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Molecular Cloning, Chromosomal Localization, and Cell Cycle-Dependent Subcellular Distribution of the A-Kinase Anchoring Protein, AKAP95

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The cyclic AMP-dependent protein kinase (PKA) type II is directed to different subcellular loci through interaction of the RII subunits with A-kinase anchoring proteins (AKAPs). A full-length human clone encoding AKAP95 was identified and sequenced, and revealed a 692-amino acid open reading frame that was 89% homologous to the rat AKAP95 (V. M. Coghlan, L. K. Langeberg, A. Fernandez, N. J. Lamb, and J. D. Scott (1994) *J. Biol. Chem.* 269, 7658–7665). The gene encoding AKAP95 was mapped to human chromosome 19p13.1-q12 using somatic cell hybrids and PCR. A fragment covering amino acids 414–692 of human AKAP95 was expressed in *Escherichia coli* and shown to bind RIIα. Competition with a peptide covering the RII-binding domain of AKAP Ht31 abolished RIIα binding to AKAP95. Immunofluorescence studies in quiescent human Hs-68 fibroblasts showed a nuclear localization of AKAP95, whereas RIIα was excluded from the nucleus. In contrast, during mitosis AKAP95 staining was markedly changed and appeared to be excluded from the condensed chromatin and localized outside the metaphase plate. Furthermore, the subcellular localizations of AKAP95 and RIIα overlapped in metaphase but started to segregate in anaphase and were again separated as AKAP95 reentered the nucleus in telophase. Finally, RIIα was coimmunoprecipitated with AKAP95 from HeLa cells arrested in mitosis, but not from interphase HeLa cells, demonstrating a physical association between these two molecules during mitosis. The results show a distinct redistribution of AKAP95 during mitosis, suggesting that the interaction between AKAP95 and RIIα may be cell cycle-dependent.

Key Words: **cAMP; protein kinase A; AKAP; anchoring protein; cell cycle; mitosis.**

INTRODUCTION

A large number of hormones and neurotransmitters utilize cAMP as an intracellular second messenger. Cyclic AMP regulates a number of key cellular processes such as metabolism, gene regulation, cell growth, cell differentiation, ion channel conductivity, and release of synaptic vesicles [1–6]. The main intracellular target for cAMP in mammalian cells is the cAMP-dependent protein kinase (PKA). This broad specificity kinase mediates discrete physiological responses following specific hormonal stimuli. However, one intriguing aspect of its action is the ability of PKA to selectively phosphorylate individual substrates in response to an array of hormonal stimuli. Selectivity in PKA action may be mediated by particular pools of kinase compartmentalized at distinct subcellular loci through interaction with A-kinase anchoring proteins (AKAPs, reviewed in [7–9]).

PKA holoenzyme complex is a tetramer consisting of two regulatory (R) and two catalytic (C) subunits. Four different regulatory subunits (RIα, RIβ, RIIα, and RIIβ) of PKA have been identified and serve to regulate catalytic activity by binding and inactivating the C subunit. The C subunit is released and activated upon the binding of four molecules of cAMP to the R subunit dimer (for review and references, see [10, 11]). Compartmentalization of PKA is mediated through R subunits. While the role of RI in subcellular targeting remains elusive, RII subunits are compartmentalized through hydrophobic interaction of the RII dimerization domain with AKAPs [12]. A number of different anchoring proteins have been identified and serve to sequester PKA type II with cytoskeletal elements such as microtubules (MAP2), postsynaptic densities, and cortical actin (AKAP79/75), filopodia (Gravin/AKAP250), and centrosomes (AKAP350) [13–17]. Furthermore, PKA type II is anchored to several organelles such as

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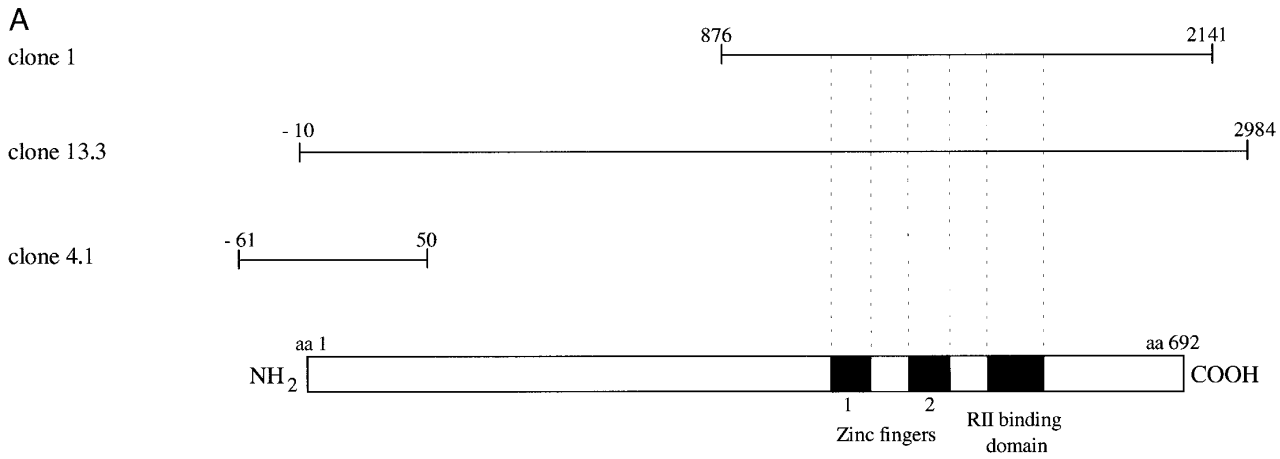

FIG. 1. (A) Cloning strategy of the human AKAP95 cDNA. Three different clones (1, 13.3, 4.1) are indicated. Numbers correspond to the nucleotide sequence, where position +1 indicates the translation initiation codon, ATG. The open box denotes the 692 amino acid (aa) open reading frame of the human AKAP95 protein. Two zinc-finger domains, 1 and 2 identified in the rat protein, and the RII-binding domain are shown as solid boxes. (B) Nucleotide sequence and deduced amino acid sequence of the human AKAP95 cDNA. Two putative zinc finger domains (underscore) and the RII-binding domain (double underscore) are indicated. The human AKAP95 cDNA nucleotide sequence data will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under Accession No. Y11997.

sarcoplasmic reticulum (AKAP100), peroxisomes (AKAP-220), Golgi (AKAP85), and mitochondria (AKAP84/149/D-AKAP1) [18–23]. A specific nuclear anchoring protein, AKAP95, was cloned from a rat pituitary library as a partial cDNA sequence and shown to interact with DNA through two zinc finger motifs [24]. Furthermore, a recent publication demonstrates that AKAP95 in contrast to AKAP79 interacts with high affinity and almost exclusively with RIIα and not RIIβ [25]. As RII is excluded from the nuclear compartment, the function of AKAP95 as an anchoring protein has been elusive. We now report the cloning of a full-length human AKAP95 cDNA that maps to a region of chromosome 19 that encodes several different zinc finger-containing DNA-binding proteins. We further demonstrate that whereas localization of AKAP95 in interphase cells is nuclear and in a separate compartment from RIIα, AKAP95 is detached from DNA during mitosis and overlap the localization of RIIα. Finally we demonstrate a physical colocalization of AKAP95 and RIIα during mitosis.

METHODS

DNA probes. An *Eco*RI–*Bgl*II fragment of rat AKAP95 covering nucleotides 1 to 2306, a PCR-generated probe to the 5′-part of AKAP95 covering nucleotides 6 to 287, and the first human AKAP95 clone (clone 1) isolated in the screening were used as probes. cDNAs were radiolabeled with [α-^{32}P]dCTP using the Megaprime DNA labeling system (Amersham, Buckinghamshire, UK) to a specific activity of approximately $1-2 \times 10^9$ cpm/μg.

Screening and isolation of cDNA clones. A total of 1×10^6 recombinant clones from a human cerebellum λgt11 cDNA library (Clontech, Cat. No. HL1128b, Palo Alto, CA) were screened by high stringency hybridization as earlier described [26, 27] using rat AKAP95 cDNA as a probe. Phage DNA was purified and the insert was subcloned into pUC18. A human testis λZAP library (Stratagene, Cat. No. 939202, La Jolla, CA) was subsequently screened with the cDNA insert from the first isolated clone in addition to a PCR probe from the most 5′end of the rat cDNA. The cDNA from positive clones were retrieved from λZAP in the plasmid pBK CMV by helper phage infection as outlined by Stratagene.

Rapid amplification of cDNA ends (RACE). The 5′-end of AKAP95 cDNA was amplified from human cerebellum Marathon RACE-ready cDNA (Clontech, Cat. No. 7401-1) using the Advantage cDNA PCR kit (Clontech, Cat. No. K1905) as described by the manufacturer. First amplification was performed using adapter primer 1 (Clontech) and oligo 1 (5′-GTCCAGCACTCCACGCCCCGTAG-3′). Thirty-five cycles were performed with a 45-s denaturation at 94°C followed by a 2-min annealing and synthesis step. Annealing and synthesis were at 72°C for the first five cycles followed by five cycles at 70°C and 25 cycles at 68°C, thereafter extension for 10 min at 68°C completed the reaction. A part of the reaction mix was then run on a 1% agarose gel and visualized using ethidium bromide staining. The product was directly subcloned from the reaction mix into the vector pCR2.1 using the TA cloning kit (Invitrogen Corp., San Diego, CA) as described by the manufacturer. The amplification and cloning were repeated, and products of two different PCR amplifications were sequenced.

DNA sequencing. cDNA clones were sequenced in both directions by the dideoxy chain termination method [28] using a combination of vector-derived and insert-specific primers and either standard protocols (Sequenase, United States Biochemicals, Colombus, CL) or automated sequencing (Eurogenetec, Seraing, Belgium). Nucleotide and amino acid sequence data were analyzed using the GCG program package, Version 8.0 [29].

Chromosomal localization. DNA from 25 somatic cell hybrids (BIOS Laboratories Inc., New Haven, CT) were used for chromosomal assignment. In addition, a human/hamster chromosome 19 mapping panel was employed to sublocalize the gene to a defined region on chromosome 19 [30]. The PCR amplifications were performed in 25 μl using 100 ng DNA, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, 10 pmol oligo 2 (5′-ACCAACGCATCCATCATCCC-3′, nucleotides 2129 to 2110), 10 pmol oligo 3 (5′-GATCCAGAAATGGAAGGAGA-3′, nucleotides 1654 to 1673), and 1.25 units Taq DNA polymerase (Perkin–Elmer, Branchburg, NJ). Amplification mixtures were denatured for

B

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                                                                -61  TGAACGCATGCGTGCTGTGGTCGCCTAGTAAACGGGGCTGCTGGTGGGCCGCGTCGAAGAC  -1

   1  ATGGACCAGGGCTACGGAGGCTACGGGGCGTGGAGTGCTGGACCTGCCAACACCCAGGGTGCATATGGAACTGGTGTGGCCAGCTGGCAAGGTTATGAAAACTACAATTACTATGGCGCC  120
   1  MetAspGlnGlyTyrGlyGlyTyrGlyAlaTrpSerAlaGlyProAlaAsnThrGlnGlyAlaTyrGlyThrGlyValAlaSerTrpGlnGlyTyrGluAsnTyrAsnTyrTyrGlyAla   40

 121  CAGAACACCAGTGTCACCACAGGCGCAACCTACAGCTACGGCCCAGCCTCGTGGGAGGCCGCCAAGGCCAATGATGGCGGCCTGGCGGCCGGGGCCCCTGCCATGCACATGGCCTCTTAC  240
  41  GlnAsnThrSerValThrThrGlyAlaThrTyrSerTyrGlyProAlaSerTrpGluAlaAlaLysAlaAsnAspGlyGlyLeuAlaAlaGlyAlaProAlaMetHisMetAlaSerTyr   80

 241  GGCCCAGAGCCATGCACCGACAATTCCGACTCCCTCATTGCCAAGATCAACCAGCGTTTGGACATGATGTCCAAGGAAGGAGGCAGGGGCGGGAGCGGCGGCGGTGGGGAGGGCATACAG  360
  81  GlyProGluProCysThrAspAsnSerAspSerLeuIleAlaLysIleAsnGlnArgLeuAspMetMetSerLysGluGlyGlyArgGlyGlySerGlyGlyGlyGlyGluGlyIleGln  120

 361  GACCGGGAGAGCTCCTTCCGCTTCCAGCCGTTCGAGTCCTATGACTCCAGGCCCTGCCTGCCGGAGCACAACCCCTACCGCCCCAGCTACAGCTACGACTATGAGTTCGACCTGGGGTCC  480
 121  AspArgGluSerSerPheArgPheGlnProPheGluSerTyrAspSerArgProCysLeuProGluHisAsnProTyrArgProSerTyrSerTyrAspTyrGluPheAspLeuGlySer  160

 481  GACCGCAATGGCAGCTTTGGGGGGCAGTACAGTGAATGCCGAGACCCAGCCCGGGAGCGGGGCTCCCTTGATGGCTTCATGCGGGGCCGGGGCCAGGGCCGCTTCCAGGACCGGAGCAAC  600
 161  AspArgAsnGlySerPheGlyGlyGlnTyrSerGluCysArgAspProAlaArgGluArgGlySerLeuAspGlyPheMetArgGlyArgGlyGlnGlyArgPheGlnAspArgSerAsn  200

 601  CCTGGCACCTTCATGCGCAGCGACCCCTTCGTGCCCCCCGCTGCGTCCTCTGAGCCCCTGTCCACGCCCTGGAACGAGCTGAACTACGTGGGTGGACGGGGCCTGGGAGGGCCCTCCCCC  720
 201  ProGlyThrPheMetArgSerAspProPheValProProAlaAlaSerSerGluProLeuSerThrProTrpAsnGluLeuAsnTyrValGlyGlyArgGlyLeuGlyGlyProSerPro  240

 721  AGCCGGCCACCTCCGTCCCTCTTCTCCCAGTCCATGGCTCCCGACTACGGCGTGATGGGCATGCAGGGGGCGGGCGGCTATGACAGCACCATGCCCTACGGATGTGGCCGCTCGCAGCCT  840
 241  SerArgProProProSerLeuPheSerGlnSerMetAlaProAspTyrGlyValMetGlyMetGlnGlyAlaGlyGlyTyrAspSerThrMetProTyrGlyCysGlyArgSerGlnPro  280

 841  CGGATGCGGGATCGGGATCGGCCCAAGAGGAGAGGGTTTGACCGCTTCGGACCAGATGGCACGGGCAGGAAACGGAAGCAGTTCCAACTTTACGAGGAGCCAGACACCAAACTGGCCCGG  960
 281  ArgMetArgAspArgAspArgProLysArgArgGlyPheAspArgPheGlyProAspGlyThrGlyArgLysArgLysGlnPheGlnLeuTyrGluGluProAspThrLysLeuAlaArg  320

 961  GTTGACAGTGAAGGAGATTTCTCCGAAAATGATGACGCAGCTGGTGACTTCCGCTCAGGAGATGAAGAATTCAAGGGTGAGGATGAACTCTGCGACTCTGGGAGGCAAAGAGGAGAGAAG 1080
 321  ValAspSerGluGlyAspPheSerGluAsnAspAspAlaAlaGlyAspPheArgSerGlyAspGluGluPheLysGlyGluAspGluLeuCysAspSerGlyArgGlnArgGlyGluLys  360

1081  GAGGACGAGGACGAGGATGTGAAGAAGAGAAGGGAAAAGCAAAGGAGAAGAGACAGGACGCGGGACCGTGCAGCCGACAGAATTCAGTTTGCCTGTTCTGTATGCAAGTTCCGTAGCTTT 1200
 361  GluAspGluAspGluAspValLysLysArgArgGluLysGlnArgArgArgAspArgThrArgAspArgAlaAlaAspArgIleGlnPheAlaCysSerValCysLysPheArgSerPhe  400

1201  GATGACGAAGAGATCCAGAAGCATCTGCAAAGCAAATTTCACAAAGAGACCCTGCGGTTCATAAGCACCAAGCTGCCCGACAAGACCGTGGAGTTCCTCCAGGAATACATTGTAAACAGA 1320
 401  AspAspGluGluIleGlnLysHisLeuGlnSerLysPheHisLysGluThrLeuArgPheIleSerThrLysLeuProAspLysThrValGluPheLeuGlnGluTyrIleValAsnArg  440

1321  AATAAGAAAATTGAGAAGCGGCGTCAGGAATTGATGGAGAAAGAAACCGCAAAACCAAAACCAGATCCTTTCAAAGGGATTGGCCAGGAGCACTTCTTCAAGAAGATCGAGGCTGCTCAC 1440
 441  AsnLysLysIleGluLysArgArgGlnGluLeuMetGluLysGluThrAlaLysProLysProAspProPheLysGlyIleGlyGlnGluHisPhePheLysLysIleGluAlaAlaHis  480

1441  TGCCTGGCCTGCGACATGCTAATTCCTGCACAGCCGCAGCTCCTCCAGCGGCACCTGCACTCCGTGGACCACAATCACAACCGCAGGTTGGCTGCTGAACAGTTCAAGAAAACCAGTCTC 1560
 481  CysLeuAlaCysAspMetLeuIleProAlaGlnProGlnLeuLeuGlnArgHisLeuHisSerValAspHisAsnHisAsnArgArgLeuAlaAlaGluGlnPheLysLysThrSerLeu  520

1561  CATGTGGCTAAGAGTGTTTTGAACAACAGACATATAGTGAAGATGCTGGAAAAATACCTCAAGGGTGAGGACCCTTTCACCAGTGAAACTGTTGATCCAGAAATGGAAGGAGATGACAAT 1680
 521  HisValAlaLysSerValLeuAsnAsnArgHisIleValLysMetLeuGluLysTyrLeuLysGlyGluAspProPheThrSerGluThrValAspProGluMetGluGlyAspAspAsn  560

1681  TTAGGAGGTGAGGATAAGAAAGAGACACCTGAGGAGGTGGCCGCGGACGTCTTAGCAGAGGTGATTACAGCAGCAGTGAGGGCCGTAGATGGGGAAGGAGCGCCCGCTCCAGAGAGCAGC 1800
 561  LeuGlyGlyGluAspLysLysGluThrProGluGluValAlaAlaAspValLeuAlaGluValIleThrAlaAlaValArgAlaValAspGlyGluGlyAlaProAlaProGluSerSer  600

1801  GGGGAGCCGGCTGAGGACGAAGGCCCCACGGACACAGCGGAGGCCGGTAGTGATCCTCAAGCCGAACAGCTGCTGGAAGAGCAGGTGCCCTGTGGAACGGCACATGAGAAGGGCGTCCCC 1920
 601  GlyGluProAlaGluAspGluGlyProThrAspThrAlaGluAlaGlySerAspProGlnAlaGluGlnLeuLeuGluGluGlnValProCysGlyThrAlaHisGluLysGlyValPro  640

1921  AAGGCCAGAAGTGAGGCTGCAGAGGCTGGAAATGGCGCCGAGACAATGGCAGCAGAGGCAGAAAGTGCCCAAACCAGAGTTGCTCCTGCCCCAGCTGCCGCGGATGCTGAAGTGGAACAA 2040
 641  LysAlaArgSerGluAlaAlaGluAlaGlyAsnGlyAlaGluThrMetAlaAlaGluAlaGluSerAlaGlnThrArgValAlaProAlaProAlaAlaAlaAspAlaGluValGluGln  680

2041  ACTGATGCAGAGTCTAAAGACGCTGTTCCCACAGAATGATGCTCATTTCCCTGTTCCAGGGAAGGCGTTGGGATGATGGATGCGTTGGTCTTTCTCCCTTGGTTTGTAAGCAGTACAAGG 2160
 681  ThrAspAlaGluSerLysAspAlaValProThrGluEnd

2161  GCGTGTGCTCCCAGAATATGCTGTAATCTAATTTTGGTGAAGAGACCCAGCGTTTCCTCCTGAGCAGTGCCTCTCACGGCTTGTCTCATGCAGTCGTGTGGCTTCTTGCCCAGGTTTCAA 2280

2281  AGCTGAAGTACATTGTCCTTAGCGGCTGTAACATGTCTCTTGACAGTAGTGCACTTGGAATAATAAAGGTTGGGTGATTATATCTTGATGATACATTACTTGTTCAATACAGCCACTGAT 2400

2401  GGAATGCTTCCTTTTTTATTTTTTTCCTTAATTTTTTTTTTTATTTGGTTGGGAACAGCTGAATACTAGGAATATATCTTGCTCTATAGAGGATTTTTTTTTGTATGTTTCAAGCTTCAG 2520

2521  CCTTTAACCTATACCTTTGTAGTGCACCATATGGTGTGTGACTTTCACAGGACTTCGCAGCACCTGGTTCACATGTGGCACTGACCGCGTCACATCCACGCACTCCCAAAGGCCAGAAGT 2640

2641  ATCTGACCGACCTACGCCACTGGAAACACACCCACCGCAACCTCAAGAACCAGACTGTGCAGAGGGCATTGCGTCCCAATCTTTAGTCCTTGCTGAATCAGTTCTCTAATATTTTACCTC 2760

2761  ATTTGTGTTCCACCTCTAGATTACTTCAGGTTTTTTTCCTTTAAAATTAGTTACTACCACTCAAATGTATTTACAAAGAGAATTTGGCCAGGCACGGTGATGCATACCTATAATCCCAGC 2880

2881  ACTTGGGGAGGCCGTGGTGAGAGGATAGCTTAAGCCCAGGAGTTCAAGACCAACCTGGACAACATAGCAAGACCCCATCTCTTAAAAAAAAAAAAAAAAAAAAA 2984
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FIG. 1—*Continued*

1 min at 94°C, annealed for 30 s at 62°C, and extended for 30 s at 72°C for 30 cycles, with a final extension of 10 min at 72°C. The amplification products were analyzed on 10% polyacrylamide gels and visualized by ethidium bromide staining.

Expression of recombinant AKAP95. A 900-bp *Eco*RI fragment of AKAP95 cDNA (nucleotides 1242 to 2141) encompassing both zinc fingers and the putative amphipathic helix RII-binding domain of AKAP95 was subcloned into the expression vector pGEX-KG. The expression and purification of the protein was as described previously [31].

Western blot analysis. Proteins were separated by SDS–PAGE and transferred by electroblotting to nitrocellulose membranes and immunoblot analysis was performed as described elsewhere [32]. Protein-G-purified rabbit anti-rat AKAP95 antibody was used at a concentration of 0.1 μg/ml [24] and biotinylated affinity-purified rabbit anti-human RIIα antibody was used at a concentration of 0.5 μg/ml [33]. Primary antibodies were detected by horseradish-peroxidase-labeled protein A (dilution 1/25 000, Amersham) and streptavidin (dilution 1/1500, Amersham) in the second layer and developed using ECL (Amersham).

RII overlays. Proteins were separated by SDS–PAGE and transferred by electroblotting to nitrocellulose membranes. Binding was detected using recombinant human RIIα labeled by phosphorylation

FIG. 2. Homology comparison of the deduced amino acid sequences of human (upper lines, indicated by h) and rat (lower lines, indicated by r) AKAP95. Only diverging residues are indicated in the rat amino acid sequence. Dots indicate gaps introduced in either sequence to optimize alignment. Two putative zinc finger domains (underscore) and the RII-binding domain (double underscore) are indicated.

with C subunit of PKA and [γ-^{32}P]ATP and used in a modified Western blot protocol as previously described [34].

Northern blots. A Northern blot containing 2 μg of poly(A)$^+$ RNA from various human tissues was purchased from Clontech. The filter was prehybridized in 5× Denhardt's solution, 5× SSC, 50 mM sodium phosphate buffer, pH 6.8, 0.1% SDS, 250 μg/ml single-stranded salmon sperm DNA, and 50% (v/v) formamide at 42°C for 3 h, and hybridized for 16 h in the same solution containing radiolabeled human AKAP95 cDNA corresponding to nucleotides 1–1088 or chicken β-actin [35]. The membranes were washed four times in 2× SSC, 0.1% SDS for 5 min at room temperature, followed by two washes using 0.1× SSC, 0.1% SDS at 50°C for 30 min. Autoradiography was performed at −70°C using Amersham Hyperfilm MP and super rapid intensifying screens (Kodak, Rochester, NY).

Immunocytochemistry. Immunofluorescence studies were performed on human Hs-68 fibroblasts (American Type Culture Collection, Rockville, MD) grown on glass coverslips and fixed in 3.7% formalin in PBS followed by extraction in −20 °C acetone. Cells were rehydrated in PBS and 1% bovine serum albumin (BSA) and then incubated with affinity-purified primary antibody to AKAP95 [24] and a newly developed purified mouse monoclonal IgG1 antibody to human RIIα (K.T. and T.J. in collaboration with Transduction Laboratories, Lexington, KY), at 1:100 dilution and 2.5 μg/ml, respectively. Following incubation at 37°C for 60 min, cells were washed three times with phosphate-buffered saline (PBS) with 1% BSA and stained for primary antibodies with fluoroscein isothiocyanate(FITC)-labeled anti-rabbit IgG or biotinylated anti-mouse IgG antibodies for 30 min. Subsequently, RIIα was visualized by staining with Texas red-conjugated streptavidin. Finally, cells were counterstained for DNA with DAPI. The cells were mounted, observed, and photographed on a Zeiss axiophot photomicroscope using a 40× plan-apochromat (1.4NA) oil immersion lens as described elsewhere [36].

Immunoprecipitation of AKAP95/RIIα complexes from 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 nonessential amino acids, 0.1 mM glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 mg/ml streptomycin. Cells were plated to 50% confluency, grown for 48 h, and either left untreated and asynchronous (approximately 2% M-phase cells and 98% interphase cells) or treated with Nocodazole (1 μM; Sigma, St. Louis, MO) for the last 24 h to block cells in mitosis. The following day, untreated cells were detached in PBS with 10 mM EDTA, whereas plates with cells treated with Nocodazole were shaken vigorously in the absence of EDTA to loosen only rounded M-phase cells. Subsequently, cells were harvested by centrifugation at 400*g*. Microscopy of Nocodazole-treated cells stained with aceto-orcein (Gibco BRL Life Technologies Ltd., Paisley, UK) revealed that more than 98% of the cells were arrested in mitosis. After several washes in PBS, 3×10^8 pelleted cells were resuspended in 1 ml buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM AEBSF, 0.5 mM DTT, 1 mM Benzamidine, 10 μM IBMX). This cell suspension was incubated at 4°C for 2 h followed by addition of NP-40 to 0.2% and homogenization with an ice-cold Dounce homogenizer and type "A" pestle. The cell lysate was centrifuged at 15,000*g* for 15 min at 4°C and total protein concentration was determined using a modified Bradford assay [37]. HeLa cell lysate (3 mg per immunoprecipitation) was precleared with 25 μl 25% (v/v) protein G–Sepharose. Thereafter, cleared supernatants were incubated overnight at 4°C with 6 μg of either affinity purified rabbit anti-AKAP95 or preimmune IgG prior to the addition of 25 μl 25% (v/v) protein G–Sepharose to precipitate the immune complexes. The precipitate was washed twice with 1 ml buffer A containing 1 M NaCl and 0.2% NP-40 followed by four washes in buffer A with 0.2% NP-40. The elution was accomplished using 150 μl 2× SDS–PAGE sample buffer and immunoprecipitates were examined for the presence of AKAP95 and RIIα by immunoblot analysis.

RESULTS

Cloning of Human AKAP95

The human homologue of AKAP95 was isolated using the rat cDNA as a probe. A cDNA (clone 1) was isolated from a human cerebellum λgt11 library. The cDNA insert of clone 1 was 1265 nucleotides in length, and

TABLE 1

Concordance and Discordance Analyses of AKAP95 Locus in Human/Hamster Cell Hybrids

Human chromosome	Concordant		Discordant		Discordancy (%)
	+/+	−/−	+/−	−/+	
1	2	17	5	1	24
2	0	17	7	1	32
3	1	15	6	3	32
4	1	17	6	1	28
5	7	3	0	15	6
6	2	16	5	2	28
7	2	18	5	0	20
8	1	14	6	4	40
9	0	15	7	3	40
10	1	16	6	2	32
11	1	16	6	2	32
12	2	16	5	2	28
13	5	17	2	1	12
14	4	15	3	3	24
15	2	16	5	2	28
16	0	15	7	3	40
17	0	16	7	2	36
18	1	15	6	3	36
19	7	18	0	0	0
20	1	16	6	2	32
21	5	16	2	2	16
22	2	16	5	2	28
X	0	15	7	3	40
Y	1	15	6	3	36

Note. Concordant (+/+ or −/−) and discordant (+/− or − +) segregation patterns of the AKAP95 gene were determined for each of the 25 cell hybrids. +/+ Indicates the presence of both the gene and the particular chromosome; −/− indicates the absence of both the gene and the chromosome; +/− indicates that the gene was detected without the chromosome being present; −/+ indicates that the gene was not detected but the chromosome was present.

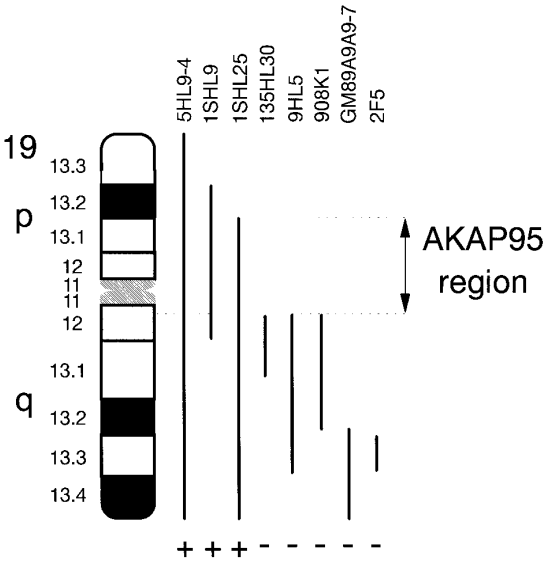


FIG. 3. Regional assignment of the AKAP95 gene by PCR on a human chromosome 19 mapping panel of somatic cell hybrids. DNA from a chromosome 19 mapping panel of somatic cell hybrids was used as a template for the specific PCR amplification of a 469-bp fragment of the human AKAP95 gene. Lines along the chromosome 19 idiogram indicate the regions of this chromosome present in the different hybrids. + or - denotes the presence or absence of the AKAP-specific PCR amplification product in the indicated hybrids. Note that the presence of region 19p13.1-q12 was necessary and sufficient for a positive PCR test.

started at position 876 compared to the rat cDNA sequence (Fig. 1A). In order to isolate the 5′end of the human AKAP95 cDNA, a random and oligo(dT)-primed human testis λZAP library was screened with a PCR-generated probe from the most 5′end of the rat cDNA (nucleotides 6 to 287) and the previously isolated clone 1 using double filter lifts. Sequencing of the positive clone 13.3 demonstrated that the clone overlapped with clone 1 and yielded 886 bp of additional 5′-sequence, including a putative translation initiation codon 10 bp from the 5′end. In order to further investigate upstream sequences, the 5′-end of the AKAP cDNA was amplified from human cerebellum cDNA using an oligonucleotide complementary to nucleotides 21 to 43 of the open reading frame of the human AKAP95 cDNA. The amplification yielded a single product of approximately 120 nucleotides as visualized by gel electrophoresis. The product was subcloned, sequenced, and shown to contain 81 nucleotides in addition to the anchor and primer sequences. Of these, 30 nucleotides were shown to be identical to the 30 most 5′ nucleotides of clone 13.3 and in continuity with the AKAP95-specific primer used for amplification, indicating that the two clones were generated from identical mRNAs. Fur-

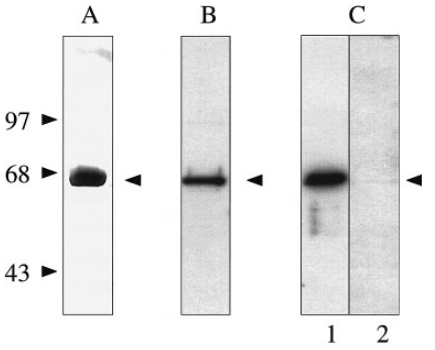


FIG. 4. Recombinant human AKAP95 binds RIIα. A fragment of the human AKAP95 cDNA (encoding residues 414–692 of the protein) was inserted into the pGEX-KG expression vector and a GST-AKAP95Δ1–413 fusion protein was purified. Purified protein (500 ng in A, 50 ng in B and C) was separated by electrophoresis in 10% SDS–poly acrylamide gels and transferred by blotting to nitrocellulose membranes. Gels were stained with Coomassie brilliant blue dye (A). The recombinant human AKAP95 fragment was detected by Western blot with affinity-purified anti-rat AKAP95 antibodies (B). RII binding was detected by a solid-phase binding assay using ^{32}P-radiolabeled RIIα (C) as a probe in absence (lane 1) or presence (lane 2) of 500 nM Ht31 anchoring inhibitor peptide. Numbered arrowheads to the left of A indicate molecular mass (kDa) of marker proteins (SDS–PAGE low range molecular markers, Bio-Rad, Richmond, CA).

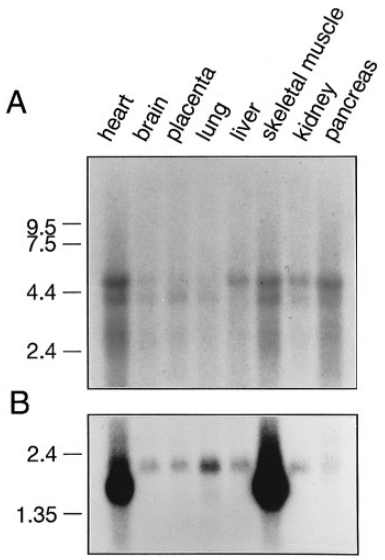


FIG. 5. Tissue distribution of AKAP95 mRNA. A Northern filter containing 2μg of poly(A)$^+$ RNA from different human tissues (human MTN, Clontech) was hybridized with a ^{32}P-radiolabeled 1.0-kb *Eco*RI–*Xho*I fragment of AKAP95 clone 13.3 (A) or β-actin (B) and subjected to autoradiography. The tissue source of each RNA is indicated above each lane. Migration of RNA markers (kb) are indicated on the left.

thermore, clone 4.1 contained additional 51 nucleotides upstream of clone 13.3. Two in-frame stop codons were located at position −33 to −31 and −36 to −34 upstream of the methionine codon, indicating that the ATG triplet at position 1 to 3 is the translation initiation site. Figure1B shows the 3045-bp composite sequence of clones 13.3 and 4.1. The nucleotide sequence is 80% identical to rat AKAP95 and encodes a 692-amino acid protein with a calculated molecular mass of 76 kDa. The deduced amino acid sequence is 89% identical to rat AKAP95, indicating that the isolated cDNA clones represent the human AKAP95 (Fig. 2). The human protein is 5 amino acids longer than the rodent counterpart due to insertions. The proposed RII-binding region (Fig. 2, double underlined) is conserved between rat and human with the exception of two conservative amino acid changes from Glu to Asp in position 4 and Lys to Arg in position 16 of a putative amphiphatic helix that can be predicted. The sequence of human AKAP95 ended in a stretch of A's, indicating that this particular clone from a combined random- and oligo(dT)-primed library originated by an oligo(dT) priming. However, this probably does not represent the poly(A) tail, as no polyadenylation signal is found in the nearby sequences.

Chromosome Localization

A PCR reaction using two primers based on sequences from the 3′ region of the human AKAP95 cDNA generated a single band when using human, but not hamster genomic DNA as a template. Sequencing demonstrated that the amplification product represented human AKAP95. Twenty-five different hamster/human hybrid cell lines were tested for the presence of the human AKAP95 gene by DNA amplification. Discordancy analysis of the entire panel of hybrid cell lines localized the human AKAP95 gene to chromosome 19, showing 100% concordancy (Table 1). The regional location of the human AKAP95 gene was determined using seven human/hamster somatic cell hybrids containing different regions of chromosome 19 (Fig. 3). This regional mapping panel is designed primarily for mapping on 19q; however, two cell lines containing p arm material (1SHL9, 1SHL25) were also included in the analysis. Together with a hybrid monochromosomal for human 19 (5HL9-4), the selected cell lines span the entire chromosome 19. PCR on DNA from the 5HL9-4 cell line monochromosomal for human chromosome 19 yielded a human AKAP95-specific PCR product, demonstrating that human chromosome 19 genomic DNA is necessary and sufficient to yield a positive PCR analysis, whereas no PCR product could be demonstrated using hamster DNA from the same source. Furthermore, DNA from two of the hybrid cell lines in the mapping panel (1SHL9 and 1SHL25) yielded a PCR product of the expected length. Figure 3 summarizes the results obtained and shows that the presence of chromosome region 19p13.1-q12 was necessary and sufficient to yield a human AKAP95-specific PCR product.

Expression and Characterization of Human AKAP95

To assess the RII binding properties of human AKAP95, a fragment of the human AKAP95 cDNA (encoding residues 414 to 692 of the protein) was inserted into the expression vector pGEX-KG and a GST-AKAP95Δ1–413 fusion protein was purified (Fig. 4A). The identity of the recombinant fusion protein was verified by Western blot analysis (Fig. 4B) using anti-rat AKAP95 antibodies. This recombinant AKAP95 protein bound ^{32}P-radiolabeled human RIIα (Fig. 4C, lane 1) as assessed by direct overlay. Solid-phase binding of RIIα was blocked in the presence of the anchoring inhibitor peptide, Ht31 [12] (Fig. 4C, lane 2).

Tissue Distribution of AKAP95 mRNA

To investigate the tissue expression of AKAP95, a Northern filter containing poly(A)$^+$ RNA from different human tissues was hybridized with a ^{32}P-radiolabeled 1.0-kb *Eco*RI–*Xho*I fragment of clone 13.3. The probe detected 4.3- and 5.0-kb mRNAs expressed at high levels in heart, liver, skeletal muscle, kidney, and pancreas (Fig. 5A). Subsequently, the filter was hybridized with β-actin as a control (Fig. 5B).

Immunolocalization of AKAP95 and PKA Regulatory Subunit RIIα

As AKAP95 has been shown to be selectively interacting with RIIα [25], the intracellular localization of AKAP95 and RIIα was examined in human fibroblasts Hs-68 cells. Immunolocalization of AKAP95 and RIIα was determined by double staining, indirect immunofluorescence, and microscopy. In contrast to the nuclear fluorescence observed for AKAP95 in interphase cells (Fig. 6A) that decorated chromatin but excludes nucleoli, the staining pattern for RIIα appeared to be primarily cytoplasmic (Fig. 6B), with perinuclear and Golgi staining. The separation of AKAP95 and RIIα into separate cellular compartments in interphase is clearly demonstrated by the discrimination of green and red in a double image overlay (Fig. 6C). These results suggests that only minor amounts of type II PKA were associated with AKAP95 in the nucleus of interphase cells.

During mitosis, the nuclear envelope is broken down and there may not be a barrier to maintain PKA and AKAP95 in separate cellular compartments. AKAP95 staining changed markedly and appeared to be excluded from condensed chromatin and exposed to cytoplasmic components and decorated an area outside but close to the metaphase plate (Fig. 6A, mitotic cell). Moreover, RIIα subunit antibody showed bright, widely distributed staining during mitosis (Fig. 6B), but with a strong intensity in the region surrounding the metaphase plate where it colocalized with AKAP95 as demonstrated by image overlay (Fig, 6C). The results suggest that AKAP95 is likely to be exposed to type II PKA during mitosis.

We further examined the distribution of AKAP95 and RIIα in different phases of mitosis (Fig. 7). The results demonstrate that AKAP95 dissociated from chromatin in prometaphase at which point the overlap with RIIα was not clear (Figs. 7A–7C). In an early forming metaphase (Figs. 7D–7F), the dissociation of AKAP95 and chromatin became clear and an overlap with RIIα was emerging. In metaphase, AKAP95 localized in a region in close proximity to, but outside the metaphase plate and overlapped with a part of the pool of RIIα (Figs. 7G–7I, upper, left cell). In anaphase, AKAP95 and RIIα started to segregate (Figs. 7G–7I, upper, middle cell), and in telophase, AKAP95 reentered the forming nucleus and was again separated from RIIα (not shown).

Colocalization of AKAP95 and RIIα

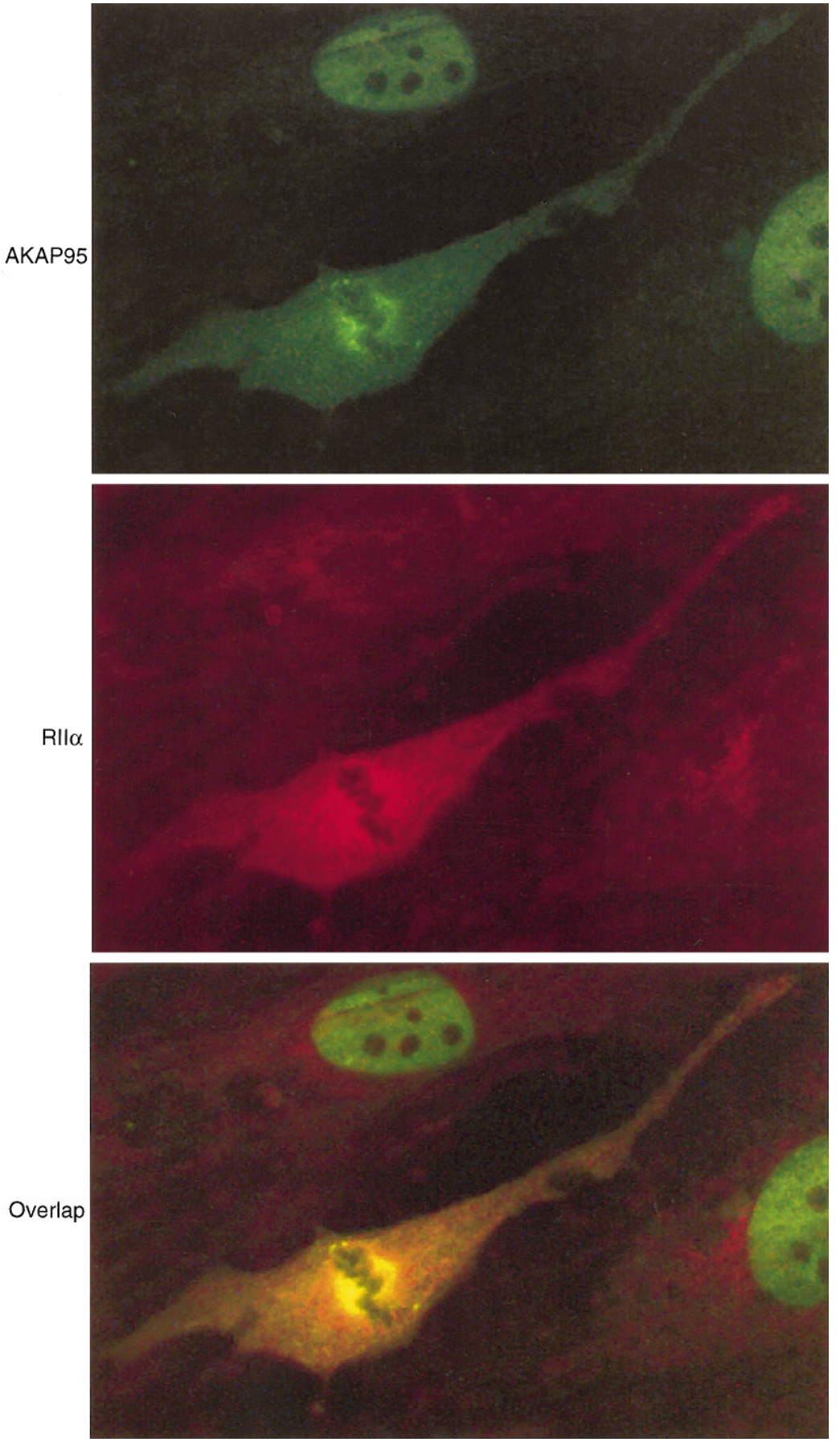
As shown in Fig. 6, AKAP95 and RIIα appeared to be in separate cellular compartments in interphase cells but showed an apparent colocalization during mitosis. For this reason, we examined the colocalization of AKAP95 with RIIα in interphase and M-phase HeLa cells. Anti-AKAP95 and anti-RIIα immunoprecipitates from lysates of asyncronous HeLa cells containing 98% cells in interphase and from lysates of Nocodazole-treated HeLa cells containing more than 98% mitotic cells were analyzed by Western blotting for the presence of AKAP95 (Fig. 8A) and RIIα (Fig. 8B). Figure 8A shows the presence of AKAP95 in immunoprecipitate from HeLa cells arrested in mitosis (lane 2, M), whereas virtually no signal was observed in immunoprecipitate from interphase cells (lane 1, I) or in the eluate from a precipitation of mitotic HeLa cell lysate with preimmune IgG (lane 3, C). A parallel anti-RIIα immunoblot in Fig. 8B showed a strong signal from RIIα coimmunoprecipitated with AKAP95 in mitotic cells (lane 2, M), whereas only low levels of RIIα were detected in AKAP95 immunoprecipitate from interphase cells or control precipitate from mitotic cells (lanes 1 and 3, respectively). A distinct proportion of RIIα was present in the supernatant following immunoprecipitation with anti-AKAP95 and apparently not associated with this anchoring protein (not shown). The lack of AKAP95 in immunoprecipitate from interphase cells is probably due to the fact that postnuclear supernatants of asynchronous HeLa cells contained very low levels of AKAP95 prior to immunoprecipitation as AKAP95 was not solubilized by the lysis buffer. This is in contrast to the majority of RIIα which was present in equal amounts in lysates of interphase and mitotic HeLa cells.

DISCUSSION

The present study reports the cloning and chromosomal localization of human AKAP95. Expression of AKAP95 mRNA is shown in human heart, liver, skeletal muscle, kidney, and pancreas. The nuclear AKAP95 is shown to redistribute and detach from condensed chromatin during mitosis. Coprecipitation of AKAP95 and RIIα is demonstrated in HeLa cells arrested in mitosis, but not in asyncronous cells.

Cloning of the human AKAP95 revealed a protein with a 692-amino acid open reading frame that was 89% homologous to the previously reported rat AKAP95. 5′ RACE cloning of human AKAP95 demonstrated several in-frame stop codons upstream of the most 5′ methionine identified, indicating that the composite human cDNA reported here as well as the rat sequence represent the complete coding sequence of AKAP95. As expected, the proposed RII-binding region and zinc-finger domains were conserved between rat and human. In addition, a putative nuclear localization sequence of basic residues, KKRREKQRRR (amino acids 368 to 377), as well as a bipartite motif for nuclear targeting, RR(X_{10})GRKRK (amino acids 290 to 306), are present in AKAP95 [38]. These sequences are conserved between the human and rat forms and may facilitate the nuclear targeting of this protein.

The gene encoding AKAP95 was located to chromo-


AKAP95
RIIα
Overlap

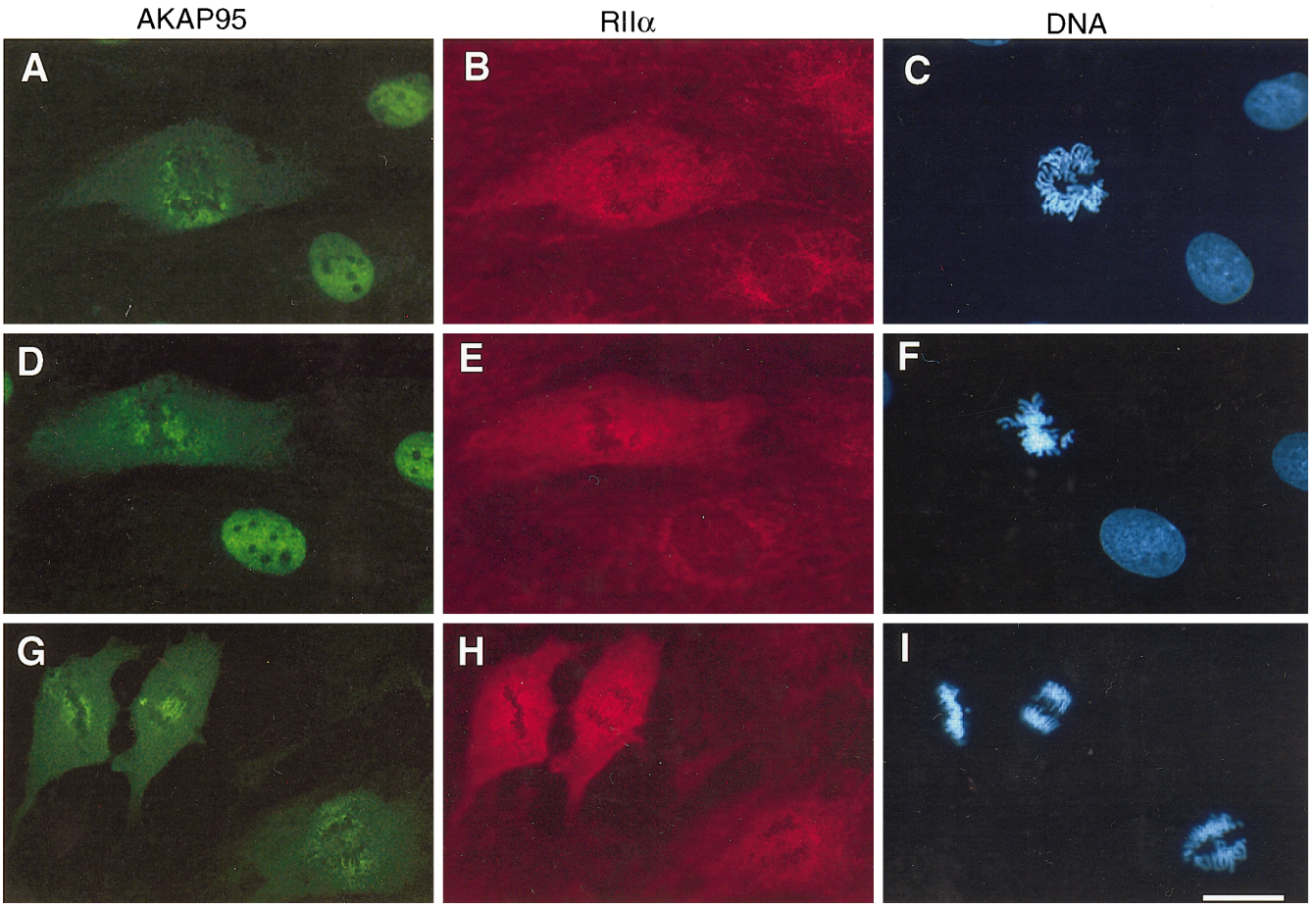


FIG. 7. Localization of AKAP95 and RIIα in different phases of mitosis. Human Hs-68 fibroblasts were double stained with antibodies to AKAP95 (A, D, G) and RIIα (B, E, H) followed by counterstaining for DNA with DAPI (C, F, I). Upper row of panels (A–C) shows a cell in prometaphase with two interphase cells at the periphery. Middle row of panels (D–F) shows a cell entering metaphase and organizing the metaphase plate with two interphase cells at the periphery. Lower row of panels (G, H, I) shows cells in metaphase (upper, left), anaphase (upper, middle), and prometaphase (lower, right). Bar, 8 μm.

some 19p13.1-q12.1 using somatic cell hybrids and a PCR reaction amplifying a human AKAP95-specific product. More than 1200 markers have an assignment overlapping 19p13.1-q12 [39]. However, this group comprises only a few characterized genes with known gene products that include the Cα subunit of PKA, the phosphodiesterase type IV A, as well as several zinc finger-DNA binding proteins [39–41]. Furthermore, several diseases including familiar hemiplegic migraine, the related hereditary peroxysomal cerebellar ataxia, and cerebral autosomal-dominant arthropathy have been mapped to this region although no candidate genes have been identified yet [42]. In addition, translocations and chromosomal aberrations involving 19p have been associated with B and T cell acute lymphoblastic leukemias, ovarian carcinomas, and benign adenomas of the pituitary and thyroid glands [43–46]. Activation of PKA by cAMP regulates proliferation in both a positive and negative fashion in different cell types [3, 47]. It is interesting to speculate that chromosomal rearrangements involving AKAP95 may disrupt normal cAMP-mediated growth regulation and lead to neoplasia.

Function of AKAP95 as an anchoring protein has been elusive as it is localized in a different compartment from the majority of RII. Several publications have demonstrated low amounts of RII associated with nuclei [48–50] but we were not able to demonstrate a

FIG. 6. Cell cycle-dependent redistribution of AKAP95 and the RIIα regulatory subunit of PKA in human Hs-68 fibroblasts. Purified antibodies were used to double stain for AKAP95 (upper panel, green) and RIIα (middle panel, red). Lower panel, shows a two-color image overlay.

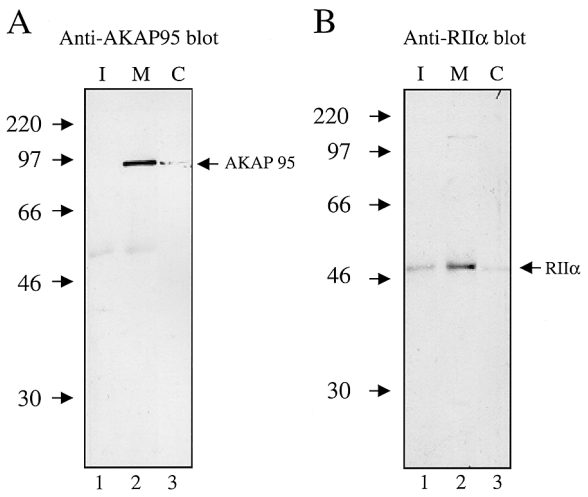


FIG. 8. RIIα coimmunoprecipitates with AKAP95 from mitotic but not from interphase HeLa cells. (A) Cell lysates from asyncronous HeLa cells containing approximately 98% interphase cells (lane 1, I) and cell lysates from HeLa cells treated with 1 μM Nocodazole for 24 h (98% of cells arrested in mitosis, lanes 2 and 3, M and C) were used for precipitation employing either an immune IgG specific to AKAP95 (lanes 1 and 2) or a preimmune IgG (lane 3, C). Precipitates were subjected to immunoblot analysis using affinity-purified rabbit anti-AKAP95 antibody. (B) Detection of RIIα in the same immunoprecipitates as in A was by Western blot using an affinity-purified, biotinylated rabbit anti-human RIIα antibody and Streptavidin in the second layer to avoid interaction with heavy chain IgG on the blot. Numbers on the left of both panels indicate molecular mass (kDa) of marker proteins (Rainbow high molecular weight range markers, Amersham).

physical colocalization of RII and AKAP95 in interphase cells. Demonstration that AKAP95 is redistributed from a nuclear localization in interphase cells to be absent from the condensed chromatin and shifted to a localization that overlaps the localization of RIIα in mitotic cells provides a possible role of AKAP95 in anchoring of RIIα during mitosis. The observation that RIIα could be coprecipitated with AKAP95 in HeLa cells arrested in mitosis, but not in interphase HeLa cells, suggests a physical colocalization of RIIα and AKAP95 during mitosis. Microinjection studies with C and PKI have demonstrated that downregulation of PKA activity is necessary for nuclear envelope breakdown [36]. However, the actual role of PKA during mitosis is not known and the function of an RII/AKAP95 complex will have to await future studies. It is also possible that AKAP95 serves to redistribute low levels of RII to the nuclear compartment at the onset of interphase although we have not been able to demonstrate RII associated with AKAP95 from nuclei. Furthermore, *in situ* RII overlays have demonstrated that the majority of free RII-binding sites *in vivo* are nuclear, indicating that the major proportion of AKAP95 is not associated with RII during interphase [25]. In addition, demonstration of RII binding to nuclear AKAP95 *in situ* in fixed and permeabilized cells indicates that nuclear targeting of AKAP95 does not inhibit concomitant binding of RII. The redistribution of AKAP95 during the cell cycle is thus not likely to be due to the regulation of the affinity between RII and AKAP95. Whether the affinity between AKAP95 and chromatin or nuclear matrix is regulated during the cell cycle remains to be shown. Phosphorylation by a cell cycle-dependent kinase to detach AKAP95 from its nuclear localization is an interesting possibility. However, several putative cdc2 phosphorylation sites in the rat AKAP95 sequence were not conserved in the human sequence and mitotic kinase extracts did not phosphorylate human AKAP95 *in vitro* (data not shown).

In summary, the present paper reports the cloning and chromosomal localization of a human homologue of rat AKAP95. A cell cycle-dependent regulation of the subcellular localization of AKAP95 is demonstrated, indicating a role for this protein in binding and targeting PKA type II during mitosis.

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