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mAKAP: an A-kinase anchoring protein targeted to the nuclear membrane of differentiated myocytes

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SUMMARY

The compartmentalization of second messenger-activated protein kinases contributes to the fidelity of hormone-mediated signal transduction events. For example, the cAMP-dependent protein kinase is tethered at specific intracellular locations through association with A-kinase anchoring proteins (AKAPs). We now report the cloning of mAKAP, an anchoring protein found predominantly in heart, skeletal muscle and brain, and whose expression is induced in neonatal ventriculocytes by treatment with hypertrophic stimuli. mAKAP is targeted to the nuclear membrane of differentiated myocytes. Analysis of mAKAP-green fluorescent protein (GFP) fusion constructs revealed that nuclear membrane targeting is conferred by two regions of the protein, between residues 772-915 and 915-1065, which contain spectrin-like repeat sequences. Heterologous expression of the mAKAP targeting sequences displaced the endogenous anchoring protein from the nuclear membrane, demonstrating that mAKAP targeting is saturable. Collectively, these data suggest that a domain containing spectrin-like repeats mediates targeting of the anchoring protein mAKAP and the cAMP-dependent protein kinase holoenzyme to the nuclear membrane in response to differentiation signals.

Key words: Protein kinase A, Heart, Spectrin repeat, Differentiation, AKAP

INTRODUCTION

The phosphorylation of intracellular proteins is a universal mechanism used to control many cellular events that occur in response to extracellular signals. Occupancy of G-protein-coupled receptors on the plasma membrane triggers the generation of second messengers such as cyclic AMP (cAMP) which, upon diffusion to their sites of action, influence the activation of protein kinases and phosphatases (Lefkowitz, 1993). In turn, the phosphorylation state of intracellular proteins is regulated by the activation or suppression of these enzymes. Since many kinases and phosphatases are widely distributed and often exhibit broad substrate specificities, additional mechanisms are in place to restrict their access to preferred substrates. Increasing evidence suggests that the subcellular location of the cAMP-dependent protein kinase (PKA) is tightly controlled (Colledge and Scott, 1999; Dell'Acqua and Scott, 1997). Moreover, activation of PKA by different extracellular stimuli results in the preferential activation of distinct processes within the same cell (Livesey et al., 1982). Isoproterenol treatment of cardiac myocytes increases cAMP levels and activates both particulate and soluble PKA (Buxton and Brunton, 1983). The resulting phosphorylation of the contractile protein troponin I and activation of glycogen phosphorylase contributes to an increase in cardiac contractility. Prostaglandin E_1 treatment of the same cells also increases cAMP levels, but in contrast, activates only soluble PKA, without effect on troponin or glycogen phosphorylase. One mechanism that explains this sophisticated level of PKA signaling is the selective activation of compartmentalized PKA pools. This view is now supported by increasing evidence illustrating that compartmentalization of the cAMP-dependent protein kinase is maintained by a family of A-kinase anchoring proteins (AKAPs) (Colledge and Scott, 1999; Dell'Acqua and Scott, 1997; Rubin, 1994).

Mammalian PKA is a heterotetramer composed of two regulatory (R) and two catalytic (C) subunits, for which there are ubiquitously expressed four R-subunit genes, RIα, RIβ, RIIα and RIIβ, and two C-subunit genes, Cα and Cβ. AKAPs bind to the regulatory subunit dimer of the PKA holoenzyme and target the PKA/AKAP complex through protein-lipid or protein-protein interactions with membranes of cellular organelles (Dell'Acqua et al., 1998; Fraser et al., 1998). In excess of 25 AKAPs have been described from diverse species, ranging from *C. elegans* and *Drosophila* to humans, all of which contain a conserved R-subunit binding site (Angelo and Rubin, 1998; Huang et al., 1997a). These binding sites form an amphipathic helix that binds to a hydrophobic pocket formed by the N termini of the R-subunit dimer (Newlon et al., 1999). AKAP specificity is conferred by a unique targeting domain that directs the PKA/AKAP complex to discrete subcellular compartments.

By screening cDNA expression libraries with radiolabeled, bacterially expressed RIIα-subunit in a protein overlay assay, we had previously isolated clones for an AKAP found in heart, skeletal muscle and brain, AKAP100 (McCartney et al., 1995). We now report the complete cloning of the rat and human isoforms of AKAP100, which we have renamed mAKAP for its presence in striated muscle. We show that mAKAP is targeted to the nuclear membrane of differentiated cardiac ventriculocytes by a spectrin repeat homology domain and is induced in expression by hypertrophic agents such as phenylephrine.

MATERIALS AND METHODS

Cloning of mAKAP

The complete cloning of mAKAP was accomplished by a modification of the Marathon Rapid Amplification of Cloned Ends (RACE) Cloning Kit (Clontech), in which rounds of polymerase chain reaction (PCR) were performed using pairs of nested primers that were based initially on the 5′ sequence of the rat ROB1 clone (Fraser et al., 1998), the overlapping human AKAP100 contig (McCartney et al., 1995) and GenBank sequence KIAA0311. Marathon brain, heart or skeletal muscle cDNA was purchased from Clontech or synthesized as per the manufacturer's protocol, except that 2.5 μg poly(A)-selected RNA were annealed to 10 pmol specific primer for the reverse transcriptase reaction, and 2 μg carrier tRNA were included during the DNA precipitation steps. RACE-PCR was performed using the 'touchdown' conditions suggested by the manufacturer using Klentaq Advantage polymerase (Clontech), nested specific primer, Marathon AP1 primer and 10 minute extension times.

RACE-PCR products were phenol-chloroform extracted, purified through a Centricon-100 filter (Amicon), and digested overnight with *Not*I and a second restriction enzyme that recognized a site near the 5′ end of the previously determined cDNA sequence. The digested products were ligated into pBSK− (Stratagene). β-galactosidase-positive *E. coli* DH5α transformants were screened by PCR with Taq polymerase and standard universal and reverse M13 primers, such that cloned RACE products on agarose gels large enough to encode new 5′ information were then subjected to automated, fluorescent dideoxynucleotide sequencing. For each of the rat and human cDNAs, final RACE reactions with multiple, different antisense oligonucleotides yielded discrete products of lengths consistent with a single 5′ end. 36 primers were designed to complete the cDNA sequencing of larger PCR clones.

Using pairs of specific oligonucleotides designed to amplify large fragments that might be used to construct full-length clones, PCR was performed with the high fidelity enzyme PfuTurbo polymerase. A varient pBSK–, pBNX, was constructed using a novel polylinker (GGC CTC GTC GAC GCG GCC GCT CCG GAC CAT GGG CCC TAG GAC GTC GCA TGC TAG CTC CGG AGA TCT AGA GGT ACC TCG AGT AGT AC) in order that PCR products might be easily subcloned and color-screened. PCR products were subcloned as above into pBNX using unique sites within the rat and human cDNA and the *Not*I site within the 5′ Marathon linker. Two human and two rat subclones and pBSK-ROB-1 were then used to assemble full-length rat and human cDNAs in pBNX. Both full-length clones were verified by complete sequence comparison to the original contigs.

Mutagenesis and construction of mAKAP expression vectors

Full-length mAKAP cDNAs (a *Not*I-*Xho*I human fragment and an *Eag*I-*Sal*I rat fragment) were subcloned into the *Not*I and *Xho*I sites in pCDNA3 (Invitrogen). A *Sal*I-*Nsi*I fragment of the rat full-length clone was subcloned into pEGFPN1 (Clontech), yielding a vector that expresses essentially full-length mAKAP (aa residues 1-2312) fused to the N terminus of green fluorescent protein (GFP). Fragments of rat or human cDNA were amplified as above using the full-length pBNX clones, PfuTurbo polymerase, and specific primers that contain an N-terminal *Bgl*II site and artificial translation site (CGG ACT CAG ATC TGC CAC CAT GGC C followed by appropriate sense mAKAP sequence) and a C-terminal *Sal*I site (CAT ATA TAT GTC GAC CC followed by appropriate antisense mAKAP sequence) or that contain an N-terminal *Xho*I site and artificial translation site (CTA ACC AAC TCG AGG GCT TGC CAC CAT GGC followed by appropriate sense mAKAP sequence) and a C-terminal *Bam*HI site (GGG CGC GGA TCC followed by appropriate antisense mAKAP sequence), which could be ligated into pEGFPN1 or pEGFPN3, respectively, to yield vectors that express mAKAP fragment-GFP fusion protein. Each pEGFP vector insert was sequenced to verify faithful construction, and expression of each fusion protein was confirmed by western blotting of transfected COS-7 cells using anti-GFP monoclonal antibody (Clontech). A point mutation I2062P was introduced into the full-length pBNX-rat mAKAP cDNA by the Quickchange method (Stratagene) using PfuTurbo polymerase and paired oligonucleotides (sense: CAG TAC ATG ACT TTG TTA AGG AAC CAA TTG ACA TGG CAT CAA CAG CCC).

Production of affinity-purified antibodies

Plasmids that express in bacteria His_6-tagged mAKAP C-terminal fragments, human aa residues 1666-2319 or rat aa residues 1401-2314, were constructed by insertion of the appropriate mAKAP cDNA sequences into pET30a (Novagen). After expression in *E. coli* BL21 DE3, recombinant proteins were purified by FPLC with a Ni-column as recommended by the manufacturer. Rabbits were injected with purified recombinant protein, rabbit VO54 with rat mAKAP 1401-2314 and rabbit VO55 and VO56 with human mAKAP 1666-2319. Bleeds were collected for antisera (Covance, Inc.). Purified rat recombinant protein was coupled to activated agarose, and all antisera, VO54, VO55 and VO56, were purified on the rat mAKAP column by batch elution with 50 mM glycine, pH 2.4.

Immunoblotting with mAKAP antibodies

Human tissues (Clontech) or rat tissues homogenized briefly by Polytron in 50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 2 μg/ml pepstatin, 2 μg/ml leupeptin, 1 mM benzamidine and 5 mM ethylenediaminetetraacetic acid (EDTA) were assayed by the Lowry method (Lowry et al., 1951), and 50-300 μg total protein were boiled in Laemmli sample buffer (Laemmli, 1970) and fractionated on SDS-PAGE gels with 3% acrylamide stacking and 5% resolving phases. Western blotting was performed using semi-dry transfer to nitrocellulose with a Biorad Transfer Unit, incubation with primary mAKAP and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Jackson Laboratories) in TTBS (Tris-buffered saline: 10 mM Tris, pH 7.5, 150 mM NaCl, with 0.03% Tween) buffer followed by chemiluminescent detection (Supersignal, Pierce). Transfected COS-7 cell extracts were similarly analyzed. For detection of native mAKAP, affinity-purified antibody diluted 1:100 in buffer was overlaid overnight, and chemiluminescence exposure to film was for at least 30 minutes. On western blots all purified antibodies, including the previously reported AKAP100 antibody (McCartney et al., 1995), recognize recombinant human mAKAP; however, only VO54 and VO56 (and weakly VO55) recognize recombinant rat mAKAP by western blotting. Size markers were (unstained) Biorad SDS-PAGE High Range Molecular Weight Standards, which include myosin, β-galactosidase, phosphorylase b, BSA and ovalbumin.

Cell culture and transfection

Rat neonatal ventriculocytes (RNV) were prepared according to the protocol of Thorburn et al. (1995). Briefly, 1- to 3-day-old rat pups

were killed under ether anesthesia by decapitation, and the hearts were removed through a sternotomy. The ventricles were trimmed free of atria, fat and connective tissue, while immersed in a neutral buffer. Myocytes were dissociated by several 20 minute cycles of collagenase/pancreatin treatment and serum neutralization. After dissociation, RNV cells were collected by centrifugation, passed through a 149 mm mesh cell filter to remove clumps, and pre-plated in culture flasks to remove fibroblasts. After 1.5 hours, the medium containing the unattached RNV cells was removed, cells were collected by centrifugation and were plated again on dual-well chamberslides previously coated with 1% gelatin and 1 mg/ml laminin solution at density varying from 13,000 to 125,000 myocytes/cm^2. Plating medium was Dulbecco's Modified Eagle Medium (DMEM) with 17% Media 199, 1% penicillin/streptomycin solution (Gibco-BRL), 10% horse serum (HS) and 5% fetal bovine serum (FBS).

After 1 day in culture, cells were transfected by the method of Chen and Okayama (1987), as follows: 2-3 hours before transfection, the medium was changed to maintenance medium (80% DMEM, 20% Media 199, 1% penicillin/streptomycin) with 4% HS. 2.5 μg total plasmid in 50 μl 0.25 M $CaCl_2$ in water was added to 50 μl 2× BS (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95), mixed, and left at room temperature for 20 minutes. The mixture was added to a single well containing 1 ml medium and left overnight in a 5% CO_2, 37°C incubator. The following day the cells were washed three times with maintenance medium and then left in maintenance medium with or without agonists for 2 days before harvesting. 100 μM phenylephrine was added to the medium in order to induce the hypertrophic response.

COS-7 and L6 rhabdomyosarcoma cells were passaged at low density and plated in 6-well dishes containing sterile coverslips at 10-75% confluence and transfected as above or with lipofectamine as per the manufacturer's protocol (Gibco-BRL). Cells were cultured in DMEM with 10% FBS, 1% penicillin/streptomycin, and L6 were differentiated in DMEM with 4% HS for a minimum of 2 days.

Immunocytochemistry

2 days after cells were transfected, cells were rinsed twice with phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde in PBS for 10 minutes, and washed again with PBS. Cells were extracted for 1 minute with cold methanol or 10 minutes with 0.3% Triton X-100 in PBS, washed once with PBS, and blocked with 0.2% bovine serum albumin in PBS (BSA/PBS) for 30 minutes. Cells were incubated with primary antibody diluted as required in 150 μl BSA/PBS for 1 hour and then washed three times with BSA/PBS for 5 minutes. Cells were then incubated with appropriate fluorescent secondary antibodies in the same manner (FITC, Texas Red and Cy5 conjugated-secondary donkey antibodies; Jackson Laboratories). After three PBS washes, the cells were rinsed with water and mounted on slides (for coverslips) or with glass coverslips (for chamberslides) with Slowfade Antifade Kit (Molecular Probes). All mAKAP staining was repeated with both the VO54 and VO56 antibodies. Rhodamine-phalloidin (Molecular Probes) was used to visualize actin fibers. Hoechst 33258 stain (10 μg/ml) was included in the last PBS wash for detection of nuclei. Either the specific indirect immunofluorescence staining or intrinsic GFP fluorescence was detected in successive focal planes by laser-scanning confocal microscopy on an MRC1024 Biorad UV/Vis system. (Dell'Acqua et al., 1998). Frozen tissue sections (Medico, Inc.) were stained as above, except that sections were permeabilized three times for 1 minute with cold methanol or for 10 minutes with 0.3% Triton X-100 in PBS.

Immunoprecipitation of mAKAP and PKA filter assay

L6 cells were washed twice with PBS and scraped into 2 ml cold PBS. Cells were collected by centrifugation at 1000 rpm for 5 minutes and lysed in 2.5 ml of fresh TBS with 0.5% Triton X-100, 1 mM DTT, 1 mM AEBSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 1 mM benzamidine and 5 mM EDTA. Extracts were clarified by centrifugation at 2000 ***g*** for 15 minutes at 4°C. 5 μl whole V054 or preimmune serum were added to 1.2 ml supernatant and incubated at 4°C for 1 hour. 100 μl pre-rinsed 20% (v/v) protein G-sepharose (Gamma-bind Plus, Pharmacia) was added and the mixture was incubated at 4°C for 2 hours, with rotation. Beads were collected by brief centrifugation at 2500 ***g***. Beads were washed twice for 5 minutes with TBS with 0.5% Triton X-100, 1 mM DTT, 1 mg/ml BSA and once with the same buffer containing 1 M NaCl. PKA C-subunit was eluted with 150 μl 1 mM adenosine 3′,5′cyclic monophosphate (cAMP) in water for 5 minutes at room temperature and assayed by the filter binding method (Corbin and Reimann, 1974). Specific PKA activity was determined by subtracting background activity found in the presence of 20 μM protein kinase inhibitor (PKI).

RIIα overlay assay

Overlay assay was performed as previously described (Carr and Scott, 1992). Briefly, COS-7 cells were transfected with pCDNA3 or pEGFP-expression vectors and were lysed into Laemmli buffer or immunoprecipitated prior to SDS-PAGE and blotting as above. Blots were blocked with Blotto (5% non-fat dried milk, 0.1% BSA in Tris-buffered saline) for 30 minutes and then incubated overnight at room temperature with 100,000 cpm/ml ^{32}P-RIIα in Blotto. Blots were washed in TTBS four times for 5 minutes and then exposed to X-ray film at −70°C with intensifier screens for 1-7 days.

RESULTS

Cloning of mAKAP

We have previously isolated cDNA clones for several A-kinase anchoring proteins (AKAPs) using an interaction cloning strategy with radiolabelled RIIα-subunit as a probe. One clone, originally called AKAP100, represented a sole human mRNA of approx. 10 kb, which was enriched in heart and skeletal muscle (McCartney et al., 1995). Additional cloning experiments isolated a rat AKAP100 homologue with an extended open reading frame at the 5′ end. Further analysis revealed that the 5′ end of the original AKAP100 cDNA was rearranged, thereby introducing an in-frame stop codon. The complete cloning of human and rat AKAP100 cDNAs was achieved by multiple rounds of a modified 5′-Rapid Amplification of cDNA Ends (RACE) procedure, and new sequence has been entered into the GenBank database (Accession numbers AF139518 and U17195). Sequence from 45 human and 47 rat RACE clones contributed to complete the double-stranded sequence for each cDNA. Although we cannot completely exclude the possibility of alternative splicing, all of the clones yielded sequences that may be attributed to a single gene product, whether derived from heart, skeletal muscle or brain cDNA. We acquired 8.8 kb of rat and 10.3 kb of human cDNA sequence, encoding proteins of 2314 and 2319 residues, respectively (Fig. 1). The predicted rat and human amino acid sequences are 79% identical. Because AKAP100 is enriched in striated muscle, we now refer to it as mAKAP (muscle AKAP).

BLAST search of the National Center for Biotechnology Information (NCBI) with mAKAP sequences found four STS-markers with identical matches to the human mAKAP cDNA sequence: WI-9732 (308 bp of identity to the human cDNA), WI-14096 (367 bp), N39150 (239 bp) and SHGC-37333 (250 bp). These markers have all have been mapped (with different resolution) to 28 cM and 60.4 cR on the genetic maps of chromosome 14q, according to information provided in the


human 1 MLTMSVTLSPLRSQDLDPMATDASPMAINMTPTVEQ--GEGEEAMKDMDSDQQYEKPPLHTGADWKIVLHLPEI 73
rat 1 MLTMSVTLSPLRSQGPDPMATDASPMAINMTPTVEQEEGEGEEAVKAIDAEQQYGKPPLHTAADWKIVLHLPEI 75
human 74 ETWLRMTSERVRDLTYSVQQDSDSKHVDVHLVQLKDICEDISDHVEQIHALLETEFSLKLLSYSVNVIVDIHAVQ 148
rat 76 ETWLRMTSERVRDLTYSVQQDADSKHVDVHLVQLKDICEDISDHVEQIHALLETEFSLKLLSYSVNVIVDIHAVQ 150
human 149 LLWHQLRVSVLVLRERILQGLQDANGNYTRQTDILQAFSEETKEGRLDSLTEVDDSGQLTIKCSQNYLSLDCGIT 223
rat 151 LLWHQLRVSVLVLRERILQGLQDANGNYTRQTDILQAFSEETTEGRLDSLTEVDDSGQLTIKCSQDYLSLDCGIT 225
human 224 AFELSDYSPSEDLLSGLGDMTSSQVKTKPFDSWSYSEMEKEFPELIRSVGLLTVAADSISTNGSEAVTEEVSQVS 298
rat 226 AFELSDYSPSEDLLGGLGDMTTSQAKTKSFDSWSYSEMEKEFPELIRSVGLLTVATEPVPSSCGEAN-EDSSQAS 299
human 299 LSVDDKGCEEDNASAVEEQPGLTLGVSSSSGEALTNAAQPSSETVQQESSSSSHHDAKNQQPVPCENATPKRTI 373
rat 300 LSDDHKGEHGEDGAPVPGQQLDSTVGMSSLDG-TLANAAEHPSETAKQDSTSSPQLGAKKTQPGPCEITTPKRSI 373
human 374 RDCFNYNEDSPTQPTLPKRGLFLKEETFKNDLKGNGGKRQMVDLKPEMSRSTPSLVDPPDRSKLCLVLQSSYPNS 448
rat 374 RDCFNYNEDSPTQPTLPKRGLFLKETQ-KNERKGSDRKGQVVDLKPELSRSTPSLVDPPDRSKLCLVLQSSYPSS 447
human 449 PSAASQSYECLHKVGNGNLENTVKFHIKEISSSLGRLNDCYKEKSRLKKPHKTSEEVPPCRIPKRGTGSGKQAKN 523
rat 448 PSAASQSYECLHKVGLGNLENIVRSHIKEISSSLGRLTDCHKEKLRLKKPHKTLAEVSLCRIPKQGGGSGKRSES 522
human 524 TKSSAVPNGELSYTSKAIEGPQTNSASTSSLEPCNQRSWNAKLQLQSETSSSPAFTQSSESSVGSDNIMSPVPLL 598
rat 523 TGSSAGPSMVSPGAPKATMRPETDSASTASGGLCHQRNRSGQLPVQSKASSSPPCSHSSESSLGSDSIKSPVPLL 597
human 599 SKHKSKKGQASSPSHVTRNGEVVEAWYGSDEYLALPSHLKQTEVLALKLENLTKLLPQKPRGETIQNIDDWELSE 673
rat 598 SKNKSQKSSPPAPCHATQNGQVVEAWYGSDEYLALPSHLKQTEVLALKLESLTKLLPQKPRGETIQDIDDWELSE 672
human 674 MNSDSEIYPTYHVKKKHTRLGRVSPSSSSDIASSLGESIESGPLSDILSDEESSMPLAGMKKYADEKSERASSSE 748
rat 673 MNSDSEIYPTYHIKKKHTRLGTVSPSSSSDIASSLGESIESGPLSDILSDEDLCLPLSSVKKFTDEKSERPSSSE 747
human 749 KNESHSATKSALIQKLMQDIQHQDNYEAIWEKIEGFVNKLDEFIQWLNEAMETTENWTPPKAEMDDLKLYLETHL 823
rat 748 KNESHSATRSALIQKLMHDIQHQENYEAIWERIEGFVNKLDEFIQWLNEAMETTENWTPPKAETDSLRLYLETHL 822
human 824 SFKLNVDSHCALKEAVEEEGHQLLELIASHKAGLKDMLRMIASQWKELQRQIKRQHSWILRALDTIKAEILATDV 898
rat 823 SFKLNVDSHCALKEAVEEEGHQLLELVVSHKAGLKDTLRMIASQWKELQRQIKRQHSWILRALDTIKAEILATDV 897
human 899 SVEDEEGTGSPKAEVQLCYLEAQRDAVEQMSLKLYSEQYTSSKRKEEFADMSKVHSVGSNGLLDFDSEYQELWD 973
rat 898 SVEDEEGTGSPKAEVQLCHLETQRDAVEQMSLKLYSEQYTSGSKRKEEFANMSKAHAEGSNGLLDFDSEYQELWD 972
human 974 CLIDMESLVMDSHDLMMSEEQQQHLYKRYSVEMSIRHLKKTELLSKVEALKKGGVLLPNDLLEKVDSINEKWELL 1048
rat 973 WLIDMESLVMDSHDLMMSEEQQQHLYKRYSVEMSIRHLKKSELLSKVEALKKGGLSLPDDILEKVDSINEKWELL 1047
human 1049 GKTLGEKIQDTMAGHSGSSPRDLLSPESGSLVRQLEVRIKELKGWLRDTELFIFNSCLRQEKEGTMNTEKQLQYF 1123
rat 1048 GKTLREKIQDTIAGHSGSGPRDLLSPESGSLVRQLEVRIKELKRWLRDTELFIFNSCLRQEKEGTS-AEKQLQYF 1121
human 1124 KSLCREIKQRRRGVASILRLCQHLLDDRETCNLNADHQPMQLIIVNLERRWEAIVMQAVQWQTRLQKKMGKESET 1198
rat 1122 KSLCREIKQRRRGVASILRLCQHLLDDRDTCNLNADHQPMQLIIVNLERRWEAIVMQAVQWQTRLQKKMGKESET 1196
human 1199 LNVIDPGLMDLNGMSEDALEWDEMDISNKLISLNEESNDLDQELQPVIPSLKLGETSNEDPGYDEEADNHGGSQY 1273
rat 1197 LNVIDPGLMDLNGMSEDALEWDETDISNKLISVHEESNDLDQDPEPMLPAVKLEETHHKDSGYEEEAGDCGGSPY 1271
human 1274 ASNITAPSSPHIYQVYSLHNVELYEDNHMPFLKNNPKVTGMTQPNVLTKSLSKDSSFSSTKSLPDLLGGSNLVKP 1348
rat 1272 TSNITAPSSPHIYQVYSLHNVELHEDSHTPFLKSSPKFTGTTQPTVLTKSLSKDSSFSSTKSLPDLLGGSGLVRP 1346
human 1349 CACHGGDMSQNSGSESGIVSEGDTETTTNSEMCLLNAVDGSPSNLETEHLDPQMGDAVNVLKQKFTDEGESIKLP 1423
rat 1347 YSCHSGDLSQNSGSESGIVSEGDNEMPTNSDMSLFSMVDGSPSNPETEHPDPQMGDAANVLEQKFKDNGESIKLS 1421
human 1424 NSSQSSISPVGCVNGKVGDLNSITKHTPDCLGEELQGKHDVFTFYDYSYLQGSKLKLPMIMKQSQSEKVHVEDPL 1498
rat 1422 SVSRASVSPVGCVNGKAGDLNSVTKHTADCLGEELQGKHDVFTFYDYSYLQGSKLKLPMIMKQPQSEKAHVEDPL 1496
human 1499 LRGFYFDKKSCKSKHQTTELQPDVPPHERILASASHEMDRISYKSGNIEKTFTGMQNAKQLSLLSHSSSIESLSP 1573
rat 1497 LGGFYFDKKSCKAKHQASESQPDAPPHERILASAPHEMGRSAYKSSDIEKTFTGIQSARQLSLLSRSSSVESLSP 1571
human 1574 GGDLFGLGIFKNGSDSLQRSTSLESWLTSYKSNEDLFSCHSSGDISVSSGSVGELSKRTLDLLNRLENIQSPSEQ 1648
rat 1572 GGDLFGLGIFKNGSDSLQRSTSLESWLTSYKSNEDLFSCHSSGDISVSSGSVGELSKRTLDLLNRLENIQSPSEQ 1646
human 1649 KIKRSVSDITLQSSSQKMSFTGQMSLDIASSINEDSASLTELSSSDELSLCSEDIVLHKNKIPESNASFRKRLT 1723
rat 1647 KIKRSVSDMTLQSSSQKMPFAGQMSLDVASSINEDSPASLTELSSSDELSLCSEDIVLHKNKIPESNASFRKRLN 1721
human 1724 RSVADESDVNVSMIVNVSCTSACTDDEDDSDLLSSSTLTLTEEELCIKDEDDDSSIATDDEIYEDCTLMSGLDYI 1798
rat 1722 RSVADESDVNVSMIVNVSCTSACTDDEDDSDLLSSSTLTLTEEELCLKDEDDDSSIATDDEIYEESNLMSGLDYI 1796
human 1799 KNELQTWIRPKLSLTRDKKRCNVSDEMKGSKDISSSEMTNPSDTLNIETLLNGSVKRVSENNGNGKNSSHTHELG 1873
rat 1797 KNELQTWIRPKLSLTREKKRSGVTDEIKVNKDGGGNEKANPSDTLDIEALLNGSIRCLSENNGNGKTPPRTHGSG 1871
human 1874 TKRENKKTIFKVNKDPYVADMENGNIEGIPERQKGKPNVTSKVSENLGSHGKEISESEHCKCKALMDSLDDSNTA 1948
rat 1872 TKGENKKSTYDVSKDPHVADMENGNIESTPERERELKPQGLPEVSENLASNVKTISESELLSEYEAVMDGSEDSSVA 1946
human 1949 GKEFVSQDVRHLPKKCPNHHHFENQSTASTPTEKSFSELALETRFNNRQDSDALKSS-DDAPSMAGKSAGCCLAL 2022
rat 1947 RKEFCPPNDRHPPQMGPKLQHPENQSGDCKPVQNPCPGLLSSEAGVGSRQDSNGLKSLPNDAPSGARKPAGCCL-L 2020
human 2023 EQNGTEENASIS-NISCCNCEPDVFHQKDAEDCSVHNFVKEIIDMASTALKSKSQPENEVAAPTSLTQIKEKVLE 2096
rat 2021 EQNETEESSASISSNASCCNCKPDVFHQKDDEDCSVHDFVKEIIDMASTALKSKSQPESEVAAPTSLTQIKEKVLE 2095
human 2097 HSHRPIQLRKGDFYSYLSLSSHDSDCGEVTNYIEEKSSTPLPLDTTDSGLDDKEDIECFFEACVEGDSDGEEPCF 2171
rat 2096 HSHRPIHLRKGDFYSYLSLSSHDSDCGEVTNYIDEKSSTPLPPDAVDSGLDDKEDMDCFFEACVEDEPVNEEAGL 2170
human 2172 SSAPPNESAVPSEAAMPLQATACSSEFSDSSLSADDADTVALSSPSSQERAEVGKEVNGLPQTSSGCAENLEFTP 2246
rat 2171 PGALPNESAIEDGAEQ-----KSEQKTASSPVLSDKTDLVPLSGLSPQKGADDAKEGDDVSHTSQGCAESTEPTT 2240
human 2247 SKLDSEKES-SGKPGESGMPEEHNAASAKSKVQDLSLKANQPTDKALH-PSPKTLTCEENLLNLHEKRHRNMHR 2319
rat 2241 PSGKANAEGRSRMQGVSATPEE-NAASAKPKIQAFSLNAKQPKGKVAMRYPSPQTLTCKEKLVNFHEDRHSNMHR 2314

Fig. 1. The primary structure of human and rat mAKAP. Human and rat amino acid sequences are aligned using the MacVector gene analysis program. Identical residues are in gray and conservative substitutions are in light gray. The putative RIIα-binding site in rat mAKAP is outlined with brackets.

National Center for Biotechnology Information UniGene GB4 database.

Detection of mAKAP protein

Recombinant fragments corresponding to residues 1666-2319 of human mAKAP or residues 1401-2314 of rat mAKAP were used as antigens for the production of polyclonal antibodies. Antisera were affinity purified and used in immunoblotting of whole tissue extracts. Two antibodies recognized a single band of approximately 300 kDa in rat heart; there was less intense recognition in skeletal muscle, but none in colon (Fig. 2A). A slightly higher band was detected in brain, which migrated with the same mobility as the full-length, recombinant rat mAKAP protein expressed in COS-7 cells (Fig. 2B). Muscle bands at 120 and 135 kDa were consistently detected with horseradish peroxidase-conjugated secondary antibody alone (not shown), whereas the detection of bands at 200-250 kDa in skeletal muscle and at 70 kDa in brain were variable and probably represent proteolytic degradation products. Similar results were obtained for human tissue extracts (data not shown).

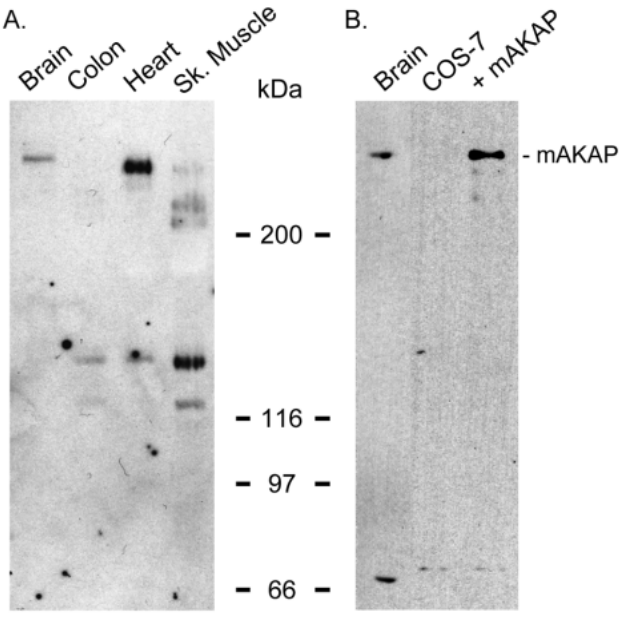


Fig. 2. Detection and expression of mAKAP. Rat tissue extracts (50 μg) were separated on 5% SDS-PAGE gels, electrotransferred to nitrocellulose and probed for mAKAP by western blot using affinity-purified VO56 (A) or VO54 (B) antibody. (A) Detection of endogenous mAKAP. The tissue source is indicated above each lane. (B) Comparison of the recombinant protein and brain mAKAP (300 μg). Whole COS-7 cell extracts were prepared from cells expressing either the pCDNA3 parent vector alone or pCDNA3-mAKAP and treated as described above. Sample sources are indicated above each lane. Relative molecular mass markers (kDa) are indicated. Blots are representative of three independent experiments.

mAKAP binds PKA holoenzyme

In order to establish that mAKAP functions as an A-kinase anchoring protein inside cells, endogenous mAKAP was immunoprecipitated from L6 rhabdomyosarcoma cells (Fig. 3). mAKAP protein of approximately 300 kDa was detected in samples immunoprecipitated with specific antibody, while no immunoblot signal was detected in control samples immunoprecipitated with non-immune antibodies (Fig. 3; insert). Kinase activity toward the heptapeptide substrate kemptide was enriched 2.6±0.6-fold (n=3, P<0.03, t-test) in mAKAP immunoprecipitates compared to that with the preimmune sera (Fig. 3). The inhibition of the mAKAP-associated kinase activity by the protein kinase inhibitor (PKI) 5-24 peptide, which is a specific inhibitor of the catalytic subunit of PKA, confirms that mAKAP can be isolated in a complex with the PKA holoenzyme from cells.

Mapping of the mAKAP PKA-binding domain

We have previously shown that the C-terminal third of mAKAP binds RIIα in vitro (McCartney et al., 1995). In order to map the RIIα-binding domain further, a series of smaller fragments fused to GFP were expressed in COS-7 cells (Fig. 4A). All constructs spanning residues 2013-2083 bound RIIα, as determined by binding in an overlay assay (Fig. 4C, right panel), whereas mAKAP fragments lacking this region were unable to interact with RIIα. As expected, several RIIα-binding proteins which are endogenous to COS-7 cells were detected in the overlay assay (Fig. 4C, right panel, data for GFP alone); therefore, the migration position of each recombinant mAKAP fragment was determined by detection with an antibody against the GFP tag (Fig. 4C, left panel).

Sequences that are predicted to form an amphipathic helix comprise the principal determinants for R-subunit binding in all AKAPs studied so far (Carr et al., 1991). A sequence between residues 2055-2072 of mAKAP fulfills this criterion (Fig. 4B). Substitution of proline for isoleucine at position

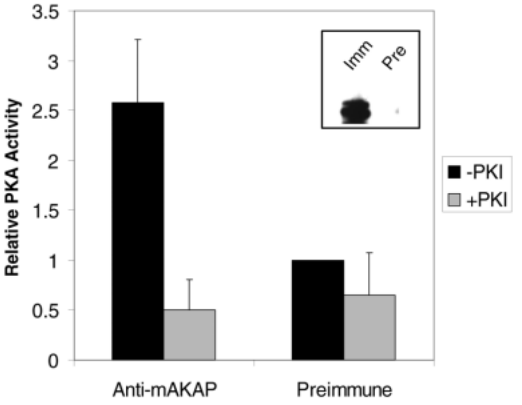


Fig. 3. mAKAP binds PKA in cells. mAKAP was immunoprecipitated from extracts of L6 rhabdomyosarcoma cells using VO54 (Anti-mAKAP) or control antisera (Preimmune). Detection of the protein was confirmed by western blot (inset). PKA activity was detected using kemptide filter assays in the presence or absence of the specific PKA inhibitor PKI. Relative kinase activity is presented as the fold increase over control ± s.e.m. Data is an amalgam of three independent experiments. Statistical significance was calculated by Student's t-test.

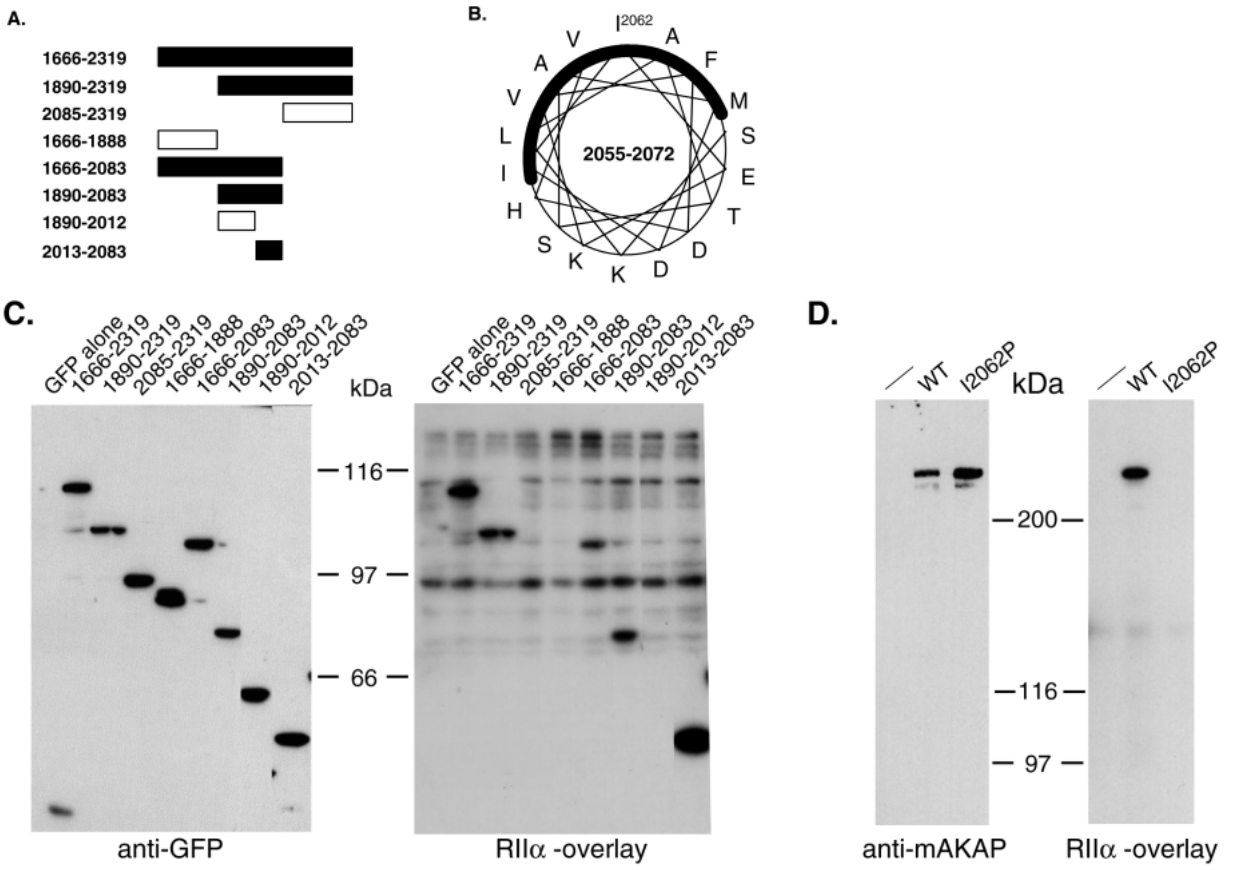


Fig. 4. Mapping the RIIα-binding site on mAKAP. (A) Schematic diagram presenting a family of epitope-tagged human mAKAP protein fragments that were expressed in COS-7 cells to map the RIIα-binding domain. The first and last residues of each fragment are indicated. RIIα-binding fragments (filled boxes) and non-binding fragments (open boxes) are indicated. (B) Helical wheel representation of residues 2055-2072 showing hydrophobic (thick line) and hydrophilic faces. (C) COS-7 cells were transfected with vectors expressing GFP-tagged human mAKAP fragments. Whole cell extracts were fractionated on 10% SDS-PAGE gels, electrotransferred to nitrocellulose, and the expressed proteins were detected by monoclonal antibodies to the green fluorescent protein (GFP) epitope tag (left panel) or by ^{32}P-RIIα overlay assay (right panel). A representative of five individual experiments is presented. The sample source is indicated above each lane. (D) Full-length mAKAP or the I2062P mutant were immunoprecipitated from COS-7 cell extracts using the VO54 antibody. Proteins were detected by western blot (left panel) and RIIα overlay (right panel) as described above. Sample sources are indicated above each lane in C and D, and relative molecular mass markers are indicated. Blots are representative of three independent experiments.

2062 is predicted to disrupt secondary structure within the putative helical region. The involvement of this region in RIIα-binding was confirmed by site-directed mutagenesis experiments (Fig. 4D). To test this hypothesis a full-length mAKAP and I2062P mutant were expressed in COS-7 cells. Both proteins were immunoprecipitated with anti-mAKAP antibodies and tested for interaction with RIIα by the overlay assay (Fig. 4D, right panel). Only the wild-type mAKAP bound RIIα, confirming that the residues surrounding Ile 2062 are determinants for PKA anchoring.

Intracellular localization of mAKAP

The intracellular location of mAKAP was examined in a variety of cell types by immunofluorescence techniques. Consistent with a previous report (McCartney et al., 1995), mAKAP was targeted to the endoplasmic reticulum in COS-7 cells (data not shown). However, the mAKAP signal was concentrated at the nuclear membrane in frozen, tissue sections of rat cardiac ventricle (Fig. 5A-C). A composite image that merges mAKAP, DNA and actin staining shows that the anchoring protein is concentrated at the nuclear membrane of some, but not all cells (Fig. 5D). Additional controls demonstrated that no staining was observed with secondary antibodies alone (Fig. 5E). Identical results were obtained with two affinity-purified mAKAP antibodies, and a similar staining pattern was detected for rat thigh skeletal muscle (data not shown). In light of the differences we observed in mAKAP localization between transformed cell lines and in tissue, we decided to examine mAKAP expression and location in primary cardiac myocyte cultures.

Rat neonatal ventriculocytes (RNV) revert to an undifferentiated phenotype when dissociated and grown at low density in low serum (Schaub et al., 1997; Sugden and Clerk, 1998). However, the cells adopt a more differentiated, hypertrophic phenotype when grown at high density or in the presence of phenylephrine, an α-adrenergic agonist. The differentiated cells are mono- or binucleate, undergo myofibrillar organization and exhibit spontaneous contractions (Thorburn et al., 1995). RNV were cultured at a range of densities to yield cells in diverse states of differentiation (Fig. 6A-F, low density;

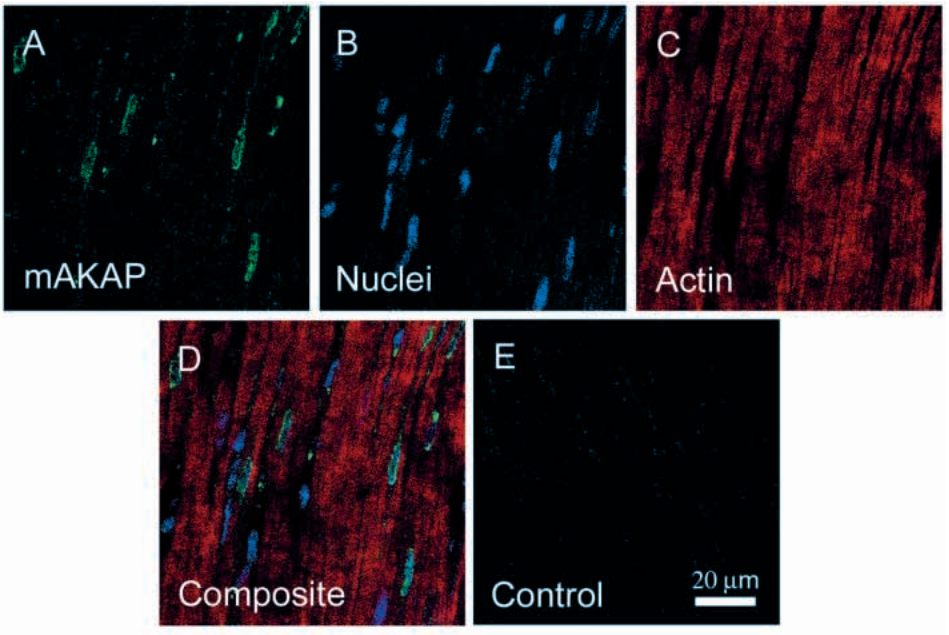


Fig. 5. The subcellular distribution of mAKAP in heart tissue. Frozen longitudinal sections of rat heart tissue were stained with anti-mAKAP VO54 and FITC-conjugated fluorescent secondary antibody (A, green), Hoechst DNA stain for nuclei (B, blue), and rhodamine-phalloidin, which detects F-actin in myofibrils (C, red). A composite is shown in (D). An FITC-channel image is shown for a control section stained identically as above, but with no mAKAP primary antibody (E). Each image is at the same scale; bar, 20 μm. All images were acquired by confocal microscopy and are representative of four independent experiments with each of the VO56 and VO54 antibodies.

H-L, high density). The differentiation state of individual cells was monitored by F-actin staining to establish the extent of myofibrillar organization (Fig. 6C,I). There was less staining of actin fibers in undifferentiated RNV, which were plated at low density and exhibited a more extended appearance (Fig. 6C). mAKAP was not detected in undifferentiated RNV permeabilized with Triton X-100 (Fig. 6A), and the intracellular distribution of RII was reticular and cytoplasmic (Fig. 6B). In contrast, differentiated RNV contained striated myofibrils that were heavily stained by rhodamine-phalloidin (Fig. 6I). Furthermore, mAKAP staining was readily apparent at the nuclear membrane and at low levels within the nucleus (Fig. 6G). Although widely distributed, RII was also more concentrated at the nuclear membrane in differentiated cells (Fig. 6H). Nuclei were detected with Hoechst 33258 (Fig. 6E,K), and control FITC-channel images are shown in Fig. 6F,L. Collectively, these results suggest that mAKAP expression is upregulated in differentiated RNV and that the net effect of these events is to concentrate PKA at the nuclear membrane.

Mapping of the mAKAP targeting domain

On the basis of our immunofluorescence studies, it was apparent that endogenous mAKAP was targeted to the nuclear membrane in heart tissue and differentiated RNV. This provided us with an assay to map a targeting domain on mAKAP that maintained the protein at the nuclear membrane. Therefore, a family of mAKAP fragments fused to GFP was constructed and expressed in RNV (Fig. 7A). Control experiments confirmed that the endogenous protein (Fig. 7B) and the full-length mAKAP-GFP-fusion protein were concentrated at the nuclear membrane (Fig. 7D). In contrast, GFP alone was distributed throughout the cell (Fig. 7C). These studies established that fusion of the GFP fluorescent tag to the C terminus of mAKAP did not affect the correct intracellular targeting of the protein.

Further expression of mAKAP-GFP fragments in RNV demonstrated that GFP-fusion proteins containing residues 585-1286 were properly targeted to the nuclear membrane (Fig. 7D,G,H). In contrast, mAKAP fragments flanking this region were broadly distributed in the cell (Fig. 7E,F). Interestingly, residues 585-1286 of the anchoring protein encompass a series of three spectrin repeat-like sequences (Fig. 8). Thus, an additional series of deletion fragments were produced to assess the contribution of individual spectrin repeats in mAKAP targeting (Fig. 7A). Fragments of mAKAP encompassing the first and second spectrin repeats were correctly targeted to the nuclear membrane (Fig. 7J,L), whereas fragments encompassing the third spectrin repeat or flanking regions were not targeted (Fig. 7I,K,M). These experiments illustrate that residues 772-915 and 915-1065 of mAKAP represent individual regions that are sufficient for nuclear membrane targeting and that the anchoring protein contains more than one targeting signal.

In order to avoid overexpression artifacts, only those RNV exhibiting low GFP-fusion protein fluorescence were selected for study in the above mapping experiments. Indeed, high level expression of these mAKAP fragments resulted in accumulation of the signal in the cytoplasm (Fig. 9A). This suggests that mAKAP targeting to the nuclear membrane is saturable. This conclusion is supported by evidence that overexpression of mAKAP targeting fragment 585-1286-GFP results in the concomitant displacement of the endogenous anchoring protein from the nuclear membrane (Fig. 9B,C). In contrast, overexpression of control GFP constructs did not displace mAKAP (Fig. 9D-F). Non-transfected cells in each field (two shown) served as internal positive controls for endogenous mAKAP targeting (Fig. 9B,E). These results suggest that the mAKAP spectrin repeats interact with a targeting locus at the nuclear membrane and can displace the endogenous anchoring protein from targeting sites inside cells.

DISCUSSION

This work describes the cloning and characterization of mAKAP, an A-kinase anchoring protein that is targeted to the nuclear membrane. Targeting is mediated by a region of the AKAP containing spectrin-like repeats and appears to be

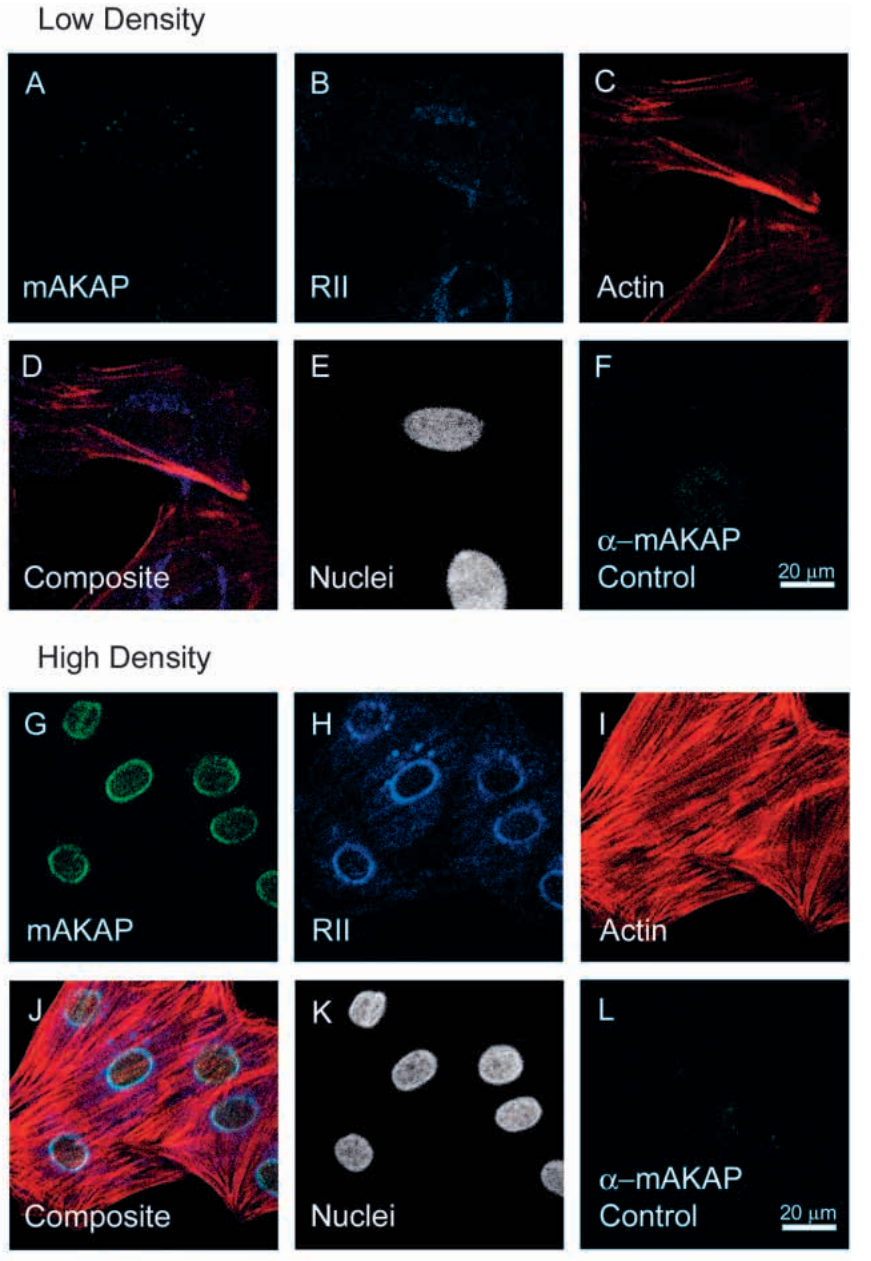
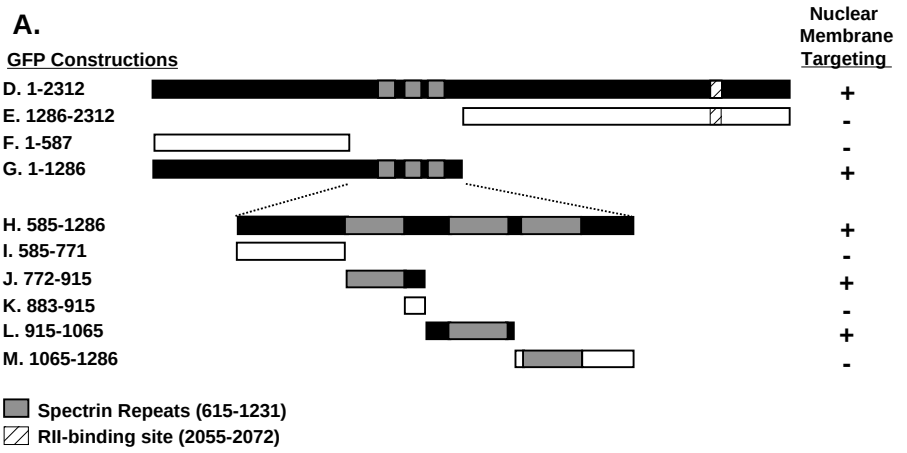


Fig. 6. Localization of mAKAP in primary cultures of rat neonatal ventriculocytes (RNV) is dependent upon state of RNV differentiation. RNV were cultured and grown on chamberslides as described in Materials and methods. (A-F) RNV cultured at low density were stained with rabbit anti-mAKAP VO54 and FITC-conjugated fluorescent secondary antibody (A, green), goat anti-PKA-RIIα and -RIIβ and Cy5-conjugated fluorescent secondary antibody (B, blue), rhodamine-phalloidin, which detects F-actin in myofibrils (C, red), and Hoechst DNA stain for nuclei (E, grayscale). A three-color composite is shown in (D). An FITC-channel image is shown for a control slide identically stained, but with no mAKAP primary antibody (F). (G-L) Primary RNV cultured at high density in the presence of 100 μM phenylephrine to induce differentiation were labeled as above: mAKAP VO54 antibodies (G), anti-PKA-RIIα and -RIIβ (H), rhodamine-phalloidin (I), composite (J), Hoechst stain (K) and FITC control (L). Each image is at the same scale; bars, 20 μm. All images were acquired by confocal microscopy and are representative of more than three independent experiments with each of the VO56 and VO54 antibodies.

related to the differentiation state of the cell. The full-length mAKAP protein is a 255 kDa, striated muscle- and brain-selective A-kinase anchoring protein, which is highly conserved between the rat and human species. Immunoblotting of whole tissue extracts revealed a single band in striated muscle that migrated in SDS-PAGE slightly faster than the single band found in brain. Analysis of the muscle and brain mAKAP mRNA species revealed no differences. Recombinant full-length mAKAP migrated with the native brain form. Thus, the difference in migration in SDS-PAGE between these forms suggests that mAKAP may be post-translationally modified. At this time it is unclear what forms of post-translational modification are responsible for the differences in mobility.

Characterization of mAKAP/ PKA interaction

AKAPs are a group of 50 or so functionally related proteins that have been classified on the basis of their ability to associate with the PKA holoenzyme inside cells (Colledge and Scott, 1999). Several lines of evidence imply that mAKAP represents a conventional anchoring protein. First, immunoprecipitation of mAKAP from L6 rhabdomyosarcoma cell extracts results in the copurification of the PKA holoenzyme and an enrichment of cAMP-dependent kinase activity. This classifies the protein as an AKAP. Second, overlay experiments detect a 255 kDa RIIα-binding band upon heterologous expression of the full-length recombinant protein. Third, we have mapped the RIIα-binding site to include residues 2013-2083 of the protein. Within this region there is a sequence (residues 2055-2072) that is predicted to form an amphipathic helix. This is another hallmark of the AKAPs, as numerous studies have suggested that an R-subunit binding site forms an amphipathic helix that participates in protein-protein interactions with a complimentary surface on the R-subunit dimer (Hausken et al., 1994, 1996; Li and Rubin, 1995). A model for the RIIα/AKAP



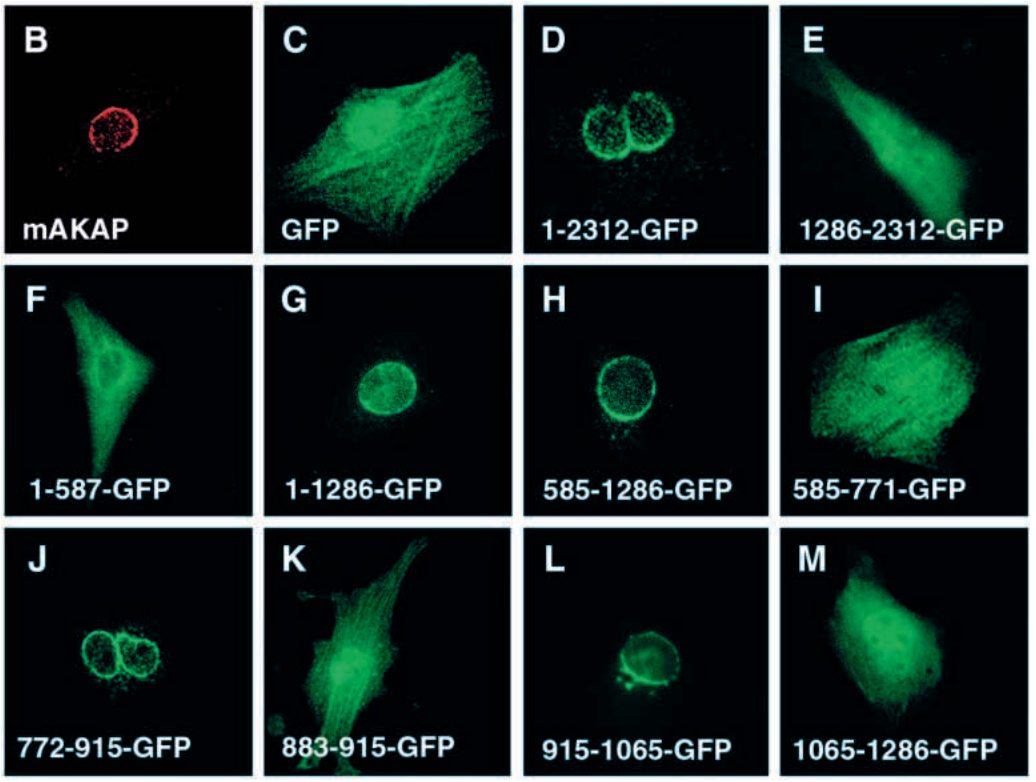


Fig. 7. Spectrin repeat-like sequences are required for mAKAP targeting. (A) Schematic diagram presenting a family of GFP-tagged human mAKAP protein fragments that were expressed in RNV to map the targeting domain. The first and last residues of each fragment are indicated. Fragments that target (filled boxes) are distinguished from those that do not (open boxes). The locations of spectrin repeats and the RIIα binding domain are indicated. (B) Control cell showing the immunofluorescence detection of endogenous mAKAP. (C) Control cell showing fluorescence detection of GFP (D-M) Detection of mAKAP-GFP fragments expressed in RNV. The first and last residues of each fragment are indicated. Representatives are shown of the multiple cells studied in each of three experiments for each construct.

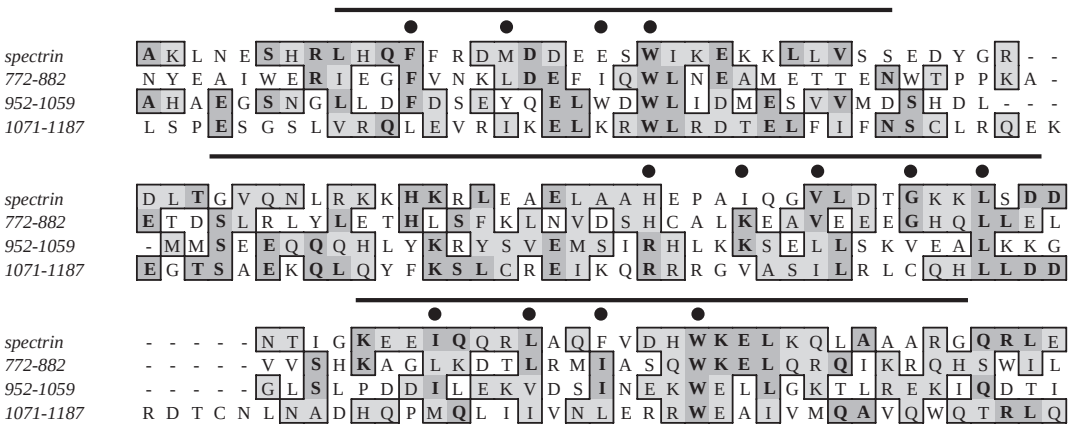


Fig. 8. Alignment of mAKAP amino acid residue sequences with the chicken brain α-spectrin 16th repeat. Dots designate residues involved in inter-helix bonding. The three helices are overlaid by bars. Structural data were derived from Pascual et al. (1996).

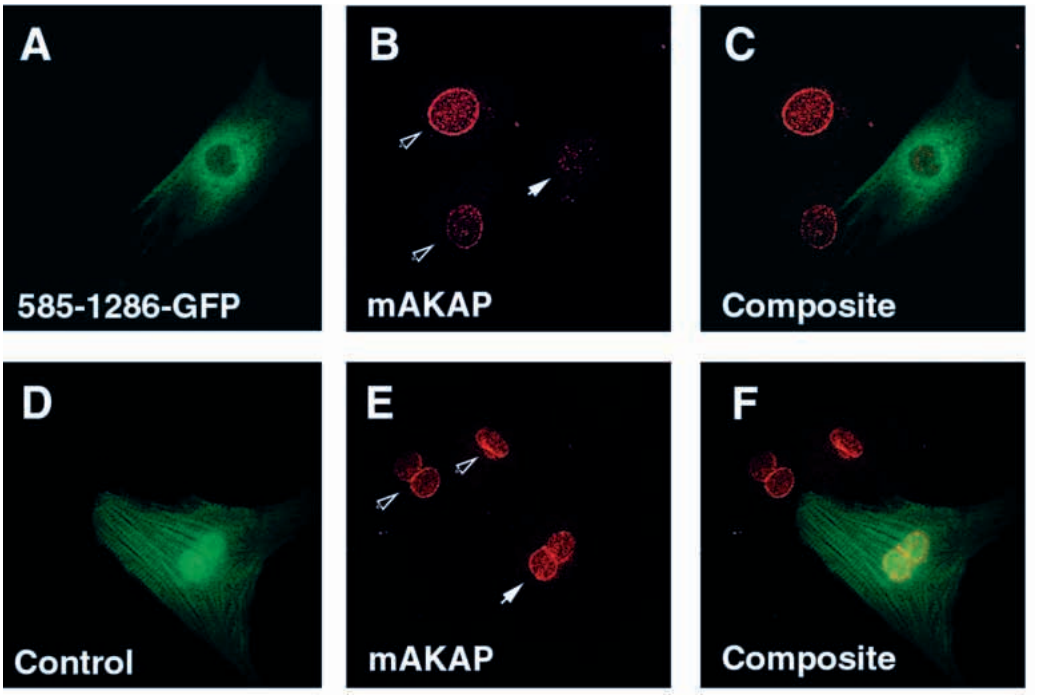


Fig. 9. Displacement of endogenous mAKAP from the nuclear membrane by overexpression of mAKAP aa 585-1286-GFP. mAKAP aa 585-1286 or GFP alone were overexpressed at high levels in phenylephrine-treated RNV (A,D). Endogenous mAKAP was detected by staining with VO54 primary and Texas-red conjugated secondary antibody (B,E). Nuclei of non-transfected cells are indicated by empty arrowheads, and nuclei of transfected cells by filled arrowheads. Composite images are shown in (C,F). Multiple cells were studied in each of eight experiments.

complex has been recently proposed based on the NMR structure of the dimeric RIIα anchoring domain. A hydrophobic face on the AKAP amphipathic helix packs onto a reciprocal surface formed by the two chains of each RIIα protomer (Newlon et al., 1997, 1999). It is likely that mAKAP binds RIIα through a similar mechanism, because substitution of isoleucine 2062 with a proline residue abolished PKA binding. It is presumed that the isoleucine-to-proline substitution perturbed the helical conformation of the RIIα-binding site on mAKAP, thereby altering the organization of hydrophobic residues.

Intracellular location of mAKAP

Each AKAP contains a unique targeting motif that directs the anchored PKA holoenzyme to precise intracellular locations (Colledge and Scott, 1999). Although AKAPs have now been identified in most cellular compartments and associated with a variety of organelles, this is the first report of an anchoring protein that can direct PKA to the nuclear membrane. The nuclear membrane is composed of two lipid bilayers separated by the perinuclear space, which is continuous with the lumen of the endo/sarcoplasmic reticulum (ER/SR). Although our immunofluorescence data of heart tissue and primary ventriculocyte cultures suggests that mAKAP is at the nuclear membrane, we cannot exclude the possibility that low levels of mAKAP are present in the SR. A recent report has also detected mAKAP in a number of myocyte compartments including the SR and the nucleus (Yang et al., 1998). Furthermore, different permeabilization conditions in immunohistochemistry can lead to varying results. For example, in undifferentiated RNV permeabilized with methanol, we found minimal levels of specific mAKAP staining within the nucleus (data not shown). Also, it is important to note that the localization of mAKAP in RNV and tissue differs from that in tumor cell lines, including embryonic cardiac H9c2, transfected COS-7 and L6 cell lines, in which mAKAP is detected at the ER/SR (McCartney et al., 1995; M. S. K., unpublished observations). This leaves open the possibility that other compartmentalized pools of mAKAP exist in certain cell types.

Another important observation from Figs 5 and 6 is that mAKAP targeting to the nuclear membrane occurs in a differentiation-dependent manner. Furthermore, our results suggest not only that the expression levels of mAKAP depend upon the state of cellular differentiation, but that these events may promote a redistribution of the PKA holoenzyme to the nuclear membranes of RNV. The induction of mAKAP expression appears coincident with an upregulation of RII protein. This would be consistent with other reports where hormones such as FSH and TSH promote selective expression of AKAPs, upregulation of RII and a redistribution of the PKA holoenzyme (Carr et al., 1993; Hunzicker-Dunn et al., 1998). However, at this stage it is not clear what differentiation signals stimulate mAKAP expression or whether the enhanced RII signal represents a stabilization of the protein through interaction with mAKAP or increased protein synthesis.

Mechanism of mAKAP targeting

Our mapping studies suggest that at least two regions of mAKAP are necessary and sufficient for targeting to the nuclear membrane. The participation of multiple targeting signals is a common theme in directing AKAPs to their correct intracellular sites. For example, the neuronal anchoring protein AKAP79 is targeted to the plasma membrane by two or three repeated motifs rich in basic and hydrophobic amino acids that can bind acidic phospholipid (Dell'Acqua et al., 1998; Glantz et al., 1993; Li et al., 1996). Likewise, AKAP18 is targeted also to the plasma membrane by the combined action of myristoylation and dual palmitoylation signals (Fraser et al., 1998; Gray et al., 1998). Alternately spliced targeting cassettes

in the amino terminus of S-AKAP84/D-AKAP-1 confer targeting to the mitochondria or endoplasmic reticulum, respectively (Chen et al., 1997; Huang et al., 1997b; Lin et al., 1995). However, a distinct feature of mAKAP targeting is highlighted by the displacement studies which demonstrate that mAKAP targeting is saturable (Fig. 9). These findings are consistent with the idea that the targeting locus or acceptor site may be another protein expressed at limiting concentrations in the nuclear membrane. This view is further supported by our evidence that spectrin repeats are determinants for mAKAP targeting. Spectrin-repeat sequences are found in the cytoskeletal proteins spectrin, dystrophin and α-actinin and participate in protein-protein interactions with cytoskeletal components such as actin and/or adducin and PDZ domain-containing proteins (Brown, 1997; Li and Bennett, 1996; Xia et al., 1997). Future studies are planned to use the spectrin-repeat region as a probe to identify the mAKAP targeting protein.

Linkage to ARVD

Although we have not yet ascribed a functional consequence of PKA targeting to the nuclear membrane of differentiated cardiac myocytes, we are intrigued by the chromosomal location of the mAKAP gene. Interestingly, a genetic locus on chromosome 14q for the autosomal dominant disorder arrhythmogenic right ventricular dysplasia (ARVD) is bounded by markers D14S262 and D14S69, which surround the human mAKAP gene (Severini et al., 1996). Familial ARVD is an autosomal dominant disorder with reduced penetrance, which has an estimated prevalence as high as 6 per 10,000 in the general population and is due to mutations at one of several genetic loci (Rampazzo et al., 1997). Although ARVD was initially described as a rare cause of right-sided heart failure, it is now recognized as an important cause of ventricular arrhythmia and sudden death in adolescents and adults. ARVD is defined pathologically as fibrofatty atrophy of the right ventricular myocardium with progressive involvement of the septum and the left ventricle (McKenna et al., 1994). mAKAP binds a protein kinase involved in signal transduction and is found at the nuclear membrane of the heart. Current studies are focused on analysis of the human mAKAP gene as a candidate gene for the Severini ARVD kinship.

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