	33712–34197
35	8650–8713	...ctctaattag	ggatcctcaa.....tgctccagtg	gtaagttata...	3	35182–35245
36	8714–8835	...tttattttag	attctggatc.....gaaaataaag	gtactaaaag...	3	35361–35482
37	8836–9027	...tggtttccag	ggattactga.....agaagccgag	gtaaccaaag...	3	36037–36228
38	9028–9216	...actttgctag	gtttatgata.....acaagaacag	gtataatgaa...	3	36770–36958
39	9217–9361	...tttgttacag	ggtgttgaat.....ccaagccaag	gtatgttgta...	3	39927–40071
40	9362–9581	...ttctgtgtag	aactcttgga.....aggctttcag	gtgtgccagg...	3	43639–43858
41	9582–9732	...tctgacttag	gttggaagtt.....agaacttgag	gtactgttat...	3	45565–45715
42	9733–10401	...ctcgtaccag	gatctgaagt.....ccttaatgag	gtaaactgac...	3	47231–47899
43	10402–10610	...aaattgccag	ccaaccacgt.....cctctgagag	gttagacttt...	3	48128–48336
44	10611–10716	...tttcttgaag	actacagttt.....aggtgaagag	gtaatacttt...	3	48651–48756
45	10717–10899	...atttttccag	cccagcttgg.....tagagataat	gtatgtccat...	3	50229–50411
46	10900–11100	...gtctttgcag	tcttccaggt.....cactttaaag	gtaggagaca...	3	51398–51598
47	11101–11333	...ctaaatatag	agaatttatg.....caatttccag	gtaaagactt...	3	53136–53368
48	11334–11419	...tcttttttag	aatgaaattt.....ctgaatcaag	gtgaagtcaa...	3	56220–56305
49	11420–11549	...gttactttag	gtgcagaaaa.....ttcagaatag	gtaagaatat...	3	57835–57964
50	11550–11689	...aatcctttag	gtacccaggc.....atacagtcag	gtgctctgag...	4	131–270
51	11690–	...tctctcatag	gttcaactac.....		4	1759–

[a]Numbered according to the AKAP450 cDNA. The first nucleotide in the ATG is defined as nucleotide 1.
[b]The DDBJ/EMBL/GenBank accession Nos of BAC sequences are: 1, AC003086; 2, AC000066; 3, AC004013; 4, AC000120.
NP, not present.

in the genomic sequence (open box in Figure 1A, line 4; Table I). This demonstrates that the AKAP450 and Yotiao cDNAs are derived from alternatively spliced mRNAs. In total, the AKAP450 gene was found to contain 51 exons, with 50 exons encoding the AKAP450 sequence presented here and an additional exon (exon 2) that previously had been shown in the Yotiao cDNA sequence (Table I). In addition, the AKAP450 protein was shown to have an amino acid (Glu1336) not present in Yotiao, indicating a polymorphism in the AKAP450 gene. Furthermore, the middle part of AKAP450 was highly similar to rabbit AKAP120. Apart from this, the protein displayed some homology to pericentrin, giantin and other structural proteins as determined by the BLASTP search program. Common to all these proteins was the presence of coiled-coil structures. Large parts of the AKAP450 protein sequence were also predicted to contain coiled-coil structures based on the algorithm by Lupas *et al.* (1991). As illustrated in Figure 1C, several areas of coiled-coil structures were identified revealing three major coiled-coil clusters, designated cc1, cc2 and cc3. Finally, as previously shown for other

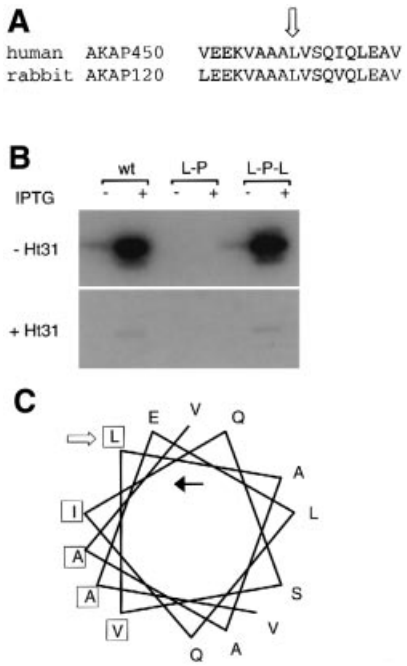


Fig. 2. Identification of an RII-binding motif in AKAP450. (**A**) Comparison of the human AKAP450 amino acid sequence to the suggested RII-binding site of AKAP120 indicated that the corresponding sequence in AKAP450 was located at amino acids 2548–2565. (**B**) A part of the wild-type AKAP450 protein (amino acids 2327–2602; wt) was expressed as a GST-fusion protein, along with a mutant where Leu2556 [indicated by an open arrow in (A) and (C)] was substituted by proline (L-P). An additional mutant where Pro2556 of the L-P mutant was changed to leucine to revert the sequence to wild-type (L-P-L) was included as a control. Bacteria were grown in the presence (+) or absence (–) of IPTG, lysed by boiling in SDS loading buffer and analyzed for RII binding by the RII overlay technique, using ^{32}P-radiolabeled RII in the absence (–, upper panel) or presence (+, lower panel) of 500 nM Ht31-derived RII-binding peptide. (**C**) Prediction of an amphipatic helix (amino acid 2552–2565) by helical wheel conformation with a putative RII-binding motif in AKAP450. Amino acid representing a putative hydrophobic surface are boxed.

AKAPs, the AKAP450 protein is highly acidic, with a calculated isoelectric point of 4.8.

Identification and characterization of an RII-binding motif in AKAP450

An RII-binding motif has previously been suggested in the rabbit AKAP120 amino acid sequence (Dransfield *et al*., 1997), and this sequence was found to be conserved in the human AKAP450 amino acid sequence, with two conservative mutations [leucine (L) to valine (V) and valine (V) to isoleucine (I); Figure 2A]. To test the RII-binding properties of this fragment of the AKAP450 protein, a cDNA encoding amino acids 2327–2602 was cloned into the bacterial expression vector pGEX-KG. Bacteria transfected with this plasmid expressed a wild-type protein following isopropyl-β-D-galactopyranoside (IPTG) induction that bound radiolabeled bovine RII in an overlay experiment (Figure 2B, wt). RII-binding was specific as the Ht31-derived competetive peptide (Carr *et al*., 1991) could prevent RII-AKAP450 interaction. To further demonstrate specificity in the interaction, the nucleotide triplet encoding leucine 2556 was mutated to encode proline. Bacteria transformed with the mutated plasmid with an L to P (L-P) sequence could be induced by IPTG to express a fusion protein of the same apparent molecular weight as the bacteria expressing the wild-type fusion protein (not shown). This protein failed to bind radiolabeled RII (Figure 2B, L-P). To assure that no other mutation in this plasmid was causing the failure to bind RII, the plasmid with the L-P mutation was mutated back to the original sequence (L-P-L). As can be seen from Figure 2B, the RII-binding capacity was restored. Also in this case, the Ht31-derived RII-binding peptide prevented RII binding, implying specificity in RII interaction with the putative RII-binding sequence. Together, these results demonstrated that an RII-binding motif is located around Leu2556 in the human AKAP450 amino acid sequence. Amphipatic helix prediction with a window of 14–18 amino acids using helical wheel projection, demonstrated a probable hydrophobic face involving Leu2556 in amino acids 2552–2565 (Figure 2C). This does not preclude the possible presence of additional RII-binding motifs in the AKAP450 sequence.

Tissue distribution of AKAP450 mRNA

To identify AKAP450 mRNAs, two similar Northern blots containing poly(A)$^+$ RNA from various human tissues were hybridized using different fragments of the AKAP450 cDNA. A fragment containing the nucleotide sequence –67 to 468 hybridized to a single RNA species of ~12 kb (Figure 3A, upper panel). Interestingly, a DNA fragment corresponding to a more 3′ part of the AKAP450 cDNA (nucleotides 5272–6078) was shown to hybridize to both the 12 kb mRNA species and a shorter mRNA species of ~8 kb (Figure 3B, upper panel). Both filters were stripped and reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (Figure 3A and B, lower panels) to control for loading differences. The levels of the 12 kb AKAP450 mRNA varied from tissue to tissue. Most tissues showed some level of expression of the 12 kb AKAP450 mRNA, but only faint signals could be detected in skeletal muscle, liver, small intestine and peripheral blood leukocytes. Since all these lanes had a prominent smear, this could be due to increased degradation in these tissues. Furthermore, kidney had a very intense signal, indicating a higher expression of AKAP450 in this organ. The 8 kb mRNA, observed only with the probe corresponding to nucleotides 5272–6078, could represent an mRNA from the same gene encoding a shorter protein. Interestingly, this mRNA showed a somewhat different tissue distribution than the 12 kb mRNA. While some tissues had prominent signal from both mRNAs, liver demonstrated a very weak 12 kb signal while having a prominent 8 kb signal. The mRNAs could be directed from two different promoters located in the same gene, thereby showing tissue differences. The protein product of the 8 kb mRNA would encode a protein shorter than the one reported here, and could be expected to have different properties. As the DNA fragment used to detect the 8 kb mRNA was derived from a part of the AKAP450 cDNA in close proximity to the part encoding the RII-binding site, this protein could be an AKAP, possibly with a different targeting motif.

AKAP450 is co-immunoprecipitated with PKA

A rabbit polyclonal antiserum designated anti-AKAP450a was raised and affinity purified against a synthetic peptide sequence identical to the N-terminal amino acids 19–33

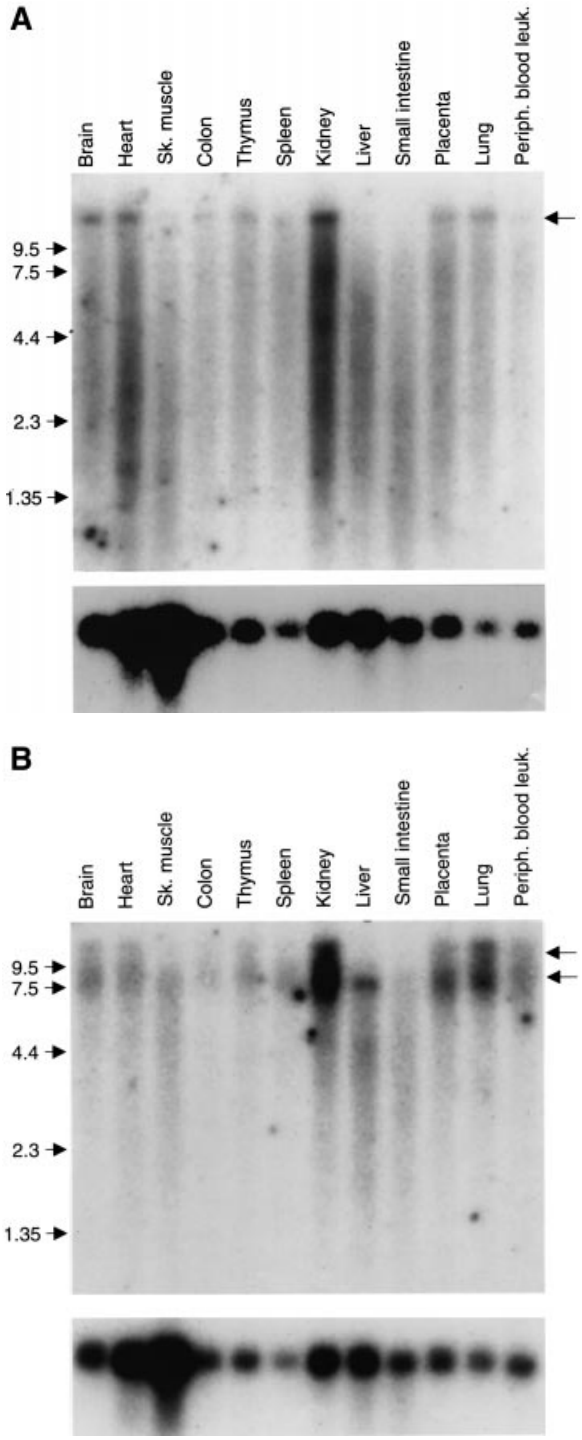


Fig. 3. Tissue distribution of AKAP450 mRNA. (**A**) A Northern filter (MTN-12, Clontech) containing poly(A)$^+$ RNA (2 μg/lane) from various human tissues was probed using a [^{32}P]cDNA fragment, corresponding to nucleotides –67 to 468 of the AKAP450 cDNA (upper panel), or GAPDH cDNA as a probe (lower panel). (**B**) A similar filter was hybridized using a [^{32}P]cDNA fragment corresponding to nucleotides 5272–6078 of the AKAP450 cDNA (upper panel) or GAPDH as a probe (lower panel). The tissue sources are indicated on the top of each lane, and the migration of molecular size markers is indicated on the left. The RNA bands recognized by the respective probes derived from AKAP450 are indicated by arrows on the right.

of the AKAP450 protein. To investigate whether RII is associated with AKAP450 in intact cells, we lysed HeLa cells in RIPA buffer and performed immunoprecipitation

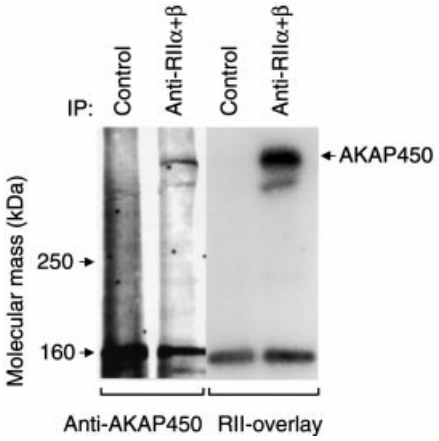


Fig. 4. Co-immunoprecipitation of AKAP450 with RII from whole-cell lysates. RIPA-buffer extracts of 1.4×10^8 HeLa cells were used for immunoprecipitation using a combination of affinity-purified anti-RIIα and anti-RIIβ antiserum or Ig fraction of irrelevant serum as a control. Samples were separated by PAGE in 4.5% gels containing 0.5 M urea, transferred to nitrocellulose and detected using either affinity purified AKAP450 antibody or radiolabeled RII in an RII overlay technique. Migration of mol. wt markers are indicated to the left. Two bands in the upper part of the gel were detected both by AKAP450 antibody and by RII overlay. The major band is indicated by an arrow.

with a combination of polyclonal anti-RIIα and anti-RIIβ antibodies (Figure 4). As control, we also precipitated with an Ig-fraction of an irrelevant Ab. Precipitates were separated by electrophoresis in 4.5% acrylamide gels containing 0.5 M urea, and blotted onto nitrocellulose. The affinity purified anti-AKAP450a antibody was used to detect AKAP450 by immunoblotting of the immunoprecipitates. The same filter was then reprobed in an overlay procedure, using radiolabeled RII. Two bands that migrated more slowly than the 250 kDa standard were detected by both methods in the sample immunoprecipitated with anti-RII. These proteins were not precipitated using control serum. Parallel control experiments demonstrate that the immunoreactive bands detected by anti-AKAP450 antibody were competed by the peptide used for immunization (not shown). This demonstrated that AKAP450 interacted with RII in intact HeLa cells.

Subcellular localization of AKAP450

To identify the subcellular localization of AKAP450, anti-AKAP450a and anti-AKAP450b (raised against peptides 2572–2586 of the AKAP450 amino acid sequence) were used in combination with anti-RII antibodies and antibodies to the centrosome to label HeLa cells and SaOS2 osteosarcoma cells by immunofluorescence. As shown in Figure 5A, anti-AKAP450a decorated one single dot in the perinuclear region of interphase HeLa cells, whereas it decorated two polarized dots in cells that were found to be in metaphase (Figure 5A). Competition with the peptide used for immunization demonstrated that the staining was specific (not shown). By dual labeling of HeLa cells with a centrosomal marker mAb CTR453 (Bailly *et al*., 1989), it appeared that both antibodies decorated the same subcellular structures (Figure 5B). Double image overlays of the anti-AKAP450a and mAb CTR453-labeling clearly demonstrated localization of AKAP450 with centrosomes (Figure 5C). Furthermore,

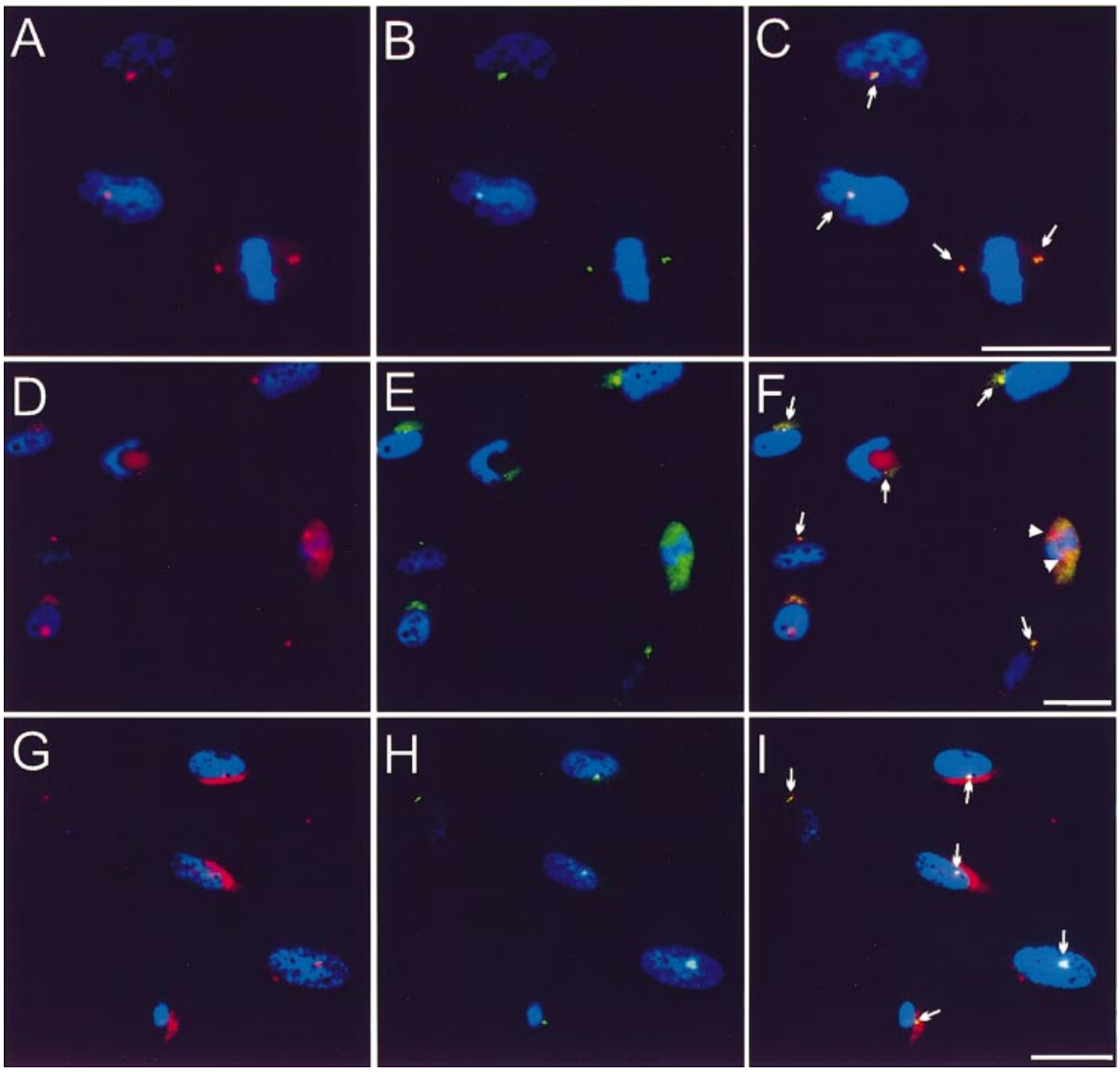


Fig. 5. Subcellular localization of AKAP450. HeLa cells (**A–C**, **G–I**) or SaOS2 osteosarcoma cells (**D–F**) were stained with affinity-purified anti-AKAP450a antibodies (red; A, C, D, F) or affinity-purified anti-AKAP450b antibodies (red; G, I) and antibodies to a centrosomal marker (mAb CTR453; green; B, C, H, I) or RIIα (green; E, F). All cells were counter-stained with DAPI (blue) to indicate DNA. For double labeling of AKAP450 with CTR453 and RIIα, images are shown separately for staining with anti-AKAP450a (A, D), mAb CTR453 (B, H), anti-RIIα mAb (E) and anti-AKAP450b (G) together with image overlays (C, F, I; co-localization indicated by yellow or purple on top of the DAPI staining). Arrows indicate position of centrosomes/AKAP450 labeling, arrow heads indicate the localization of AKAP450 in cells undergoing mitosis (D–F). Bars, 12 μm.

when staining SaOS2 osteosarcoma cells with anti-AKAP450a, we observed a similar staining as seen in HeLa cells (Figure 5D). In these cells, dual staining with a monoclonal antibody to human RIIα demonstrated that anti-RIIα prominently decorated the perinuclear area including centrosomes (Figure 5, arrows) and Golgi compartments. In contrast, the anti-RIIα antibody staining was either not observed in the centrosome region of cells in metaphase or present at mitotic poles only at low levels compared with the staining of RIIα at interphase centrosomes, consistent with what we have observed recently (Keryer *et al.*, 1998). Localization of mitotic poles/centrosomes in Figure 5D are indicated by arrowheads in Figure 5F. Image overlays of the anti-AKAP450a and the anti-RIIα staining demonstrated a distinct centrosomal co-localization of AKAP450 and RIIα in interphase cells. Anti-AKAP450b decorated one single dot in the perinuclear region of HeLa cells (Figure 5G), that by dual labeling with mAb CTR453 and double image overlays (Figure 5I) were demonstrated to be centrosomes (arrows). Furthermore, anti-AKAP450b stained a larger area than CTR453, resembling Golgi structures. To examine biochemically the association of AKAP450 with centrosomes, we purified centrosomes from KE37 lymphoblast cells. Centrosomes were loaded in adjacent lanes, resolved by PAGE in 4% separating gels containing 2 M urea and transferred to a nitrocellulose filter that was subsequently cut in parallel strips. Strips were incubated with radiolabeled RII (Figure 6, lane 1), a previously characterized rabbit antiserum to human centrosomes designated 0013 (Gosti-Testu *et al.*, 1986) (lane 2) and anti-AKAP450a (lane 3). Radiolabeled RII bound two proteins in the

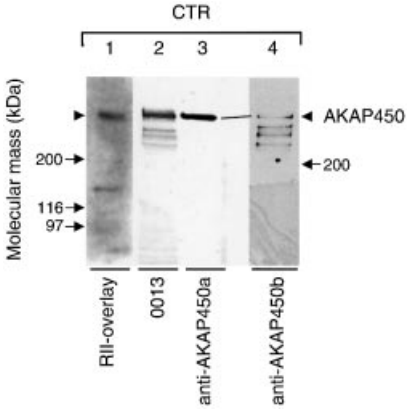


Fig. 6. AKAP450 is present in centrosomes. Purified centrosomes (5×10^7 in each lane) were separated by PAGE with 2 M urea in the separating gel, transferred to nitrocellulose and subjected to detection using radiolabeled RII in an overlay procedure (lane 1), or by Western blotting using either 0013 anti-centrosomal antibody (lane 2), anti-AKAP450a (lane 3) or anti-AKAP450b (lane 4). Lanes 1–3 were from the same gel, while lane 4 was from a different gel. Cross-probing of the filters demonstrated that the upper band in lane 4 comigrated with the upper bands in lanes 1–3 (indicated by slanted line), demonstrating that this protein band represents AKAP450.

centrosomal extract. Furthermore, the centrosomal antiserum 0013 recognized several proteins, of which the upper major band comigrated with the slowly migrating protein recognized by radiolabeled RII. In addition, anti-AKAP450a recognized one distinct band that comigrated with the upper protein band recognized both by radiolabeled RII and the 0013 antiserum. Finally, probing of a parallel blot with centrosome preparations using anti-AKAP450b, four distinct bands were identified (Figure 6, lane 4). Cross-probing of filters with different antibodies identified the upper band in lane 4 as comigrating with the band detected by 0013 and anti-AKAP450a in Figure 6, lanes 2 and 3 (indicated by slanted line). This band was not detected in soluble and insoluble Triton X-100 fractions from the same centrosome preparation (data not shown). The three faster migrating bands appeared to comigrate with bands detected by the 0013 antiserum.

Discussion

In the present study we report the cloning of a cDNA encoding a centrosomal protein capable of binding PKA, designated AKAP450. A previously identified human cDNA encoding the Yotiao protein (Lin *et al*., 1998) is almost identical to the 5′ end of the cDNA sequence presented here. Furthermore, the rabbit AKAP120 cDNA (Dransfield *et al.*, 1997) is highly similar to the middle part of the AKAP450, indicating that rabbit AKAP120 cDNA is derived from the rabbit homologue of the human AKAP450 gene. Whether the human Yotiao and rabbit AKAP120 represent splice variants or cloning artifacts of the human and rabbit AKAP450 genes remains to be determined.

No in-frame stop codon could be identified upstream of the translation initiation signal found in the AKAP450 cDNA. However, the first in-frame ATG matched the translation initiation consensus sequence (Kozak, 1986). Furthermore, repetitive RACE-amplification did not reveal any in-frame start codons upstream of this ATG (data not shown). In addition, the genomic sequence upstream of the first AKAP450 exon contained no predicted exons, except for exons encoding mitochondrial transcription termination factor, a previously characterized gene (Fernandez-Silva *et al*., 1997). Together with the fact that the genomic region upstream of exon 1 contained a GC-rich area which is typical of mammalian promoters, it is likely that the cDNA presented here contains the entire protein-coding sequence of AKAP450.

The Northern blot analyses indicated that there are different mRNAs derived from the AKAP450 gene. A 12 kb mRNA is expected to be the origin of the cDNA reported here, based on the observation that it hybridized to both 5′ and 3′ probes, and that its apparent molecular size matched the size of the cDNA. The 8 kb mRNA contains only parts of the cDNA sequence reported here, as it was shorter than the sequenced cDNA and did not hybridize to the 5′-based probe and may represent an alternatively spliced or initiated transcript. As the probe that recognized the 8 kb mRNA was derived from a part of the AKAP450 cDNA close to the part encoding the identified RII-binding site, the resulting protein would also be expected to encode an AKAP.

The antibody raised against the N-terminal part of AKAP450, anti-AKAP450a, would be expected not to recognize a protein translated from the 8 kb mRNA. This is in contrast to anti-AKAP450b, which was raised against a more C-terminal sequence in the AKAP450 protein and would therefore be expected to recognize proteins translated from both the 8 and 12 kb mRNAs. We could show that purified centrosomes contain a protein with an apparent mol. wt >200 kDa. This centrosomal protein was recognized by anti-AKAP450a, anti-AKAP450b, the rabbit anti-centrosomal antibody 0013, and bound radiolabeled RII, indicating that this is the previously characterized centrosomal AKAP with an apparent mol. wt of 350 kDa (Keryer *et al.*, 1993). This, together with the fact that the anti-AKAP antisera were made against a protein with a predicted mol. wt of 453 kDa and that both AKAP450 antisera decorate centrosomes, strongly indicates that the anti-AKAP450a and anti-AKAP450b recognize the same centrosomal protein, AKAP450. Despite this, we cannot rule out the possibility of other variants of AKAP450 that are transcribed from the AKAP450 gene and are present in centrosomes. Furthermore, the anti-AKAP450b antiserum also decorated the perinuclear region that resembles Golgi structures. In addition, this antibody recognized four distinct bands migrating above 200 kDa in the centrosome immunoblots. The two bands with the highest apparent mobility were also detected in the Triton X-100 soluble fraction from the same centrosomal preparation and may indicate these proteins to be associated with membrane structures (data not shown). Altogether, this may suggest alternative splice variants with differential localization that will have to be investigated in future studies.

We could demonstrate three major clusters (cc1, cc2 and cc3) of predicted coiled-coil structure in AKAP450. The presence of coiled-coil structures is consistent with what has previously been observed for several other centrosomal proteins (Doxsey *et al*., 1994; Bouckson-Castaing *et al*., 1996; Fry *et al*., 1998a). It is known that coiled-coil structures take part in the structural framework

in the pericentriolar matrix, implying that one or more of the AKAP450 coiled-coil clusters may serve this role. Which cluster that participates in anchoring AKAP450 to the centrosome remains to be seen. Moreover, to assure proper segregation of chromosomes at mitosis, centrosome duplication and spindle formation are tightly regulated processes. Thus, in cycling cells ubiquitous expression of centrosomal proteins is expected. The observation of differential tissue expression of AKAP450 (12 kb mRNA) may correlate with the amount of dividing versus differentiating cells in various tissues.

We demonstrate that RIIα and AKAP450 co-localize in centrosomes, and that RIIα binds to a recombinant protein containing a part of the AKAP450 protein. We have also demonstrated an interaction between RIIβ and the recombinant AKAP450 fragment (data not shown). Although not shown here, centrosomes also bind with high affinity the majority of RIIβ when expressed in differentiated or neoplastic cells (G.Keryer, B.S.Skålhegg, B.F.Landmark, V.Hansson, T.Jahnsen, K.Tasken, submitted). Furthermore, both RIIα and RIIβ have been demonstrated to co-purify with centrosomes as demonstrated by Keryer *et al.* (1993). Whether the RII-binding motif(s) has any difference in affinity for the different RII isoforms will have to await further studies. Despite this, it is interesting that contrary to RIIα, which is phosphorylated by $p34^{cdc2}$ and dissociates from centrosomes at mitosis (Keryer *et al.*, 1998), the centrosomal association of RIIβ is not regulated.

Both microtubule organization and dynamics seem to be regulated by extracellular signals and activation of kinases such as MAP kinases and PKA. Interphase–metaphase transition of microtubule arrays can be induced by addition of mitogen-activated MAP kinase to interphase extracts of *Xenopus* eggs (Gotoh *et al*., 1991), whereas PKA could be involved in maintenance of the interphase microtubule network (for a review see Fernandez *et al*., 1995). It has been shown that PKA switches off the effect of stathmin (Gradin *et al*., 1998), a phosphoprotein family (Gavet *et al*., 1998), on microtubule dynamics. Moreover RNA localization along the antero-posterior axis of the *Drosophila* oocyte requires PKA to direct normal microtubule polarity organization (Lane and Kalderon, 1994). Inhibition of PKA catalytic activity seems to impair the minus-end redistribution of microtubules from the posterior pole towards the anterior pole of stage 7 *Drosophila* oocytes. Thus, centrosomal anchoring of PKA might be important for stabilizing the minus-end of microtubules that originate from the centrosome. Several other kinases have also been shown to be associated with centrosome although their centrosomal substrates are not known and their function(s) remains to be determined (Bailly *et al*., 1989; Bischoff *et al*., 1998; Fry *et al*., 1998b; Zhou *et al*., 1998). An anchoring protein as big as AKAP450 could act as a scaffold protein, able to cluster several different signaling molecules together in centrosomes.

Although we have demonstrated that AKAP450 is located in the centrosome in HeLa and osteosarcoma cells, we can not exclude the possibility that the same protein or other splice forms of it may have other functions. For example, the pericentriolar matrix and microtubule nucleating activity are distributed differently in differentiated cells and AKAP450 may thus localize differently in these cells. Furthermore, other splice variants potentially with different targeting motifs may certainly have different functions. Indeed, the Yotiao protein, containing the N-terminal part of AKAP450, was shown to bind to the NMDA receptor in a yeast two-hybrid system. Antibodies raised against Yotiao, using a partial amino acid sequence identical to the one found in AKAP450, were shown by dual immunofluorescence staining to co-localize with NMDA receptor subunit NR1 (Lin *et al*., 1998), which may then be regulated by PKA. Furthermore, the anti-Yotiao antibody was found to stain post-synaptic densities and T tubules of rat skeletal muscle, indicating a role in postsynaptic signaling. The description of AKAP450 will facilitate further studies into the role of PKA in centrosome functions, microtubule stability and cell cycle regulation and promote studies of other gene products from the AKAP450 gene.

Materials and methods

Antibodies

Two anti-AKAP450 antisera, designated anti-AKAP450a and anti-AKAP450b, were made by immunizing rabbits with hemocyanine-coupled synthetic peptides (Ag EP980409, NH_2-CQFRQRKAQSDG-QSPS-$CONH_2$; and EP98408, NH_2-CQDNQTISSEPERTN-$CONH_2$; Eurogentec, Seraing, Belgium) corresponding to amino acids 19–33 and 2572–2586 of AKAP450, respectively. Anti-AKAP450 antisera were enriched for IgG on protein A columns and subsequently affinity purified on columns with peptides coupled to CNBr-activated Sepharose-4B (Pharmacia, Stockholm, Sweden). Affinity purified antibody was diluted to 10 μg/ml for immunoblot analyses, and used at a concentration of 5 μg/ml for immunofluorescence studies. For competition studies, peptides were used at 300-fold molar excess. A spontaneous rabbit antiserum, 0013, had previously been demonstrated to recognize human centrosomes (Gosti-Testu *et al*., 1986), and was used at a concentration of 1:500 for Western blotting.

Two polyclonal antisera, anti-human RIIα and anti-human RIIβ, were used for immunoprecipitation at 1:30 and 1:60 dilution, respectively (Skålhegg *et al*., 1992; Keryer *et al*., 1993; Tasken *et al*., 1993; G.Keryer, B.S.Skålhegg, B.F.Landmark, V.Hansson, T.Jahnsen, K.Tasken, submitted). A monoclonal anti-human RIIα antibody (Eide *et al*., 1998) (IgG1, Transduction Laboratories, Lexington, KY) was used at a concentration of 250 μg/ml for immunofluorescence studies. Monoclonal antibody CTR453 (IgG2b) was obtained from a library of monoclonal antibodies against centrosomes isolated from human lymphoblasts. It has been characterized as a specific marker of centrosomes (mAb CTR453; Bailly *et al*., 1989) and was used at a concentration of 140 ng/ml. Secondary antibodies used in immunofluorescence labeling were Texas Red-conjugated anti-rabbit immunoglobulins and FITC-conjugated anti-mouse immunoglobulins, both diluted 1:200 (Jackson ImmunoResearch Laboratories, West Grove, PA).

Screening of phage library

Approximately 10^6 recombinant clones of an expression library containing human Jurkat-cell cDNA in λZAP Express/*Eco*RI (Stratagene) was screened using ^{32}P-labeled human RIIα as described previously (Bregman *et al*., 1989). Plasmids containing recombinant cDNAs were generated by *in vivo* excision as described by the manufacturer and sequenced using Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Buckinghamshire, UK).

RT–PCR

Human T-lymphocyte total RNA was reverse-transcribed using an oligo(dT) primer and Expand™ Reverse Transcriptase as described by the manufacturer (Boehringer Mannheim). The resulting cDNA was amplified using Expand™ Long Template PCR System according to Boehringer Mannheim. The reaction was performed using primer 5′-CGACCTTTCCTTTCTATCCCCAACCAC-3′, corresponding to nucleotides 96529–96555 in BAC1 with DDBJ/EMBL/GenBank accession No. AC003086, and primer 5′-TTAGGCAAAACATGTAACTTCAGTAGC-3′ corresponding to nucleotides 2177–2151 in BAC4 with DDBJ/EMBL/GenBank accession No. AC000120. After denaturation for 2 min at

94°C, 30 cycles of PCR were performed with 10 s at 94°C and 10 min (elongated with 10 s for each cycle from cycle 11 to 30) at 68°C, and a final extension step for 7 min at 68°C. The resulting 12.1 kb amplification product was visualized by agarose gel electrophoresis and ethidium bromide staining, excised from the gel and cloned into the pCR-XL-TOPO vector as described by the manufacturer (Invitrogen, San Diego, CA). This clone was used for further amplification of four overlapping PCR clones that were subcloned into pCR2.1-TOPO vector from Invitrogen and sequenced by Medigenomix (Martinsried, Germany). Sequence analyses were performed using the UWGCG program package (Program Manual for the Wisconsin Package, version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, WI), and nucleotide database searches were performed using the 2.0 version of BLAST (Altschul *et al*., 1997).

5′/3′ RACE

5′- and 3′-RACE was performed using human prostate and human brain Marathon RACE-ready cDNA (Clontech, Palo Alto, CA) and the Advantage KlenTaq Polymerase Mix (Clontech) as described by the manufacturer. All amplifications were carried out using two rounds of amplification, first with adapter primer 1 (Clontech) and a gene specific primer, then using a dilution of the first PCR as template with a nested gene-specific primer and adapter primer 2 (Clontech). The following PCR conditions were used: denaturation at 94°C for 1 min, 5 cycles of 30 s at 94°C and 4 min at 72°C; followed by 5 cycles of 30 s at 94°C and 4 min at 70°C; and finally 25 cycles of 30 s at 94°C and 4 min at 68°C. The resulting PCR products were cloned into pCR2.1-TOPO vector from Invitrogen as instructed by the manufacturer and sequenced by the dideoxy chain-termination method using Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham). A contig was made by 5′-RACE from nucleotides 6344–4744 of AKAP450 cDNA by using primer 5′-CTCTAGTTTGATGTTCACTGATAGGCT-3′ as gene-specific primer and then, in succession, primers 5′-TCATGTTCTCTGT-CAATGGCTTG-3′, 5′-GCTGTCTTAAATCCATTAGTTCA-3′, 5′-AG-GGACTCTGTTGCTTCTTGTTT-3′ and 5′-TTCATTTTCAGGGTC-TATTTCAG-3′ as nested gene-specific primers. A contig was also made by 3′-RACE using primer 5′-ACTGCATGCACAAATGAATGGTA-3′ as first gene specific primer, and then nested PCR using gene-specific primer 5′-GTGGTTGCTGAACTGAAGAGTGA-3′. The PCR products were subcloned and sequenced as above.

Expression of recombinant wild-type and mutated AKAP450

Two primers (5′-CCATGGATCTTGAAACCCAAATAGAATGT-3′ and 5′-AAGCTTCTATATATCTGACCCCAACTCATCTTC-3′) were used to amplify a part of the cDNA encoding AKAP450 (amino acids 2327–2602) from reverse-transcribed (as above) human T-cell total RNA. The resulting PCR product was cloned into pCR2.1-TOPO vector, sequenced, and subcloned into the *Nco*I–*Hin*dIII sites of the bacterial expression vector pGEX-KG. This plasmid (wild-type) was then mutated as described by the manufacturer using the Quick-change site directed mutagenesis kit (Stratagene, La Jolla, CA) using primers 5′-AGTGGCTGCTGCTCCGGTCAGT-CAAATCCAAC-3′ and 5′-GTTGGATTTGACTGACCGGAGCAGCA-GCCACT-3′. The resulting mutant (L-P) was subsequently mutated back to wild-type sequence using primers 5′-AGTGGCTGCTGCTCTTGTCA-GTCAAATCCAAC-3′ and 5′-GTTGGATTTGACTGACAAGAGCA-GCAGCCACT-3′, as described above, resulting in a plasmid encoding the wild-type sequence (L-P-L). *Escherichia coli* BL21*pLysS* were transformed using these plasmids, grown in the presence or absence of IPTG and lysed directly in SDS loading buffer. The lysates were separated by SDS–PAGE, transferred to nitrocellulose membranes and subjected to an RII overlay using radiolabeled bovine RII as previously described (Bregman *et al*., 1989) in the absence or presence of 500 nM Ht31 derived RII-binding peptide (NH_2-Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile-Glu-Gln-Val-Lys-Ala-Ala-Gly-Ala-Tyr-COOH).

Northern blots

DNA probes were radiolabeled with [α-^{32}P]dCTP to a specific activity of ~10^9 c.p.m./μg DNA according to the manufacturer (Amersham, Buckinghamshire, UK) using the Megaprime DNA labeling system. Two Northern blots containing 2 μg of poly(A)$^+$ RNA from various human tissues were purchased from Clontech, and hybridized using two different AKAP450 cDNAs corresponding to nucleotides –67 to 468 and 5272–6078 of the AKAP450 cDNA and human GAPDH cDNA (as a control) in ExpressHyb Solution, according to the manufacturer. Autoradiography was performed at –70°C using Amersham Hyperfilm MP and super rapid intensifying screens (Kodak, Rochester, NY).

Immunoprecipitation of AKAP450/RII from HeLa cells

Human HeLa cells (American Type Culture Collection) were grown in Eagle's minimum essential medium (EMEM) containing 10% newborn calf serum and supplemented with non-essential amino acids, 0.1 mM glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 mg/ml streptomycin. Cells were plated to 50% confluence, grown to 80–90% confluence and then harvested. The cells were detached in phosphate-buffered saline (PBS) with 10 mM EDTA, and washed several times in PBS. Cells (1.5×10^8) were resuspended and lysed for 30 min at 4°C in RIPA buffer [150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% deoxycholate, 0.1% SDS and 50 mM Tris–HCl pH 8.0] with 10 μg/ml protease inhibitors (chymostatin, leupeptin, antipain and pepstatin A; Peninsula Laboratories Inc., Belmont, CA). After sonication and centrifugation, the lysates were incubated overnight at 4°C with a combination of anti-human RIIα and anti-human RIIβ (affinity-purified antibodies, 1:30 and 1:60 dilution, respectively), and Ig-fraction of irrelevant serum (1:100 dilution) as negative control. The samples were incubated with protein A/G Plus–agarose (Santa Cruz Biotechnology, CA) for 2 h at 4°C. After three washes in PBS the immunoprecipitates were boiled in SDS loading buffer.

Centrosome isolation

Centrosomes were isolated from the T lymphoblastic cell line KE37 (Mayer *et al*., 1982) as previously described (Bornens *et al*., 1987). This preparation included treatment of cells with microtubule- and microfilament-disrupting drugs prior to lysis at low ionic strength in the presence of a neutral detergent. After DNase treatment to eliminate chromatin contamination, centrosomes were purified by sucrose gradient centrifugation.

Electrophoresis and immunoblotting

Isolated centrosomes, Triton X-100-insoluble or Triton X-100-soluble proteins of KE37 cells and immunoprecipitates of human HeLa cells were separated in 4–4.5% polyacrylamide gels containing 0.5 or 2 M urea in the separating gel with a 4.5% stacking gel without urea (Bloom *et al*., 1985). Electrophoresis was performed at 80 V in SDS running buffer at room temperature over night. The proteins were transferred by electroblotting to nitrocellulose or PVDF membranes at room temperature for 6 h at 800 mA/h in Towbin's buffer. The filters were blocked in 5% bovine serum albumin (BSA) in PBS for 3 h and incubated overnight at 4°C with affinity purified anti-AKAP450a, anti-AKAP450b or with crude 0013 serum in blocking buffer. Filters were washed for 6 h in PBS containing 0.05% Tween 20 and 0.3% Triton X-100 at room temperature, and subsequently incubated with horseradish-peroxidase-labeled protein A (dilution 1:25 000, Amersham, UK), horseradish peroxidase-labeled goat anti-rabbit IgG (dilution 1:5000) or alkaline phosphatase labeled anti-rabbit IgG (dilution 1:5000). For RII overlay, filters were subsequently subjected to an RII overlay procedure using radiolabeled bovine RII as described previously (Eide *et al*., 1998).

Immunocytochemistry

Human HeLa cells and SaOS2 osteosarcoma cells for immunocytochemistry were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin and 10% fetal calf serum and plated at 10^4 cells/ml for immunocytochemistry. Cell lines were from American Type Culture Collection (Rockville, MD). Cells were grown on culture-treated slides for 2 days and washed once with PHEM buffer (45 mM PIPES, 45 mM HEPES, 10 mM EGTA, 5 mM $MgCl_2$, 1 mM PMSF, pH 6.9), extracted with detergent by incubating for 1 min with PHEM buffer with 0.5% Triton X-100, washed in PHEM buffer and subsequently fixed with cold 100% methanol for 6 min. Following fixation, cells were rinsed with PBS containing 0.1% Tween-20 (PBST). Primary antibodies diluted in PBS containing 3% BSA were added and incubated for 1 h at room temperature. Cells were then washed three times in PBST to remove unbound antibodies followed by incubation with fluorochrome-conjugated secondary antibodies for 1 h at room temperature. Finally, cells were mounted in Vectashield mounting media containing DAPI (Vector Laboratories, Inc., Burlingame, CA), examined and photographed with an Olympus BX60 microscope (×63 plan neofluor objectives). Pictures were taken using a Hamamatsu digital camera C 4741-95 and acquired on the computer with the Vision Explorer LabView (Graftek, S.A., France) acquisition program.

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