Regulation of anchoring of the RIIα regulatory subunit of PKA to AKAP95 by threonine phosphorylation of RIIα: implications for chromosome dynamics at mitosis

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SUMMARY

CDK1 phosphorylates the A-kinase regulatory subunit RIIα on threonine 54 (T54) at mitosis, an event proposed to alter the subcellular localization of RIIα. Using an RIIα-deficient leukemic cell line (Reh) and stably transfected Reh cell clones expressing wild-type RIIα or an RIIα(T54E) mutant, we show that RIIα associates with chromatin-bound A-kinase anchoring protein AKAP95 at mitosis and that this interaction involves phosphorylation of RIIα on T54. During interphase, both RIIα and RIIα(T54E) exhibit a centrosome-Golgi localization, whereas AKAP95 is intranuclear. At mitosis and in a mitotic extract, most RIIα, but not RIIα(T54E), co-fractionates with chromatin, onto which it associates with AKAP95. This correlates with T54 phosphorylation of RIIα. Disrupting AKAP95-RIIα anchoring or depleting RIIα from the mitotic extract promotes premature chromatin decondensation. In a nuclear reconstitution assay that mimics mitotic nuclear reformation, RIIα is threonine dephosphorylated and dissociates from AKAP95 prior to assembly of nuclear membranes. Lastly, the Reh cell line exhibits premature chromatin decondensation in vitro, which can be rescued by addition of wild-type RIIα or an RIIα(T54D) mutant, but not RIIα(T54E, A, L or V) mutants. Our results suggest that CDK1-mediated T54 phosphorylation of RIIα constitutes a molecular switch controlling anchoring of RIIα to chromatin-bound AKAP95, where the PKA-AKAP95 complex participates in remodeling chromatin during mitosis.

Key words: Mitosis, Chromosome Condensation, Phosphorylation, PKA, AKAP95

INTRODUCTION

Intracellular effects of cAMP are conveyed by cyclic AMP-dependent protein kinase (PKA) types I and II in eukaryotic cells. The PKA type II holoenzyme consists of two catalytic subunits and two regulatory subunits (RIIα or RIIβ) that bind to and inactivate the catalytic subunits (Tasken et al., 1997). PKA is activated by the binding of two cAMP molecules to each R subunit, which promotes release of the catalytic subunits from the R-cAMP complex. Active catalytic subunits phosphorylate specific substrates and can be translocated to the nucleus, where they activate cAMP-responsive genes (Riabowol et al., 1988).

The specificity of responses to cAMP is mediated by targeting of the RII subunit of PKA to organelles, cytoskeleton or membranes through associations with A-kinase-anchoring proteins (AKAPs) (Colledge and Scott, 1999). AKAP95 is a 95 kDa AKAP that is exclusively intranuclear during interphase (Coghlan et al., 1994; Eide et al., 1998). AKAP95 co-fractionates primarily with the nuclear matrix (Collas et al., 1999), but as no RIIα has been detected in interphase nuclei (Eide et al., 1998), the role of AKAP95 in the nucleus remains elusive. Nevertheless, recent studies have shown that AKAP95 is required for chromosome condensation at mitosis (Collas et al., 1999) by acting as a receptor for the condensin complex (Steen et al., 2000). AKAP95 mostly co-fractionates with mitotic chromosomes and associates with RIIα to form a complex necessary to maintain chromosomes in a condensed form throughout mitosis (Collas et al., 1999). These observations suggest that the interaction between AKAP95 and RIIα is cell-cycle dependent, but what regulates this interaction is unknown.

The subcellular localization and activity of protein kinases is often altered by protein phosphorylation. In contrast to RI, RII can be autophosphorylated by the catalytic subunit of PKA (Erlichman et al., 1983). Human RIIβ is also phosphorylated on threonine 69 (T69) by the mitotic kinase CDK1 (Keryer et al., 1993). Deletion and mutation analyses have shown that human RIIα is also autophosphorylated on S99 throughout the cell cycle, and is hyperphosphorylated on T54 at mitosis and by purified CDK1 in vitro (Keryer et al., 1998). Furthermore, whereas RIIα is associated with the centrosome-Golgi area during interphase, it is dislocated from this region at metaphase (Keryer et al., 1998). RIIα is also solubilized from a particulate fraction of interphase cell extracts upon exposure to CDK1, suggesting that CDK1

phosphorylation of RIIα at mitosis alters its subcellular localization (Keryer et al., 1998).

We report here that, at mitosis and in a mitotic cell extract, association of human RIIα with chromosome-bound AKAP95 requires phosphorylation of RIIα on T54. Disrupting AKAP95-RIIα anchoring or depleting RIIα elicits premature chromatin decondensation, suggesting that the AKAP95-RIIα complex plays a role in maintaining chromosomes condensed during mitosis. Nuclear reconstitution in vitro is accompanied by threonine dephosphorylation and dissociation of RIIα from decondensing chromatin prior to reassembly of nuclear membranes. Our results suggest that T54 phosphorylation of RIIα constitutes a molecular switch controlling anchoring of RIIα to chromatin-bound AKAP95 at mitosis and its dissociation at mitosis exit.

MATERIALS AND METHODS

Cell lines

The pre-B lymphoblastic leukemia cell line Reh cannot differentiate and does not express RIIα (Tasken et al., 1993). Stably transfected single cell clones expressing moderate levels of wild-type RIIα (RIIα) or of the RIIα(T54E) mutants were generated by electroporation and selection on hygromycin B in RPMI 1640 medium (GibcoBRL) containing 10% fetal calf serum (Carlson et al., 2001; Tasken et al., 1994). The rat PC12 neuronal cell line was grown in DMEM (GibcoBRL) containing 5% fetal calf serum and 10% horse serum. Cells were synchronized in M phase with 1 μM nocodazole for 17 hours.

Antibodies and recombinant proteins

Affinity-purified rabbit polyclonal antibodies against rat AKAP95 (Upstate Biotechnology) and monoclonal antibodies (mAbs) against human AKAP95 (mAb47; Transduction Laboratories) were described previously (Coghlan et al., 1994; Collas et al., 1999). Anti-human RIIα and anti-human RIIβ mAbs were from Transduction Laboratories (Eide et al., 1998) and cross-react with rat RIIα. Polyclonal antibodies against human lamin B receptor (LBR; a gift from J.-C. Courvalin, Institut Jacques Monod, Paris, France) were described previously (Collas et al., 1996). The anti-phosphothreonine mAb (anti-pT) was from New England Biolabs and the rabbit anti-phosphoserine (anti-pS) antibody was from Zymed.

The Ht31 peptide derived from the AKAP Ht31 was used as a specific inhibitor of AKAP-RII interaction (Carr et al., 1991). Control Ht31-P peptides contained two isoleucines mutated to prolines, disrupting the amphipathic helix structure of Ht31. Recombinant human wild-type RIIα and the RIIα(T54A), RIIα(T54D), RIIα(T54E), RIIα(T54L) and RIIα(T54V) mutants were expressed and purified as described (Keryer et al., 1998; Tasken et al., 1993).

Interphase nuclei and mitotic chromatin

To isolate interphase Reh nuclei, Reh cells (2×10^6 ml^{-1}) were suspended in 1 ml of hypotonic buffer (10 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT, 1 mM PMSF and a protease inhibitor cocktail). NP-40 was added to 0.5% and nuclei sedimented at 1000 ***g*** for 5 minutes. Nuclei were resuspended in ice-cold buffer N (hypotonic buffer with 250 mM sucrose), sedimented at 1000 ***g*** and resuspended in buffer N to ~2×10^7 nuclei ml^{-1}. Nuclei were used fresh or frozen at −80°C in buffer N containing 70% glycerol.

A soluble mitotic chromatin fraction was prepared from mitotic Reh or PC12 cells essentially as described previously, by digestion of chromosomes with 5 U ml^{-1} of micrococcal nuclease (MNase; Sigma) in TKM buffer (Collas et al., 1999). Released solubilized chromatin was separated from MNase-insoluble material by sedimentation and held on ice until use. Chromatin masses obtained in mitotic extract were retrieved by sedimentation at 1000 ***g*** through 1 M sucrose, washed in TKM buffer and solubilized with MNase as above.

Mitotic extracts

Mitotic Reh, Reh-RIIα or RIIα(T54E) cells, as indicated, were washed twice in ice-cold lysis buffer (20 mM Hepes, pH 8.2, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT, 20 μg ml^{-1} cytochalasin B and protease inhibitors) and sedimented. The cell pellet was resuspended in 1 volume of lysis buffer and incubated for 30 minutes on ice before Dounce homogenization using a tightly fitting glass pestle. The lysate was centrifuged at 10,000 ***g*** and the supernatant cleared at 200,000 ***g*** to produce a mitotic cytosolic extract (Collas et al., 1999). Extracts were aliquoted, frozen in liquid nitrogen and stored at −80°C.

In vitro nuclear disassembly and reconstitution

A nuclear disassembly assay consisted of 20 μl mitotic Reh, Reh-RIIα or Reh-RIIα(T54E) extract, as indicated, and 1 μl nuclear suspension (10^5 nuclei). Reactions were started by the addition of an ATP-generating system and allowed to proceed at 30°C for 2 hours (Collas et al., 1999). Mitotic extracts supported chromatin condensation without DNA degradation (Steen et al., 2000). Chromatin condensation was monitored by staining aliquots with 0.1 μg ml^{-1} Hoechst 33342 and assessed by irregular and compact morphology of the chromatin. For immunofluorescence analysis of chromatin, chromatin masses were washed at 1000 ***g*** through a 1 M sucrose cushion.

The in vitro nuclear reassembly assay was adapted from that of Burke and Gerace (Burke and Gerace, 1986). Mitotic Reh-RIIα cells were Dounce homogenized in an equal volume of KHM (78 mM KCl, 50 mM Hepes (pH 7.0), 4 mM $MgCl_2$, 10 mM EGTA, 8.4 mM $CaCl_2$, 1 mM DTT, 20 μM cytochalasin B and protease inhibitors). The lysate was supplemented with the ATP-generating system and incubated for up to 75 minutes at 30°C. Chromosome decondensation was assessed after DNA labeling by the expanded and nearly spherical morphology of the chromatin. Nuclear envelope reformation was monitored by immunofluorescence using anti-LBR antibodies.

Premature chromatin decondensation assay

A condensed Reh-RIIα chromatin substrate prepared in mitotic Reh-RIIα extract was recovered by sedimentation through sucrose, washed in lysis buffer and incubated for up to 3 hours in 20 μl of fresh Reh-RIIα mitotic extract containing 500 nM Ht31. Alternatively, condensed Reh, Reh-RIIα or Reh-RIIα(T54E) chromatin were obtained during a 2 hour incubation in the relevant mitotic extracts, and incubated in the same extracts for another 2 hours. Chromatin morphology was examined by Hoechst staining. 'Premature chromatin decondensation' (PCD) referred to swelling of chromatin into ovoid or spherical objects with no discernable chromosomes while the extract remained mitotic, as judged by elevated histone H1 kinase activity (Collas et al., 1999).

Alkaline phosphatase treatment

Mitotic Reh chromatin was solubilized with MNase and incubated for 45 minutes at room temperature with either 100 U ml^{-1} calf intestinal alkaline phosphatase (APase; Promega) or as control 100 U ml^{-1} APase plus 20 mM of the phosphatase inhibitor sodium vanadate (Sigma). APase- and APase+sodium-vanadate-treated chromatin fractions were used for immunoprecipitations of RIIα and AKAP95.

Immunological procedures

SDS-PAGE and immunoblotting were performed as described earlier (Collas et al., 1999) with 30 μg protein per lane and using the following antibodies: anti-AKAP95 mAb47 (1:250 dilution), anti-RIIα mAb (1:250), anti-pS (1:250) and anti-pT (1:5000). Blots were revealed by enhanced chemiluminescence. Immunoprecipitations were preformed as reported earlier (Collas et al., 1999). Whole cells

or whole in vitro nuclear disassembly or reassembly reaction mixtures were sonicated in immunoprecipitation (IP) buffer (10 mM Hepes, pH 8.2, 10 mM KCl, 2 mM EDTA, 1% Triton X-100 and protease inhibitors) and the lysate centrifuged at 15,000 ***g***. The supernatant was pre-cleared with protein A/G agarose and immunoprecipitations were carried out with relevant antibodies (1:50 dilutions). Protein of immune complexes were eluted in SDS sample buffer. RIIα and AKAP95 were also immunoprecipitated from cytosolic fractions and mitotic chromatin solubilized with MNase. Immunofluorescence analysis of cells or chromatin masses was done as described (Collas et al., 1996). Antibodies were used at a 1:100 dilution and DNA was stained with 0.1 μg ml^{-1} Hoechst 33342. Observations were made and photographs taken as described previously (Collas et al., 1999).

RESULTS

Cell cycle distribution of AKAP95 and RIIα in Reh cells expressing wild-type RIIα and the RIIα(T54E) mutant

The AKAP95 and RIIα contents of interphase and mitotic Reh cells and Reh transfectants expressing wild-type RIIα ('Reh-RIIα cells') or the RIIα(T54E) mutant ('Reh-RIIα(T54E) cells') were examined by immunoblotting. All cell types contained similar levels of AKAP95 during interphase and mitosis (Fig. 1A). Moreover, whereas Reh cells did not harbor any RIIα, as expected, Reh-RIIα and Reh-RIIα(T54E) cells expressed similar levels of RIIα and RIIα(T54E), respectively, during interphase and at mitosis (Fig. 1A).

The subcellular localization of AKAP95 and RIIα was examined in each cell type by double immunofluorescence using antibodies against AKAP95 and RIIα. In accordance with previous observations in HeLa cells (Collas et al., 1999), AKAP95 was restricted to the nucleus during interphase (Fig. 1B, green label), whereas RIIα and RIIα(T54E) were detected in the centrosome-Golgi area in Reh-RIIα and Reh-RIIα(T54E) cells (Fig. 1B, red label). At mitosis, AKAP95 co-localized with chromosomes over a diffuse cytoplasmic background (Fig. 1C). Variable amounts of RIIα staining were seen on chromosomes in addition to cytoplasmic labeling in mitotic Reh-RIIα cells (Fig. 1C). By contrast, most RIIα(T54E) labeling was punctate and did not decorate chromosomes or the cytoplasm, nor did it co-localize with AKAP95 (Fig. 1C). Further analysis using anti-RIIα mAb and an antibody against the centrosome marker, AKAP450 (Witczak et al., 1999), indicated that RIIα(T54E) labeling was restricted to the centrosome area at mitosis (data not shown; Carlson et al., 2001). Similar observations were made in interphase and mitotic mouse A9 fibroblasts but rat RIIα did not appear to be released from centrosomes of rat peritubular cells at mitosis (data not shown; Carlson et al., 2001).

The differential distribution of human RIIα and RIIα(T54E) at mitosis was confirmed by immunoblotting analysis of fractionated cells. Mitotic Reh-RIIα and Reh-RIIα(T54E) cells were subjected to Dounce homogenization and centrifuged at 15,000 ***g***. The 15,000 ***g*** pellet (P15) was extracted with 1% Triton X-100, sedimented, suspended, digested with MNase and sedimented again to produce a soluble chromatin fraction and a particulate fraction. The 15,000 ***g*** supernatant (S15) was fractionated at 200,000 ***g*** into soluble (S200) and particulate (P200; membrane-enriched) fractions. In agreement with immunofluorescence data, AKAP95 was detected in all fractions, except P200, of both cell types, albeit primarily in the MNase-soluble chromatin fraction (Fig. 2A, MNase[S]). Both RIIα and RIIα(T54E) were detected in particulate (P15) and soluble (S200) fractions. However, most of the particulate RIIα co-fractionated with MNase-soluble chromatin, whereas essentially all particulate RIIα(T54E) was found in MNase-

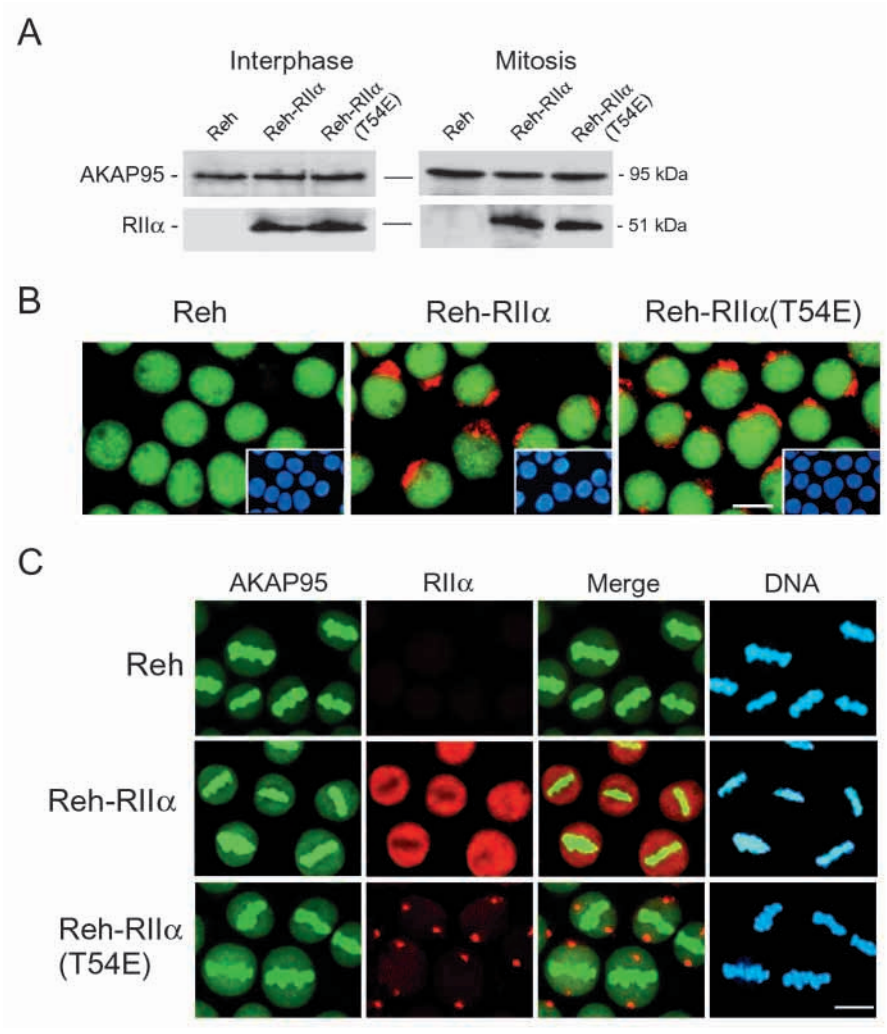

Fig. 1. Subcellular distribution of AKAP95 and RIIα in Reh, Reh-RIIα and Reh-RIIα(T54E) cells. (A) Assessment of AKAP95 and RIIα in Reh, Reh-RIIα and Reh-RIIα(T54E) cells. Lysates from interphase and mitotic cells (10^6 cells per lane) were analyzed by immunoblotting using mAbs against human AKAP95 (mAb47) and human RIIα. (B,C) Immunofluorescence analysis of interphase (B) and mitotic (C) cells using affinity-purified anti-AKAP95 polyclonal antibodies (green) and anti-RIIα mAbs (red). DNA was counterstained with Hoechst 33342 (blue). Bars, 10 μm.

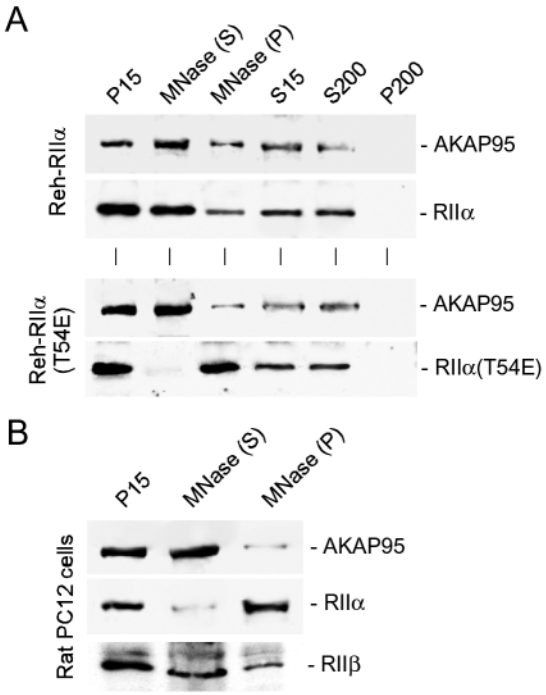

Fig. 2. Wild-type human RIIα, but not RIIα(T54E), co-fractionates with chromatin at mitosis. (A) Mitotic Reh-RIIα cells (upper panels) and mitotic Reh-RIIα(T54E) cells (lower panels) were homogenized and lysates centrifuged at 15,000 ***g***. The sedimented material (P15) was extracted with 1% Triton X-100 and the Triton-X-100-insoluble material was treated with MNase and sedimented to produce MNase-soluble (MNase (S)) and MNase-insoluble (MNase (P)) fractions. The 15,000 ***g*** supernatants from the first centrifugation (S15) were fractionated at 200,000 ***g*** into soluble (S200) and particulate (P200) fractions. Fractions were immunoblotted using anti-AKAP95 and anti-RIIα mAbs. (B) Mitotic rat PC12 cells were fractionated into P15, MNase-soluble (S) and MNase-insoluble (P) fractions, and each fraction was immunoblotted as in (A) and using anti-RIIβ mAbs.

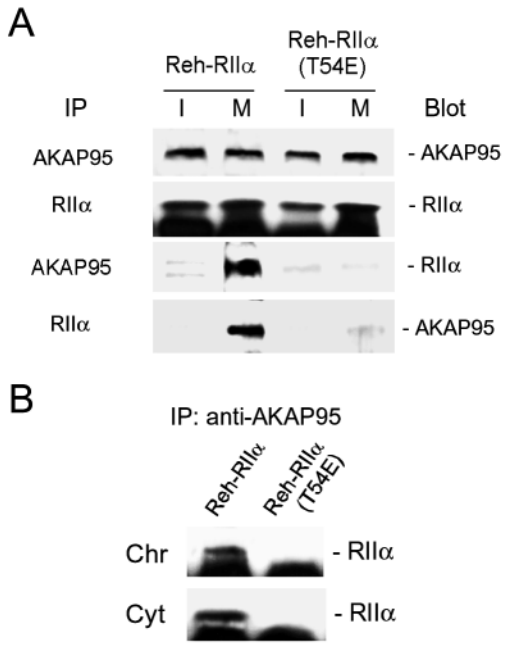

Fig. 3. Association of AKAP95 and RIIα at mitosis. (A) AKAP95 and RIIα were immunoprecipitated (IP) from interphase (I) or metaphase (M) lysates of Reh-RIIα and Reh-RIIα(T54E) cells, and immunoprecipitates were immunoblotted using anti-AKAP95 and anti-RIIα mAbs (Blot). (B) MNase-soluble chromatin (Chr) and a cytosolic fraction (Cyt) were prepared from mitotic Reh-RIIα or Reh-RIIα(T54E) cells. AKAP95 was immunoprecipitated and the precipitates immunoblotted using anti-RIIα mAbs.

insoluble sediments (Fig. 2A, MNase(P)). Fractionation of mitotic rat PC12 neuronal cells corroborated our immunofluorescence observations. Whereas both AKAP95 and RIIα were detected in a P15 fraction, rat RIIα did not co-fractionate with AKAP95 in the MNase-soluble mitotic chromatin fraction but remained in the MNase particulate fraction (Fig. 2B). RIIβ did partly co-fractionate with the MNase-soluble chromatin fraction (Fig. 2B).

Collectively, these results indicate that human RIIα and RIIα(T54E) fractionate differently at mitosis. RIIα primarily co-fractionates with MNase-soluble chromatin and with AKAP95, whereas most RIIα(T54E) remains in the centrosome area. Moreover, similarly to AKAP95, both RIIα and RIIα(T54E) also exist in a minor soluble form at mitosis. Rat RIIα behaves like the human RIIα(T54E) mutant in that it does not co-fractionate with chromatin-bound AKAP95 at mitosis.

RIIα, but not RIIα(T54E), interacts with chromatin-associated AKAP95 at mitosis

We have previously reported the interaction of RIIα with AKAP95 in a mitotic HeLa cell chromatin fraction (Collas et al., 1999). Immunoprecipitations of AKAP95 or RIIα from interphase and mitotic Reh-RIIα or Reh-RIIα(T54E) cells showed that RIIα and AKAP95 co-precipitated only at mitosis (Fig. 3A). By contrast, RIIα(T54E) and AKAP95 did not co-precipitate. Immunoprecipitations performed with fractionated cells revealed that AKAP95 and RIIα co-precipitated from MNase-soluble mitotic chromatin and from cytosol (Fig. 3B). AKAP95 and RIIα(T54E) did not co-precipitate from either fraction (Fig. 3B), despite the presence of both proteins in the cytosol of Reh-RIIα(T54E) cells (Fig. 2). Therefore, only wild-type RIIα is capable of interacting with AKAP95 in a mitotic chromatin fraction.

Association of human RIIα with AKAP95 on chromatin in vivo and in vitro correlates with T54 phosphorylation of RIIα

Mitotic association of human RIIα, but not of RIIα(T54E), with AKAP95 suggests that the T54 residue of RIIα is critical for this interaction. CDK1 phosphorylates RIIα on T54 at mitosis (Keryer et al., 1998), so it is possible that T54 phosphorylation of RIIα is involved in the AKAP95-RIIα interaction. To test this hypothesis, we first assessed threonine phosphorylation of RIIα during interphase and mitosis by immunoblotting anti-RIIα immune precipitates using anti-pT antibodies. Fig. 4A indicates that, whereas RIIα was threonine phosphorylated at mitosis, RIIα(T54E) remained unphosphorylated. No threonine phosphorylation of AKAP95 was detected on a blot of interphase or mitotic anti-AKAP95 immune precipitates (Fig. 4A). Reprobing the anti-RIIα and anti-AKAP95 immunoblots with anti-pS antibodies indicated that, whereas RIIα (wild-type and mutant) was serine

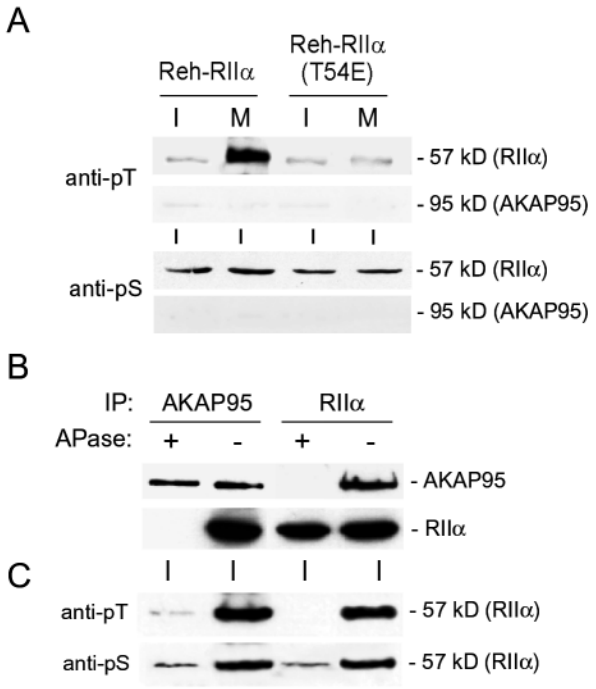

Fig. 4. Alkaline phosphatase disrupts the mitotic AKAP95-RIIα interaction. (A) Anti-RIIα (first panel) and anti-AKAP95 immunoprecipitates (second panel) from interphase (I) and mitotic (M) Reh-RIIα and Reh-RIIα(T54E) cells were immunoblotted using anti-pT and anti-pS antibodies. (B) Soluble chromatin was prepared from mitotic Reh-RIIα cells and treated with 100 U ml^{-1} APase (APase+) or 100 U ml^{-1} APase plus 20 mM sodium vanadate (APase–). AKAP95 or RIIα were immunoprecipitated (IP) and immune precipitates immunoblotted using anti-AKAP95 and anti-RIIα mAbs. (C) Duplicates of blots were immunoblotted using anti-pT and anti-pS antibodies.

phosphorylated during both interphase and mitosis, AKAP95 was not (Fig. 4A, anti-pS). These data confirm the interphase and mitotic S99 autophosphorylation of RIIα (and RIIα[T54E]) previously reported (Erlichman et al., 1983). They also imply that AKAP95 is neither serine nor threonine phosphorylated during the cell cycle.

We next determined whether protein dephosphorylation affected the AKAP95-RIIα interaction. Mitotic Reh-RIIα chromatin was solubilized with MNase and incubated with 100 U ml^{-1} of the serine-threonine phosphatase, APase or, as a control, APase plus 20 mM of the phosphatase inhibitor sodium vanadate. The effect of APase on AKAP95-RIIα interaction was assessed by immunoprecipitating AKAP95 and RIIα, and blotting the precipitates with both antibodies. With sodium vanadate, RIIα and AKAP95 co-precipitated with either antibody (Fig. 4B, APase–), confirming the AKAP95-RIIα interaction reported in Fig. 3B. However, APase treatment of the chromatin clearly disrupted this association as the proteins did not co-precipitate (Fig. 4B, APase+). Probing duplicate anti-AKAP95 and anti-RIIα blots with anti-pT and anti-pS antibodies, indicated that APase effectively threonine dephosphorylated RIIα and, to a lesser extent, also serine dephosphorylated RIIα and RIIα(T54E) (Fig. 4C). As AKAP95 is neither threonine nor serine phosphorylated, the release of RIIα from AKAP95 by APase correlates with the dephosphorylation of RIIα. Moreover, as only RIIα, and not RIIα(T54E), is threonine phosphorylated at mitosis, we argue that T54 phosphorylation of RIIα is essential for mitotic association of RIIα with AKAP95. This contention is supported by the lack of association of AKAP95 with rat RIIα, which lack a CDK1 phosphorylation site corresponding to T54 of human RIIα.

The dynamics of the chromatin-associated human AKAP95-RIIα complex was examined using a cell-free extract that mimics mitotic nuclear disassembly. Purified interphase Reh nuclei (devoid of RIIα) were incubated in mitotic extracts

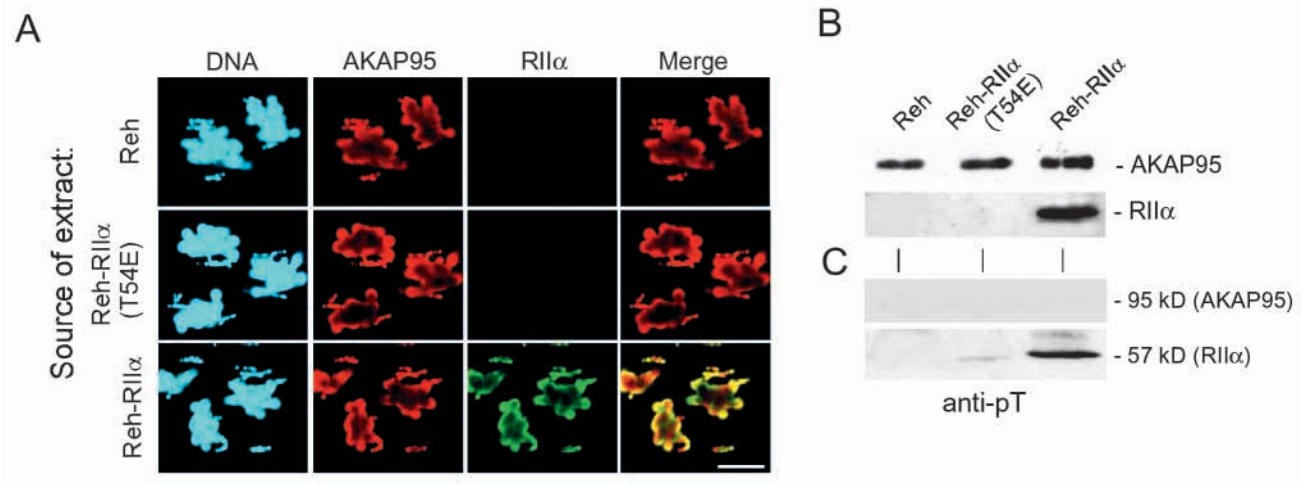

Fig. 5. Recruitment of RIIα onto Reh chromatin in mitotic extract derived from Reh-RIIα, but not Reh-RIIα(T54E), cells. Purified Reh nuclei were incubated for 2 hours in extract derived from mitotic Reh, Reh-RIIα(T54E) or Reh-RIIα cells to elicit chromatin condensation. (A) Localization of AKAP95 (red) and RIIα (green) was assessed by immunofluorescence analysis of condensed chromatin. Bar, 10 μm. (B) Condensed chromatin masses were recovered by sedimentation through sucrose and proteins immunoblotted using anti-AKAP95 and anti-RIIα mAbs. (C) AKAP95 and RIIα were immunoprecipitated from each condensed chromatin fraction after solubilization with MNase, and immune precipitates were immunoblotted using anti-pT antibodies.

derived from Reh, Reh-RIIα or Reh-RIIα(T54E) cells. Within 2 hours, the chromatin condensed into compact masses as judged by DNA staining (Fig. 5A). As expected from in vivo data, AKAP95 was detected on chromatin by immunofluorescence in all extracts (Fig. 5A). RIIα, absent in Reh extract, was detected on chromatin condensed in Reh-RIIα extract and co-localized with AKAP95 (Fig. 5A). However, no RIIα labeling occurred on chromatin condensed in Reh-RIIα(T54E) extract. The location of wild-type RIIα on chromatin was verified by immunoblotting analysis of purified chromatin fractions (Fig. 5B). Moreover, AKAP95 and RIIα co-immunoprecipitated from condensed chromatin fractions solubilized with MNase, suggesting that, as in vivo, AKAP95 and RIIα were associated (data not shown). Finally, when threonine phosphorylation of chromatin-bound AKAP95 and RIIα was examined, we found that as in vivo, AKAP95 was not phosphorylated, whereas RIIα was threonine phosphorylated (Fig. 5C). As RIIα(T54E) was not threonine phosphorylated, this implies that RIIα was threonine phosphorylated solely on T54 in the mitotic extract. Collectively, these results indicate that in vitro, RIIα is recruited on chromatin where it interacts with AKAP95, whereas RIIα(T54E) remains soluble. As in vivo, RIIα recruitment to chromatin correlates with T54 phosphorylation.

Dissociating RIIα from chromatin-bound AKAP95 induces premature chromosome decondensation

To address the significance of the AKAP95-RIIα association at mitosis, we used an assay developed in our laboratory for the decondensation of mitotic chromosomes in a mitotic extract

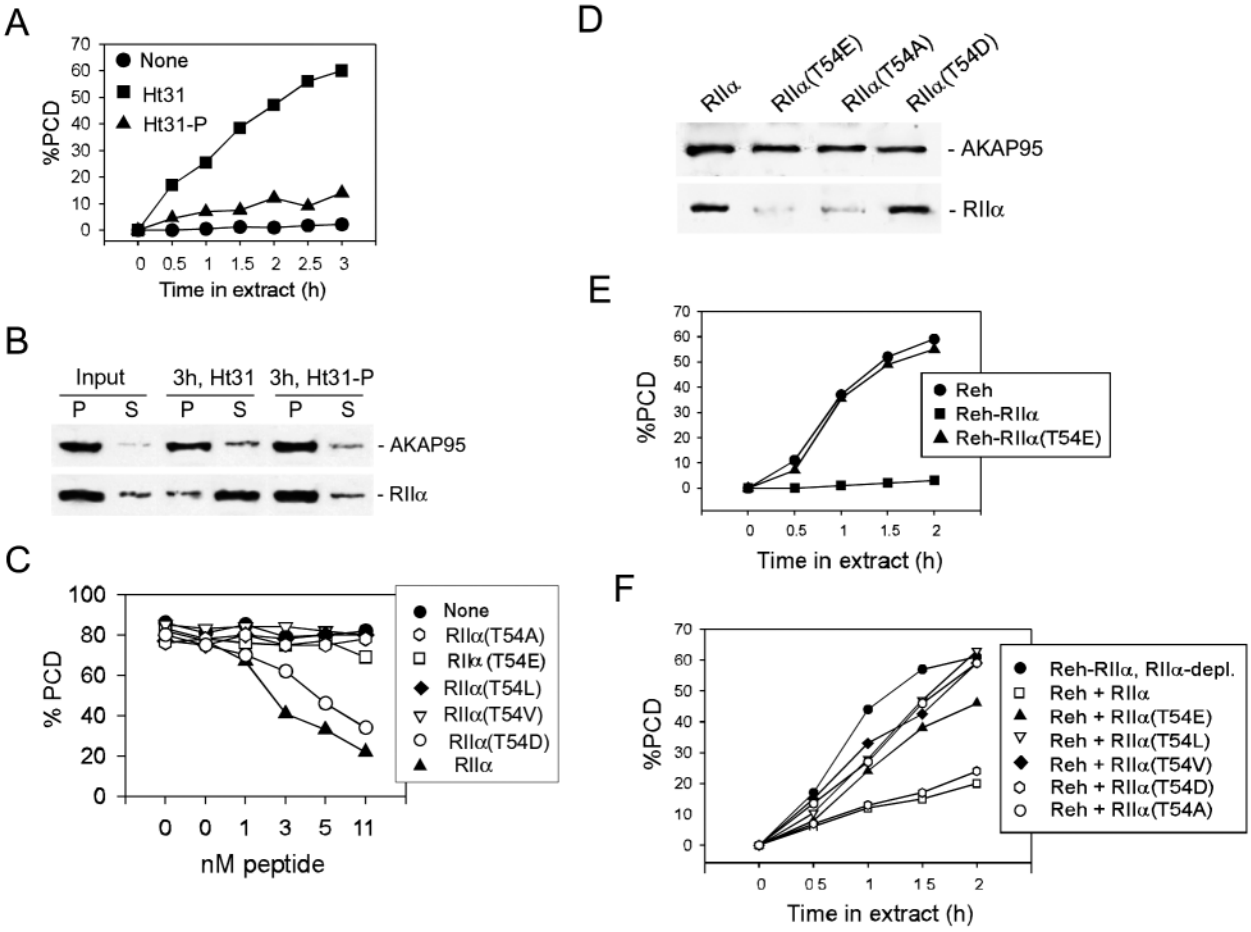

Fig. 6. Disruption of AKAP-RIIα anchoring induces reversible premature chromatin decondensation. (A) Reh-RIIα chromatin condensed in mitotic Reh-RIIα extract was recovered and incubated in fresh mitotic Reh-RIIα extract containing 500 nM Ht31, 500 nM control Ht31-P peptide or no peptide. Percentage of PCD was monitored over 3 hours after DNA staining. (B) Chromatin (P) and reaction supernatants (S) before (Input) and after a 3-hour incubation in extract containing Ht31 or Ht31-P as in (A) were analyzed by immunoblotting using anti-AKAP95 and anti-RIIα mAbs. (C) Prematurely decondensed Reh-RIIα chromatin was recovered from Ht31-containing extract and resuspended in fresh mitotic Reh extract containing no peptide or increasing concentrations of recombinant wild-type RIIα or indicated mutants. Proportions of PCD were monitored after 1.5 hours by DNA staining. (D) Chromatin masses obtained at the end of incubation with wild-type RIIα or RIIα mutants were isolated and immunoblotted using anti-AKAP95 and anti-RIIα antibodies. (E) Reh nuclei were condensed for 2 hours in mitotic Reh extract. Condensed chromatin was recovered and incubated (*T*=0 hours) in mitotic extract of Reh, Reh-RIIα or Reh-RIIα(T54E) cells. The percentage of PCD was evaluated over 2 hours after DNA staining. (F) Reh nuclei were condensed for 2 hours in mitotic Reh extract as in (E). Condensed chromatin was recovered and incubated (*T*=0 hours) in Reh-RIIα mitotic extract immunodepleted for RIIα, or in Reh extract containing 11 nM of either wild-type RIIα or RIIα(T54E). The percentage of PCD was evaluated over 2 hours after DNA staining.

(Collas et al., 1999). This assay, referred to as premature chromatin decondensation (PCD) assay, relies on the dissociation of RIIα from AKAP95 in a mitotic extract, elicited by incubation with the AKAP-RII anchoring the competitor peptide Ht31 (Carr et al., 1991). Using the PCD assay, the effect of disrupting AKAP95-RIIα anchoring on chromatin morphology in vitro was investigated. Purified Reh-RIIα nuclei were condensed in mitotic Reh-RIIα extract. Chromatin masses were recovered, incubated in fresh mitotic Reh-RIIα extract containing 500 nM Ht31, 500 nM of non-competing peptide Ht31-P or no peptide. In the extract, Ht31 elicited PCD of 60% of chromatin masses within 3 hours, whereas Ht31-P was ineffective (Fig. 6A). Immunoblotting analysis of chromatin (P) and reaction supernatants (S) after 3 hours of incubation with Ht31 or Ht31-P showed that, whereas AKAP95 remained associated with chromatin, most RIIα was displaced and solubilized with Ht31, but not by Ht31-P (Fig. 6B). Thus, PCD correlates with the dissociation of RIIα from chromatin-bound AKAP95 rather than from the redistribution of AKAP95.

The reversibility of Ht31-induced PCD was subsequently analyzed. Prematurely decondensed Reh-RIIα chromatin obtained in Ht31-containing extracts was recovered and incubated in fresh mitotic Reh extract (devoid of RIIα). Wild-type RIIα or RIIαT54 mutants were added to the extract and chromatin morphology examined after 1.5 hours. Fig. 6C shows that chromosome condensation was restored by wild-type RIIα in a concentration-dependent manner, and was associated with association of RIIα with chromatin (Fig. 6D). RIIα(T54A, E, L or V) mutants were incapable of rescuing condensation (Fig. 6C). Nevertheless, the RIIα(T54D) mutant, which mimics constitutive phosphorylation of T54, was found partially to restore chromosome condensation and to bind chromatin (Fig. 6C,D). These results indicate that (1) dissociation of RIIα from AKAP95 with Ht31 elicits PCD, (2) AKAP95 remains bound to chromatin during PCD and (3) wild-type RIIα or the RIIα(T54D) mutant can restore condensation. We concluded from these experiments that the AKAP95-RIIα interaction is essential to maintain chromatin in a condensed form in mitotic extract.

The PCD assay was subsequently used to determine whether the T54E mutation of RIIα affected chromatin structure in mitotic extract. Reh chromatin was condensed in mitotic Reh extract (devoid of RIIα), recovered and incubated for another 2 hours in Reh, Reh-RIIα or Reh-RIIα(T54E) mitotic extract. Whereas chromatin remained condensed in Reh-RIIα extract, it underwent PCD in Reh extract and Reh-RIIα(T54E) extract (Fig. 6E). Thus, both absence of RIIα in the extract and the T54E mutation of RIIα correlate with PCD.

A role of wild-type RIIα in preventing PCD was shown by the induction of PCD in Reh-RIIα extract immunodepleted of RIIα (Fig. 6F). PCD was largely inhibited in Reh extract containing 11 nM RIIα, whereas 11 mM of RIIα(T54A, E, L or V) were ineffective (Fig. 6F). By contrast, RIIα(T54D) was capable of inhibiting PCD (Fig. 6F), an observation consistent with our previous data. In any situation, H1 kinase activity remained at mitotic levels, indicating that none of the treatments promoted exit of the extracts from mitosis (data not shown). These results indicate that PKA type II plays an essential role in the regulation of chromatin dynamics during mitosis. The data also suggest that mitotic T54 phosphorylation and subsequent association of RIIα with AKAP95 on chromosomes is critical to maintain chromosomes condensed during mitosis.

Nuclear reassembly correlates with threonine dephosphorylation of RIIα and dissociation of RIIα from AKAP95 and chromosomes

Our results suggest that T54 phosphorylation of RIIα controls AKAP95-RIIα association on chromatin, which in turn affects chromatin dynamics during mitosis. According to this hypothesis, the reformation of nuclei at the end of mitosis should correlate with threonine dephosphorylation of RIIα and/or dissociation of RIIα from its chromatin-bound anchor.

To test this possibility, we used a cell-free system reminiscent of that developed by Burke and Gerace (Burke and Gerace, 1986), which supports nuclear reconstitution, and determined whether the AKAP95-RIIα interaction was altered during nuclear reassembly. Crude lysates of mitotic Reh-RIIα cells were supplemented with an ATP-generating system and incubated at 30°C. Chromosome decondensation and reformation of the nuclear envelope were monitored by DNA staining and by immunofluorescence labeling of lamin B receptor (LBR), a marker of the inner nuclear membrane (Worman et al., 1990). LBR assembles onto chromosomes as early as anaphase (Chaudhary and Courvalin, 1993) and thus constitutes a reliable marker of early nuclear membrane reassembly. Distribution of RIIα was monitored simultaneously by immunofluorescence.

Decondensation of the mitotic chromosomes took place within 75 minutes (Fig. 7A). Remarkably, RIIα staining (green) disappeared as LBR (red) gradually assembled on the chromatin surface. By 30 minutes, most RIIα was removed from chromatin, whereas nuclear membranes had not fully reformed. By 75 minutes, nuclear membranes were reassembled and no RIIα was detected on chromatin. Immunoblotting analysis of soluble and sedimented chromatin fractions before and after nuclear reassembly showed that, whereas AKAP95 remained associated with insoluble material, all detectable RIIα was solubilized (Fig. 7B). Moreover, within 10 minutes of nuclear reassembly, RIIα was fully threonine dephosphorylated, whereas strong threonine phosphorylation was detected in a control incubation in extract for 75 minutes without the ATP-generating system (M, 75 min).

Collectively, these data indicate that the dissociation of human RIIα from chromatin-bound AKAP95 is completed before nuclear membranes reform and does not result from the establishment of a barrier separating AKAP95 and RIIα. Second, RIIα is threonine dephosphorylated prior to, or concomitantly with, its disassembly from chromatin and chromatin decondensation, strongly suggesting that this event is required for RIIα relocalization and for chromatin remodeling at the end of mitosis.

DISCUSSION

Using a human lymphoblastic leukemic cell line stably transfected with wild-type RIIα or an RIIα(T54E) mutant, or that transiently expresses additional RIIαT54 mutants, we report a cell-cycle-dependent interaction between AKAP95 and PKA-type II regulatory subunit mediated by RIIα

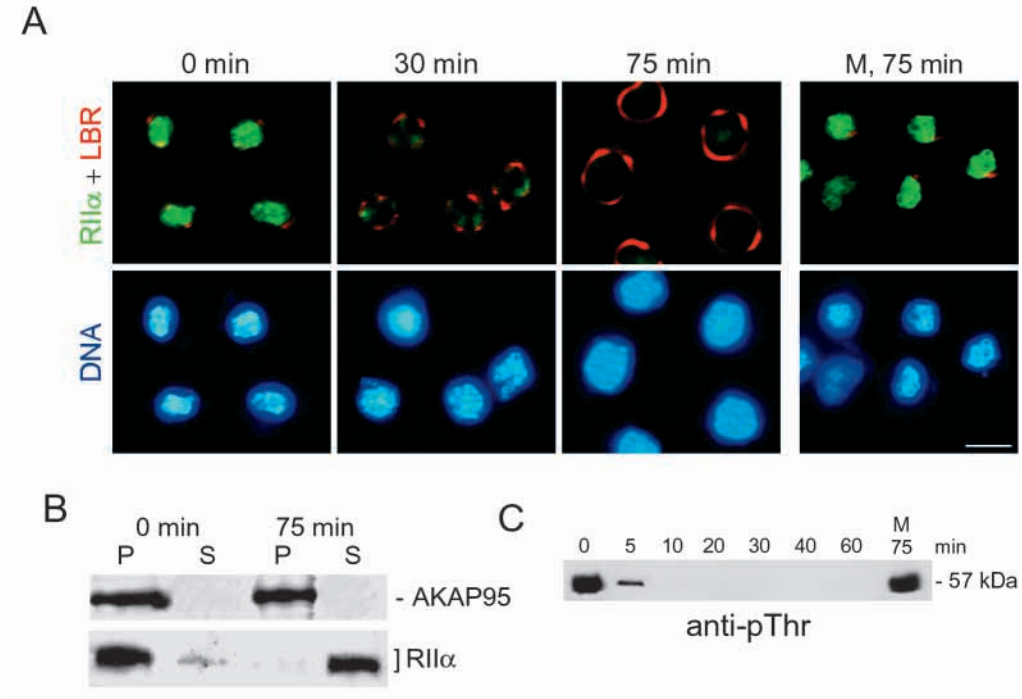

Fig. 7. Nuclear reassembly in vitro is accompanied by dissociation of RIIα from chromatin-bound AKAP95 and threonine dephosphorylation of RIIα. Mitotic Reh-RIIα cells were lysed by Dounce homogenization and nuclei were allowed to reform by incubating the lysate at 30°C. (A) At the indicated time points, solubilization of RIIα and reassembly of nuclear membranes were monitored by immunofluorescence using anti-RIIα mAbs (green) and affinity-purified antibodies against LBR, an integral protein of the inner nuclear membrane (red); merged images are shown (upper panels). DNA was labeled with Hoechst 33342 (lower panels). Bar, 10 μm. (B) Input (0 minutes) and decondensed chromatin fractions (75 minutes) were sedimented and pellets (P) and supernatants (S) immunoblotted using anti-AKAP95 and anti-RIIα mAbs. (C) Entire nuclear reassembly reactions were homogenized at the indicated time points, RIIα was immunoprecipitated and precipitates were immunoblotted using anti-pT antibodies. RIIα was also immunoprecipitated after a 75-minute control incubation of chromatin in mitotic lysate without an ATP-generating system (M 75 min).

phosphorylation, and a functional significance for this interaction. Several lines of evidence indicate that AKAP95-RIIα interaction is modulated by RIIα phosphorylation. (1) Human RIIα is phosphorylated on T54 at mitosis and by the major mitotic kinase CDK1 in vitro (Keryer et al., 1998). (2) RIIα, but not RIIα(T54E), co-fractionates with chromosome-bound AKAP95 in mitotic extract. (3) The T54D mutation (but not T54A, T54E, T54L or T54V), which mimics constitutive T54 phosphorylation of RIIα, enables binding of RIIα to chromosomes and restores recondensation of prematurely decondensed chromosomes in vitro. (4) Phosphatase treatment of mitotic chromatin threonine dephosphorylates RIIα and disrupts the AKAP95-RIIα interaction. (5) Rat RIIα, which lacks a CDK1 phosphorylation site corresponding to T54 of human RIIα, does not associate with AKAP95 at mitosis. (6) Finally, reconstitution of nuclei in vitro is preceded by threonine dephosphorylation of RIIα and dissociation of RIIα from AKAP95.

We propose a model in which threonine phosphorylation of RIIα constitutes a molecular switch controlling the association of RIIα with AKAP95, which in turn affects chromosome dynamics during mitosis (Fig. 8). During interphase, AKAP95 and RIIα are located in distinct compartments separated by the nuclear envelope. At mitosis entry, AKAP95 associates with chromatin (Steen et al., 2000) and RIIα is phosphorylated on T54 by CDK1. This turns on a molecular switch eliciting binding of, presumably, the PKA holoenzyme to AKAP95 via RIIα (switch ON). Anchoring of RIIα to chromatin-bound

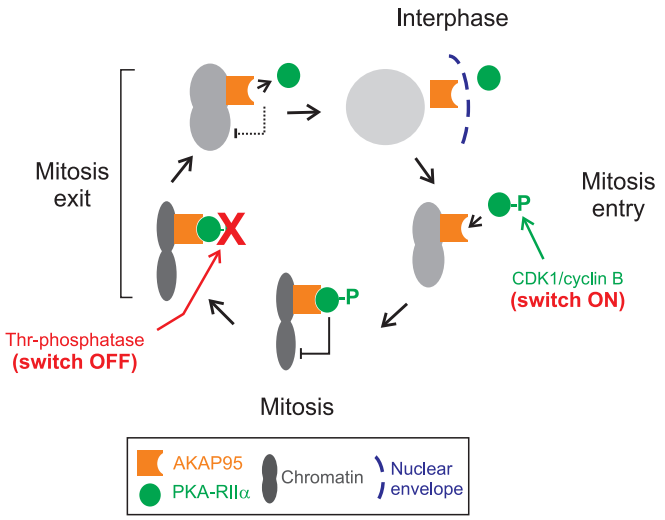

Fig. 8. Model for how reversible AKAP95-PKA interaction (mediated by RIIα phosphorylation) controls chromatin structure during mitosis. During interphase, AKAP95 and PKA-RIIα localize to distinct compartments, separated by the nuclear envelope. At mitosis entry, AKAP95 associates with condensing chromatin. RIIα is phosphorylated by the CDK1-cyclin-B complex. RIIα phosphorylation turns on a molecular switch promoting RIIα anchoring to AKAP95 and maintenance of condensed chromosomes during mitosis. (Although anchoring of RIIα to AKAP95 has been demonstrated, anchoring of the catalytic subunit of PKA is only suggested.) Throughout mitosis, anchoring of phosphorylated RIIα to chromatin-bound AKAP95 is required to prevent PCD. At mitosis exit, dephosphorylation of RIIα by a threonine phosphatase induces RIIα dissociation from chromatin-bound AKAP95. This relieves inhibition of chromatin decondensation, allowing chromosome decondensation as the nuclear envelope reassembles.

AKAP95 is required to prevent premature chromatin decondensation during mitosis. At mitosis exit, dephosphorylation of RIIα by a threonine phosphatase (switch OFF) elicits dissociation of RIIα from AKAP95 before the nuclear envelope reforms. This relieves the inhibition of chromatin decondensation imposed by the RIIα-AKAP95 complex (switch OFF), enabling chromosome decondensation as the nucleus reassembles. In vivo, released RIIα is presumably targeted back to the centrosome-Golgi area, where it is predominantly anchored during interphase (Eide et al., 1998). Moreover, during decondensation, AKAP95 remains associated with chromatin until it is redistributed to the nuclear matrix upon nuclear reformation (P.C., unpublished). Thus, the release of RIIα precedes that of AKAP95, arguing that the AKAP95-RIIα complex is disrupted rather than being detached as a whole from the chromatin.

In contrast to human, bovine or mouse, rat RIIα does not harbor any CDK1 phosphorylation site corresponding to T54 of human RIIα. Because no association of rat RIIα (using a specific antibody) with AKAP95 has been reported, this supports our model of phosphorylation-mediated RIIα-AKAP95 association. However, this might also question the significance of AKAP95-RIIα interaction as a general mechanism for regulating mitotic chromosome condensation. In an attempt to address this issue, we have shown in this paper, on a western blot of MNase-soluble and -insoluble fractions of PC12 cells, that a fraction of RIIβ co-fractionates with chromatin and AKAP95 at mitosis (Carlson et al., 2001). Although RIIα appears to be a preferred ligand over RIIβ for AKAP95 in other species (Hausken et al., 1996; Herberg et al., 2000), association of RII proteins in the rat in vivo (using α- or β-specific antibodies) has not been reported. Thus, we hypothesize that, in the rat, RIIβ might substitute for RIIα to regulate chromosome dynamics at mitosis together with AKAP95.

Modulation of RII-AKAP binding by RII phosphorylation is probably not specific for AKAP95. We show in another paper (Carlson et al., 2001) that the interaction of human or bovine PKA with the centrosomal AKAP450 is disrupted by T54 phosphorylation of RIIα, whereas T54 dephosphorylation at mitosis exit promotes its reassociation. Interestingly, RIIβ phosphorylation also lowers its ability to anchor MAP-2 in neurons (Keryer et al., 1993).

What is the function of the chromatin-associated RII-AKAP95 complex? We have recently shown that AKAP95 is required for both assembly and structural maintenance of mitotic chromosomes (Collas et al., 1999; Steen et al., 2000). Curiously, PKA activity is dispensable for chromosome condensation per se, but is required for maintenance of chromosomes in a condensed form during mitosis. A putative target for PKA is AKAP95 (Eide et al., 1998) but we have not detected any serine or threonine phosphorylation of AKAP95 during interphase or mitosis. Another possibility is that PKA phosphorylates subunits of the condensin complex required for chromosome condensation (Hirano et al., 1997). Additional proteins implicated in chromosome condensation or structural chromosomal proteins could also serve as PKA substrates (Hirano and Mitchison, 1993; Van Hooser et al., 1998). Although we do not formally demonstrate the existence of the PKA catalytic subunit on mitotic chromosomes, our previous (Collas et al., 1999) and present data suggest that PKA activity must be concentrated around chromosomes for maintenance of condensation, as disruption of PKA-AKAP95 anchoring causes premature decondensation of chromosomes.

If the chromatin-bound AKAP95-RIIα complex is important for regulating mitotic chromosome dynamics then how do Reh cells proceed through mitosis? As in the rat, RIIβ might substitute for RIIα in Reh cells (Tasken et al., 1993). Moreover, as most cancer cell lines, the Reh cell line is aneuploid and chromosome segregation defects at mitosis might result, in part, from abnormal chromatin dynamics. Proliferation of Reh cells in the absence of RIIα also suggests that the cell cycle control machinery of these cells might not be entirely functional. Finally, the incidence of PCD of Reh cell chromosomes in vitro suggests that this cell line might be prone to chromosome segregation defects at mitosis.

In summary, we demonstrate a cell-cycle-dependent interaction of RIIα with AKAP95 in human cells. The phosphorylation status of RIIα on T54 appears to regulate this association. It would be of interest to determine whether PKA anchoring to other AKAPs is also regulated by phosphorylation.

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