Protein Kinase A-anchoring Protein AKAP95 Interacts with MCM2, a Regulator of DNA Replication*

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Protein kinase A (PKA)-anchoring protein AKAP95 is localized to the nucleus in interphase, where it primarily associates with the nuclear matrix. A yeast two-hybrid screen for AKAP95 interaction partners identified the minichromosome maintenance (MCM) 2 protein, a component of the pre-replication complex. AKAP95-MCM2 interaction was mapped to residues 1–195 of AKAP95 and corroborated by glutathione S-transferase precipitation and immunoprecipitation from chromatin. Disruption of AKAP95-MCM2 interaction with an AKAP95-(1–195) peptide within HeLa cell nuclei abolishes initiation of DNA replication in G1 phase and the elongation phase of replication in vitro without affecting global nuclear organization or import. Disruption of the C-terminal zinc finger of AKAP95 reduces efficiency of replication initiation. Disruption of the PKA-binding domain does not impair replication in G1- or S-phase nuclei, whereas a PKA inhibitor affects the initiation but not the elongation phase of replication. Depleting AKAP95 from nuclei partially depletes MCM2 and abolishes replication. Recombinant AKAP95 restores intranuclear MCM2 and replication in a dose-dependent manner. Our results suggest a role of AKAP95 in DNA replication by providing a scaffold for MCM2.

Eukaryotic DNA replication involves at least 20 different proteins and is a process largely conserved from yeast to human (1). A key player in the regulation of DNA replication is the hexameric minichromosome maintenance (MCM) complex. MCMs are proteins identified initially for their role in plasmid maintenance and cell cycle progression (2). Six of the MCM proteins, including MCM2, are highly conserved and play a direct role in both initiation of DNA synthesis (3) and DNA chain elongation (1, 3). Once the MCM complex is assembled, S-phase cyclin-dependent kinase activity and the Cdc7-Dbf4 protein kinase (an MCM2-7 kinase) trigger firing of DNA synthesis (5). After origin firing, the pre-RC is dissociated, and it is not reassembled until the next cell cycle. A proportion of MCM proteins are phosphorylated and believed to be released from chromatin, although a DNA unwinding or helicase function of MCM proteins has been shown during elongation (6). Moreover, depletion of MCMs after initiation of DNA replication irreversibly blocks progression of replication forks in Saccharomyces cerevisiae, indicating that MCM proteins are essential for elongation (7).

We and others have cloned and characterized a 95-kDa protein kinase A (PKA, or cAMP-dependent kinase)-anchoring protein, AKAP95 (8, 9). AKAP95 is a nuclear AKAP that in interphase primarily co-fractionates with the nuclear matrix, a nonionic detergent-, nuclease-, and salt-resistant structure, whereas a minor fraction associates with chromatin (8, 10). At mitosis, and upon nuclear envelope breakdown in vitro, AKAP95 dissociates from the matrix and binds chromatin (10, 11), onto which it has been proposed to recruit the condensin complex and thereby play a role in chromosome condensation (11, 12). Mapping of chromatin condensation and condensin targeting functions of AKAP95 has shown that these processes are independent of PKA binding but require both the ZF1 and ZF2 zinc finger motives in the C-terminal half of AKAP95 (12). A yeast two-hybrid screen has identified p88 RNA helicase as a binding partner for AKAP95, suggesting a putative scaffolding role of AKAP95 in the assembly of transcription complexes (13). The c-Myc-binding protein, AMY-1, also binds AKAP95 near the PKA-RII-binding domain (14). AMY-1 can form a ternary complex with AKAP95 and RII that prevents targeting of the PKA catalytic subunit (14). Any other putative function of AKAP95 in the interphase nucleus has remained unexplored.

DNA replication has been connected to the nuclear matrix, based on indications that many DNA polymerases are immobilized on an insoluble intranuclear network in so-called replication factories (15, 16). Here, we present evidence for a direct interaction between AKAP95 and MCM2. Disruption of this interaction abolishes initiation and elongation phases of replication in vitro. Removing AKAP95 from nuclei partially depletes MCM2 and abolishes replication. Recombinant AKAP95 restores intranuclear MCM2 and replication. Our results suggest a role of AKAP95 as an MCM2 anchor and an involvement of the AKAP95-MCM2 association in DNA synthesis.
**EXPERIMENTAL PROCEDURES**

Antibodies and Peptides—Anti-AKAP95 polyclonal antibodies were from Upstate Biotechnology (10). Monoclonal antibodies (mAbs) against AKAP95 (22), MCM2 (Bm28) were from Transduction Laboratories. Anti-GST, anti-Orc2, anti-Cdc6, and anti-MCM2 polyclonal antibodies were from Santa Cruz Biotechnology. mAbs against Gal4AD or Gal4BD domains were from Clontech. Antibodies against lamina-associated polypeptide 2β and B-type lamins were from Dr. J.-C. Courvalin (17). Anti-HA95 antibodies were as described previously (18). Full-length AKAP95 or AKAP95-(1-387) was amplified by PCR using AKAP95-cDNA (9) as template to generate restriction sites for subcloning into pGEX-KG, expression from which yielded a GST-AKAP95-(1-692) fusion protein (9). This expression vector was used as template to generate deletion constructs and for site-directed mutagenesis (QuickChange; Stratagene) (12). GST- AKAP95 peptides produced were AKAP95-(1-692), AKAP95-(1-195), AKAP95-(387-692), AKAP95-(387-602), AKAP95-(357-568), AKAP95-(387-524), and AKAP95-(387-450) and peptides with a mutation in zinc fingers ZF1 and ZF2 or in the FHA-binding domain as described in “Results” (12). MCM2-(398-543) was ligated into pGEXSK-X3 (Amer- sham Biosciences) to produce a GST-MCM2-(388-543) protein. All constructs were sequenced. Expression and purification of GST-tagged proteins were done as described previously (19). Protein concentrations were determined by the Bradford method.

**Cells—**HeLa cells were grown in Eagle’s minimal essential medium/10% fetal calf serum (Life Technologies, Inc.). Cells were synchronized in M phase with 1 μM nocodazole for 18 h (10). To allow cell cycle reentry, mitotic cells were harvested and replated at 2.5 × 10^6 cells/162-mm flask. G1- and S-phase cells were harvested 2 and 12 h, respectively, after release from mitotic arrest.

Nuclei and Chromatin—Intact nuclei were isolated from unsynchronized and G1- or S-phase HeLa cells, as indicated, by Dounce homogenization (21) and used for immunoprecipitation and replication assays, respectively. Concentration of nuclei was determined using an automated cell counter (Coulter).

Purified S-phase nuclei were depleted of AKAP95 by loading anti-NuMA antibodies and subsequent exposure to a mitotic HeLa cell extract as described previously (11). To reconstitute nuclear architecture, AKAP95-depleted chromatin masses were recovered from the extract by sedimentation through 1 m sucrose and incubated for 1.5 h in a nuclear reassembly extract containing cytosol, membrane vesicles, ATP-regenerating system, and GTP to promote nuclear envelope formation (20). Reconstituted nuclei were sedimented at 1,000 × g through 0.5 m sucrose for analysis. To prepare chromatin, confluent interphase HeLa cells were harvested and suspended in hypotonic buffer (10 mM Tris, pH 7.6, 10 mM NaCl, 3 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture) (10). Nuclei were isolated by sedimentation, resuspended in CSK buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 3 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture) (10) and used for immunoprecipitation and replication assays.

DNA Replication Assay—Replication extracts were prepared from S-phase HeLa cells collected 15 h after release from nucodazole-induced mitotic arrest. Cells were lysed by Dounce homogenization (20) and briefly sonicated on ice to lyse nuclei and release soluble nuclear components. The lysate was sedimented at 15,000 × g for 15 min and then at 200,000 × g for 2 h at 4 °C. Protein concentration of the extract was 15 mg/ml.

Replication was assayed by incorporation of [α-32P]dCTP (20, 22, 23). Purified G1- or S-phase nuclei, loaded with GST-AKAP95 peptides or GST alone as control, were incubated for 3 h at 5,000 nuclei/μl in 40 μl of replication extract containing an ATP-regenerating system (1.2 μl), 100 μM GTP (0.4 μl), buffered deoxynucleotide triphosphates (40 mM Hepes, pH 7.8, 7 mM MgCl₂, and 0.1 μM each of dATP, dGTP, dTTP, and dCTP; 2 μl), and [α-32P]dCTP (3,000 Ci/mmol; Nycomed-Amersham, Brinkmann Instruments). At the end of incubation, samples were mixed with 1 volume of 20 mM Tris (pH 7.5) and digested for 2 h at 37 °C with 1 mg/ml protease K. Samples were mixed by pipetting, and aliquots were electrophoresed through 0.8% agarose. Gel loading was assessed by ethidium bromide staining. Signals were detected by autoradiography.

**RESULTS**

Isolation of MCM2 as an AKAP95-binding Protein by Two-hybrid Screening—A full-length AKAP95 clone was fused to the C terminus of Gal4-DNA-BD (16 kDa), expressed in yeast and detected as a ~120-kDa product (Fig. 1A). Screening of a pre-transformed HeLa cell library (5 × 10^6 clones) yielded 290 candidate clones after quadruple drop out and β-galactosidase selection, of which 7% were lacZ⁺, His⁺, and Ade⁺ in the absence of bait and eliminated. One hundred and twenty positive clones were sequenced. Whereas most clones were excluded as being false positives or artifacts because the open reading frame was out of frame or in the wrong orientation with respect to the fused Gal4 activation domain, nine clones appeared to be putative interaction partners. Of these, one
AMY-1 was used as positive control, and empty vectors (pAS2-1 and AKAP95 and MCM2, respectively) were mated. Diploids were restreaked on quadruple drop out medium (left panel), and β-galactosidase activity was analyzed (right panel). AMY-1 was used as positive control, and empty vectors (pAS2-1 and pGADGH) were used as negative controls.

represented the AKAP95-binding protein AMY-1 (14), and seven are currently subject to further investigation. One clone encoded residues 398–543 of MCM2, a component of the pre-RC, with a mobility of 30 kDa when fused to the 12-kDa Gal4AD domain (Fig. 1B). This clone was lacZ”, His”, and Ade” only in presence of AKAP95, but not with Gal4-DNA-BD alone (Fig. 1C, left panel). AMY-1, detected in the screen, was used as positive control, and pGADGH was used as negative control (Fig. 1C). Interaction of MCM2 and AMY-1 with AKAP95 was confirmed by β-galactosidase assay (Fig. 1C).

GST Precipitation of [35S]-AKAP95 and MCM2 and Mapping of Interaction Domain—To verify the interaction between AKAP95 and MCM2, in vitro-translated [35S]Met-labeled AKAP95 was incubated with GST-MCM2 or GST alone, and GST-protein complexes were precipitated. [35S]-AKAP95 co-precipitated with GST-MCM2, but not with GST alone (Fig. 2A). Furthermore, a GST-AKAP95(1–692) fusion protein, but not GST alone, co-precipitated endogenous MCM2 from a HeLa cell extract, as shown by anti-MCM2 immunoblot analysis (Fig. 2B). This indicates that AKAP95 interacts directly with MCM2.

We next mapped the MCM2 interaction domain of AKAP95. In vitro-translated [35S]Met-labeled MCM2 was incubated with GST-AKAP95(1–692) deletion peptides. GST precipitations show that AKAP95(1–692) and AKAP95(1–195) interacted with MCM2, whereas interaction was nearly abolished with AKAP95(387–692) (Fig. 2C). This result was confirmed in a precipitation of GST-AKAP95 peptides from HeLa nuclear extracts. GST-AKAP95(1–195) or GST-AKAP95(1–692) co-precipitated endogenous MCM2, whereas GST-AKAP95(397–692) (or shorter deletions thereof; data not shown) was ineffective (Fig. 2D). Notably, neither AKAP95 peptide co-precipitated Orc2 or Cdc6, two other components of the pre-RC (Fig. 2D), arguing toward specificity of the association between AKAP95(1–195) and MCM2. Collectively, the data argue that the first 195 residues of AKAP95 are involved in the interaction with MCM2.

AKAP95 Peptides Dissociate MCM2 from AKAP95 Immune Complexes—AKAP95 and MCM2 co-immunoprecipitated from HeLa cell extracts (Fig. 3A). Fractionation of purified HeLa nuclei further showed that AKAP95 co-fractionated with both nuclear matrix and chromatin, whereas MCM2 co-fractionated primarily with chromatin (Fig. 3B). AKAP95 and MCM2 co-immunoprecipitated from the chromatin fraction (data not shown), suggesting that both proteins reside in a same complex associated with chromatin.

Whether GST-AKAP95 peptides would disrupt the interaction between AKAP95 and MCM2 was determined. Anti-AKAP95 immune precipitates from HeLa nuclei were incubated for 1 h with 100 µM GST-AKAP95 fragments, immune precipitates were sedimented, and dissociation of MCM2 from AKAP95 was monitored by immunoblotting. AKAP95(1–195) dissociated most MCM2 from the AKAP95 immune precipitates, whereas AKAP95(387–692), shorter C-terminal AKAP95 fragments (data not shown), or GST alone was ineffective (Fig. 3C). This result corroborates the interaction of AKAP95 with MCM2 via residues 1–195 and raises the possibility of disrupting this interaction in nuclei for functional studies.

Disruption of the AKAP95-MCM2 Interaction in G1 Inhibits Initiation and Elongation Phases of DNA Replication—To explore a putative role of the AKAP95-MCM2 interaction on DNA replication, dissociation of MCM2 from AKAP95 was elicited intranuclearly, and the effect of this disruption on replication in a cell-free system was investigated. GST-AKAP95 peptides or, as control, GST alone was introduced into nuclei isolated from G1-phase HeLa cells after gentle permeabilization of the nuclei with lysolcithin. Control and peptide-loaded nuclei were incubated for 3 h in a nuclear and cytosolic extract from S-phase HeLa cells containing [α-32P]dCTP, deoxynucleotide triphosphates, GTP, and an ATP-regenerating system to promote replication. In the absence of peptide or with GST alone, nuclei synthesized DNA, and replication was inhibited by 50 µM aphidicolin in the extract (Fig. 4A, lanes 1 and 2). AKAP95-(1–692) and AKAP95(1–195) abolished replication, whereas AKAP95(387–692), AKAP95(387–602), AKAP95(387–569), and AKAP95(387–543) were ineffective (Fig. 4B).
AKAP95-MCM2 Interaction

Fig. 3. GST-AKAP95-(1–195) dissociates MCM2 from AKAP95 immune precipitates. A, AKAP95 or MCM2 was immunoprecipitated from HeLa cell extracts using polyclonal antibodies. Rabbit IgGs were used as negative controls. Immune complexes were immunoblotted using anti-AKAP95 and anti-MCM2 mAbs. B, HeLa nuclei (Nuc) were fractionated into NaCl-washed nuclear matrix (Mtx), the NaCl wash, and micrococcal nuclease-soluble chromatin (Chr). Each fraction was immunoblotted using anti-AKAP95 or anti-MCM2 antibodies. C, AKAP95-IPs were incubated for 1 h with no peptide (-), 100 μM GST-AKAP95-(1–195), 100 μM GST-AKAP95-(387–692), or GST alone. Immune precipitates were sedimented, and pellets (P) and supernatants (S) were immunoblotted using anti-MCM2 or anti-AKAP95 antibodies.

and AKAP95-(387–524) only partially, but significantly, affected replication (Fig. 4A, lanes 3–8). In contrast, AKAP95-(387–450) did not affect replication (Fig. 4A, lane 9). Disruption of AKAP95 zinc finger ZF1 in AKAP95-(387–692) by mutation of two of the zinc-chelating cysteines (C392S and C395S) or similarly mutating ZF1 in AKAP95-(387–450) did not alter the effect of these peptides on replication (Fig. 4A, ZF1*, lanes 11 and 13). In contrast, mutation of the C-terminal zinc finger ZF2 (C481S and C484S) in AKAP95-(387–692) restored a control level of DNA synthesis (Fig. 4A, ZF2*, lane 12), supporting an effect of a region containing ZF2 on replication. Disruption of the PKA-binding domain of AKAP95 by an I582P mutation did not alter the effect of AKAP95-(387–692) on replication efficiency (Fig. 4A, PKA*, lane 14).

To provide evidence that 32P labeling in G1 nuclei was due to initiation of replication, we showed that replication was inhibited with 10 μM olomoucine, an inhibitor of the cyclin A-cyclin-dependent kinase 2 complex required for S phase entry (22) (Fig. 4B). We also excluded the possibility that the DNA synthesis signal in G1 nuclei represented an elongation phase in already replicating nuclei because G1 nuclei incubated in a G2–phase extract did not replicate (data not shown). Furthermore, we showed that the localization of proteins of the inner nuclear membrane (lamina-associated polypeptide lamina-associated polypeptide 2β), nuclear lamina (B-type lamins), and endogenous AKAP95 was not altered in G1 nuclei containing GST-AKAP95 peptides (Fig. 4C). Lastly, G1 nuclei containing GST-AKAP95 peptides were capable of importing the replication factor Cdc6 in vitro (Fig. 4C).

The results indicate that AKAP95 peptides containing the MCM2-binding domain abolish DNA replication in G1 nuclei in vitro without affecting global nuclear architecture or import. Partial initiation of replication also occurs with AKAP95-(387–524) but not AKAP95-(387–450). This suggests that two regions of AKAP95 are implicated in replication, namely, amino acids 1–195 and a critical domain between residues 450 and 524 that includes ZF2.

Whether the elongation phase of DNA replication was also affected by AKAP95 peptides was determined. Nuclei isolated from S-phase HeLa cells were loaded with GST-AKAP95 peptides and incubated in S-phase extract under conditions promoting replication. AKAP95-(1–195) largely inhibited DNA synthesis (Fig. 4D); however, in contrast to G1 nuclei, replication in S-phase nuclei was not affected by any other AKAP95 fragment including those bearing mutations in ZF1, ZF2, or the PKA-binding domain (data not shown). Note that some DNA synthesis occurred in S-phase nuclei incubated in extract from G0 cells, reflecting the replicating state of these nuclei (data not shown). We concluded that, as with initiation of DNA replication, elongation is inhibited by AKAP95-(1–195). However, C-terminal AKAP95 peptides have no effect on the elongation phase of DNA replication.

PKI Abolishes Initiation of Replication in Vitro—To address the involvement of PKA in DNA replication, the effect of the AKAP-RII disruptor, Ht31 (24), and of the PKA inhibitor PKI, was examined. Replication in G1 nuclei was not altered with 500 nM Ht31 in the extract (Fig. 4E, lane 2). However, pre-incubation of G1 nuclei in extract with 1 μM PKI before adding the ATP and GTP (to start replication) drastically reduced replication efficiency (Fig. 4E, lane 3). However, 1 μM PKI did not affect elongation in S-phase nuclei (Fig. 4E, lane 4). These results suggest an involvement of PKA in the initiation phase of DNA replication.

Recombinant AKAP95 Restores DNA Replication in AKAP95-depleted S-phase Nuclei—Whether nuclei depleted of AKAP95 were capable of replicating DNA was investigated. Purified S-phase HeLa nuclei were loaded with anti-NuMA antibodies and exposed to a mitotic extract to produce AKAP95-depleted chromatin (11). Control (mock-depleted) nuclei were produced using an irrelevant rabbit IgG (11). Chromatin was sedimented through sucrose, and nuclei were reconstituted in a nuclear reassembly assay consisting of CaCl2-containing mitotic cytosol, membrane vesicles, an ATP-regenerating system, and GTP. This promoted the reassembly of a nuclear envelope (Fig. 5A). AKAP95- and mock-depleted nuclei were not distinguishable by phase-contrast microscopy or by immunofluorescence analysis of B-type lamins (Fig. 5A). Western blotting analysis confirmed removal of AKAP95 and the presence of a nuclear lamina (Fig. 5B). Notably, AKAP95-depleted nuclei harbored reduced levels of MCM2 (Fig. 5B; see also Fig. 5D). Levels of HA95, a chromatin-associated protein related to AKAP95 (18), were not affected by elimination of AKAP95, suggesting that gross nuclear architecture was not affected. AKAP95-depleted nuclei also supported import of karyophilic substrates (data not shown).

Replication in “S-phase” AKAP95-depleted nuclei was inhibited (Fig. 5C). This was anticipated because some MCM2 was also removed from the nuclei. Nevertheless, a role of AKAP95 in replication was illustrated in an add-back experiment. Nuclei reassembled from “S-phase” AKAP95-depleted chromatin were permeabilized with lysolecithin and incubated with 1, 10, or 100 μg/ml full-length GST-AKAP95 for ∼2 h. After washing, nuclei were incubated for 3 h in S-phase extract under conditions promoting replication. AKAP95 restored replication in these nuclei in a dose-dependent manner (Fig. 5D). Western blotting analysis showed that increasing amounts of GST-AKAP95 resulted in increasing intranuclear MCM2...
levels (Fig. 5D). Cdc6 and HA95 levels were not affected by AKAP95 depletion or restoration (Fig. 5D). MCM2 was imported from the S-phase replication extract because an antibody against nucleoporins (mAb 414) prevented the restoration of intranuclear levels of MCM2 upon addition of recombinant AKAP95 (Fig. 5D). Furthermore, mAb 414 inhibited replication rescue by AKAP95 (Fig. 5D), indicating that restoring AKAP95 alone was necessary but not sufficient to promote DNA syn-

Fig. 4. AKAP95-(1–195) inhibits initiation and elongation phases of DNA replication in vitro. A, G1-phase HeLa nuclei loaded with the indicated GST-AKAP95 peptides were incubated for 3 h in S-phase extract containing [α-32P]dCTP under conditions promoting replication. [α-32P]dCTP incorporation into synthesized DNA was analyzed by agarose gel electrophoresis (DNA) and autoradiography (32P). Aphid., 50 μM aphidicolin was added to the extract. Star indicates a mutation in ZF1, ZF2, or the PKA-binding domain of AKAP95. B, DNA synthesis in G1 nuclei incubated in S-phase extract containing 0 or 1 mM olomoucine. C, GST-AKAP95 peptides do not disrupt global nuclear architecture in vitro. G1 nuclei were loaded with indicated GST-AKAP95 peptides, incubated in S-phase extract for 3 h, and labeled with antibodies against AKAP95, lamina-associated polypeptide 2β, and B-type lamins. Import of Cdc6 was visualized by immunofluorescence using anti-Cdc6 antibodies. Bars, 10 μm. D, nuclei purified from S-phase cells and loaded with the indicated GST-AKAP95 peptides were incubated in S-phase extract, and incorporation of [α-32P]dCTP was analyzed as described in A. E, G1 nuclei (lanes 1–3) were exposed to S-phase extract containing 500 nM Ht31 or 1 μM PKI for 15 min before addition of the ATP-regenerating system and GTP to promote DNA synthesis. Replication was analyzed as described in A. Lane 4, replication of S-phase nuclei in S-phase extract containing 1 μM PKI.

Fig. 5. Recombinant AKAP95 restores replication in AKAP95-depleted nuclei. A, phase-contrast analysis and B-type lamin immunofluorescence labeling of HeLa nuclei (Input), AKAP95-depleted nuclei, and mock-depleted nuclei (Control). Nuclei were in S phase at the time they were manipulated. B, Western blotting analysis of Input, AKAP95-depleted, and control nuclei using indicated antibodies. C, nuclei as described in A were incubated in S-phase extract, and replication was analyzed by autoradiography. D, AKAP95-depleted nuclei were incubated with no (+) or 1, 10, and 100 μg/ml GST-AKAP95-(1–692) for ~2 h, washed, and exposed to S-phase extract for replication analysis (top panel). Nuclei were also sedimented and analyzed by immunoblotting using indicated antibodies (Blots, bottom panel).
thesis under these conditions. Altogether, these results illustrate a relationship between AKAP95 content and (i) intranuclear MCM2 level (at least when import is permitted) and (ii) replication efficiency.

**DISCUSSION**

Several lines of evidence argue for a direct or indirect role of AKAP95 in DNA replication. (i) AKAP95 directly interacts with the pre-RC component, MCM2, in vitro and in vivo via residues 1–195. (ii) Disruption of this interaction with a competitor AKAP95 peptide inhibits replication initiation and elongation phases in vitro. Inhibition of replication is not due to overt alterations in nuclear architecture or import functions. (iii) Replication initiation (but not elongation) is also affected by an AKAP95 fragment containing the C-terminal zinc finger ZF2. (iv) Nuclei depleted of AKAP95 do not replicate, and such nuclei also display reduced levels of MCM2. (v) However, restoration of intranuclear AKAP95 with a recombinant protein reestablishes MCM2 levels and replication ability in these nuclei. AKAP95-mediated restoration of replication is dependent on nuclear import of MCM2. These observations suggest that AKAP95 is important for DNA replication by providing a scaffold for MCM2 and possibly other MCM proteins.

Two regions of AKAP95 appear to be involved in replication, at least in vitro (Fig. 6). The first 195 amino acids of AKAP95 interact with MCM2 and were shown to disrupt DNA synthesis in G1- and S-phase nuclei exposed to an extract supporting replication. A C-terminal domain (residues 387–524) is also essential for initiation of replication. Because AKAP95(387–450) does not affect replication efficiency, the C-terminal AKAP95 domain involved in replication may tentatively be restricted to amino acids 451–524. This region contains the zinc-chelating motif ZF2. ZF2 structure is important for replication because a double disruptive mutation in ZF2 (C481S and C484S), expected to inhibit zinc chelation, abolishes the inhibitory effect of a peptide containing ZF2 (AKAP95(387–892)) on initiation of DNA synthesis in G1 nuclei. How the ZF2-containing region of AKAP95 affects replication is currently unclear but may involve interaction of AKAP95 with chromatin (12) or DNA (8). A hypothesis to be tested is that AKAP95(1–195) and a ZF2-containing domain are brought together in the vicinity of the pre-RC in interphase to promote replication.

The MCM complex is a likely candidate to act as the replicative helicase during elongation, but modifications by the two S-phase promoting kinases, Cdc7-Dbf4 and cyclin A-cyclin-dependent kinase 2, appear to be required for conversion to an active helicase complex (4, 25). In contrast, MCM2 appears to inhibit MCM-associated helicase activity (26), but phosphorylation of MCM2 (5, 27) or other MCM proteins by Cdc7 could possibly release this inhibition. After initiation, ORC and Cdc6 can be removed from the pre-RC without affecting retention or activity of the MCM complex, suggesting an independent mechanism of anchoring. This independence is further illustrated by binding of AKAP95 to MCM2 but not Orc2 or Cdc6, retention of Cdc6 in nuclei depleted of AKAP95 and thereby of most MCM2, and rather constant levels of Cdc6, whereas the amount of intranuclear MCM2 rises with increasing concentrations of recombinant AKAP95 introduced in depleted nuclei. Recent reports suggest that Cdc7-Dbf4 recruitment to chromatin is dependent on the MCM complex but that Cdc7 and Dbf4 associate with chromatin independently of MCMs and Cdc6 (28, 29). One possibility is that AKAP95 targets MCM2 to the Cdc7 kinase on chromatin to obtain proximity for phosphorylation of MCM2.

It was recently shown that activation of PKA was required for progression into S phase by phosphorylation of Cdc6 followed by release of the protein from chromatin (30). Participation of AKAP95 in the MCM complex by interaction with MCM2 may provide a possible mechanism for this regulation because AKAPs provide docking sites for the PKA holoenzyme via the regulatory RII subunit dimer (31). However, this hypothesis implies some level of intranuclear PKA holoenzyme, which has yet to be shown. Nevertheless, should some PKA holoenzymes be retained in the nucleus, AKAP95 could serve to optimally target the kinase for its role in DNA replication. Alternatively, AKAP95 may serve as an anchoring protein for the catalytic (C) subunit of PKA in the vicinity of MCM2. This remains to be explored, in light of the discovery that the AKAP95-like protein, HA95 (18), is capable of binding PKA-C in nuclei of lymphoid Bjab cells (32) and that formation of a ternary complex between AKAP95, RII, and AMY-1 prevents targeting of PKA-C (14). Assembly of the pre-RC can be blocked with 6-dimethylamino purine, an inhibitor of serine/threonine protein kinases (33). Replication deficiency caused by 6-dimethylamino purine is rescued by a crude preparation of MCM proteins (34). One can speculate that PKA anchored in the vicinity of MCM2 by AKAP95 is inhibited by 6-dimethylaminopurine, contributing to the inhibition of DNA synthesis.

We did not see an effect of mutating the PKA-binding domain of AKAP95 on DNA replication, indicating that the AKAP95-RII interaction is dispensable for this process. However, the PKI reduced the efficiency of replication initiation (but not elongation). Thus, it is possible that an excess of soluble PKA-C in the vicinity of replication initiation foci acts to initiate replication independently of anchoring to AKAP95. PKI might inhibit this PKA activity by binding to the PKA-C and exporting it out of the nucleus.

We have recently unraveled a role of HA95 in DNA replication initiation (20). HA95 presents homology to AKAP95 but is not an AKAP (18). Interestingly, both proteins interact with the pre-RC, but whereas AKAP95 associates directly with

**Fig. 6. Interaction and functional domains of AKAP95.** Identified binding domains and functions (in bold) of human AKAP95. Numbers refer to amino acid positions. NMTS, nuclear matrix targeting sequence; biNLS, bipartite nuclear localization signal; NLS, nuclear localization signal; ZF, zinc finger; PKA, PKA-binding domain. +, Ref. 13; §, Ref. 12; #, Ref. 35; ||, this paper.
MCM2, HA95 co-immunoprecipitates with Cdc6 (20). Interaction of HA95 with lamina-associated polypeptide 2 proteins appears to be important for protecting Cdc6 from degradation by the proteasome during G1. Thus, it seems that HA95 plays a protective role toward the pre-RC, whereas AKAP95 provides a structural basis for pre-RC function in initiation of replication.

The increasing number of functional and interaction domains identified in AKAP95 (Fig. 6) suggests that AKAP95 serves multiple functions during the cell cycle. Nuclear matrix association of AKAP95 was shown to be mediated by an N-terminal 30-amino acid signal (13) contained within the MCM2-binding region. Residues 109–201 of AKAP95 were also shown to bind the p68 RNA helicase in the nuclear matrix, suggesting a role of AKAP95 as a scaffold for coordinating the assembly of transcription complexes (13). This region partially overlaps with the MCM2 interaction region (Fig. 6); nevertheless further mapping is required to determine whether these regions are related or independent. A region sufficient to bind chromosomes has been mapped to residues 387–450 of AKAP95 and requires the zinc finger ZF1 (12), whereas a functional ZF2 is necessary for recruitment of the condensin complex and AKAP95 function in chromosome condensation at mitosis (11, 12, 35). These observations argue that ZF2 is involved in activities affecting chromatin or DNA dynamics (i.e. chromatin condensation and replication). It also appears from these studies that the N-terminal region of AKAP95 is involved in interphase localization and anchoring of AKAP95 to, primarily, the nuclear matrix. The C-terminal region, by mediating chromatin-interaction, mediates mitotic localization of AKAP95 to chromosomes. AKAP95, therefore, emerges as an important player in the regulation of chromatin structure during replication, transcription, and chromosome condensation.

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Protein Kinase A-anchoring Protein AKAP95 Interacts with MCM2, a Regulator of DNA Replication

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