CDK1-mediated phosphorylation of the RIIα regulatory subunit of PKA works as a molecular switch that promotes dissociation of RIIα from centrosomes at mitosis

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SUMMARY

Protein kinase A regulatory subunit RIIα is tightly bound to centrosomal structures during interphase through interaction with the A-kinase anchoring protein AKAP450, but dissociates and redistributes from centrosomes at mitosis. The cyclin B-p34^{cdc2} kinase (CDK1) has been shown to phosphorylate RIIα on T54 and this has been proposed to alter the subcellular localization of RIIα. We have made stable transfectants from an RIIα-deficient leukemia cell line (Reh) that expresses either wild-type or mutant RIIα (RIIα(T54E)). When expressed, RIIα detaches from centrosomes at mitosis and dissociates from its centrosomal location in purified nucleus-centrosome complexes by incubation with CDK1 in vitro. By contrast, centrosomal RIIα(T54E) is not redistributed at mitosis, remains mostly associated with centrosomes during all phases of the cell cycle and cannot be solubilized by CDK1 in vitro. Furthermore, RIIα is solubilized from particular cell fractions and changes affinity for AKAP450 in the presence of CDK1. D and V mutations of T54 also reduce affinity for the N-terminal RII-binding domain of AKAP450, whereas small neutral residues do not change affinity detected by surface plasmon resonance. In addition, only RIIα(T54E) interacts with AKAP450 in a RIPA-soluble extract from mitotic cells. Finally, microtubule repolymerization from mitotic centrosomes of the RIIα(T54E) transfectant is poorer and occurs at a lower frequency than that of RIIα transfectants. Our results suggest that T54 phosphorylation of RIIα by CDK1 might serve to regulate the centrosomal association of PKA during the cell cycle.

Key words: Centrosome, PKA, AKAP450, Mitosis, CDK1

INTRODUCTION

The cAMP-dependent protein kinase (protein kinase A; PKA) is involved in the regulation of key cellular processes such as gene expression, metabolism and cell growth and division. PKA is a tetrameric enzyme consisting of two catalytic (C) subunits and one regulatory subunit (R) dimer, to which cAMP binds, thereby dissociating and activating the C subunits (Beebe and Corbin, 1986; Francis and Corbin, 1994). The PKA isozymes differ mainly in their R subunit composition and are divided into type I ($RI\alpha_2C_2$ and $RI\beta_2C_2$) and type II ($RII\alpha_2C_2$ and $RII\beta_2C_2$). The regulatory subunits RIα and RIIα are present in almost all cell types, whereas expression of RIβ and RIIβ is cell- and tissue-specific (Scott, 1991; Skålhegg and Taskén, 1997).

Tethering of PKA to subcellular loci via A-kinase anchoring proteins (AKAPs) is important to mediate the effects of cAMP. AKAPs target PKA close to its substrates and in this way contribute specificity in the cAMP-PKA signaling system. Many AKAPs have been identified that are located in different cellular organelles and membranes (Colledge and Scott, 1999). Recently, two new AKAPs have been characterized that target PKA to centrosomes: AKAP350/AKAP450/CGNAP (Schmidt et al., 1999; Takahashi et al., 1999; Witczak et al., 1999) and pericentrin (Diviani et al., 2000). Whereas functions of AKAP450 are under investigation, pericentrin is a highly conserved component of the centrosomal matrix implicated in the organization of the mitotic spindle. Another AKAP (AKAP220) has also been localized to the centrosome area in developing germ cells and sperm (Reinton et al., 2000).

Centrosomes are major microtubule-organizing centers (MTOCs). During S phase, the cell duplicates its centrosome and, as prophase begins, the two daughter centrosomes separate and move to opposite positions in the cell. Each centrosome organizes its own array of microtubules. As the cell enters mitosis, the microtubule dynamics increase, enabling a rapid assembly and disassembly of the mitotic spindle. PKA modifies the microtubule dynamics and organization (Lamb et

al., 1991), and it is anticipated that the centrosomal and microtubular localization of PKA are implicated in these functions. It has been shown that PKA switches off the effects of stathmin (Gradin et al., 1998), a centrosome- and microtubule-associated phosphoprotein involved in the regulation of microtubule dynamics.

Both RIIα and RIIβ are found in the pericentriolar matrix of the centrosome during interphase (Keryer et al., 1999). At the onset of mitosis, the mitotic kinase CDK1 is associated with centrosomes (Bailly et al., 1989; Bailly et al., 1992) and RIIα is phosphorylated by CDK1 on T54 and concomitantly dissociates from its centrosomal anchor (Keryer et al., 1998). Although RIIβ contains a CDK1 phosphorylation site (T69; Keryer et al., 1993), it does not detach from the centrosome at mitosis. Together with the observation that normal differentiated cells and cancer cells have centrosomal RIIβ (whereas normal dividing cells only express RIIα), cell cycle-dependent redistribution of RIIα is interesting (Keryer et al., 1999). To study the mechanisms of redistribution of RIIα and the functional implications of the detachment of RIIα from centrosomes at mitosis, we made cell lines stably expressing wild-type and mutated RIIα(T54E) on an RIIα-deficient background (Reh cells; Taskén et al., 1993). Mutated RIIα(T54E) was not phosphorylated by CDK1 and was retained at the mitotic centrosomes of the transfectants. CDK1 phosphorylation of wild-type RIIα lowered the affinity for AKAP450 in vitro and dissociated RIIα from purified centrosomes. This suggests that CDK1 phosphorylation serves as a molecular switch that regulates RIIα association with centrosomal AKAPs.

MATERIALS AND METHODS

Cell growth and synchronization

Reh cells were grown at 37°C in RPMI medium (Gibco BRL) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin, streptomycin, 1mM sodium pyruvate and nonessential amino acids. Reh-RIIα(T54E) cells (see below) were arrested in mitosis with 10 μM nocodazole for 20 hours. Reh-RIIα cells (see below) were accumulated by thymidine block (2.5 mM) for 20 hours and released for 9 hours before a second thymidine block and release for 9 hours. Then the cells were incubated with 1 μM nocodazole for 4 hours. At least 50-60% of the cells were in mitosis without apoptosis after this treatment. The mouse A9 fibroblastic cell line was grown in α-Eagle's minimum essential medium (MEM) supplemented with antibiotic/antimycotic, 0.1% tylosine tartrate, 40 mM β-mercaptoethanol, 2 mM L-glutamine and 10% fetal calf serum at 37°C. Primary cultures of fibroblast-like peritubular cells were prepared from rat testes and cultured in MEM with the addition of antibiotic/antimycotic, L-glutamine (2 mM) and 10% fetal calf serum at 32°C in a humidified atmosphere with 5% CO_2.

Transfection and GFP constructs

Wild-type RIIα was mutated (Thr54 to Glu) as previously described (Keryer et al., 1998). The wild-type and mutated cDNAs were cloned into the expression vector pMEP4 (Invitrogen) as a *Kpn*I/*Bam*HI fragment and placed under the control of the human metallothionein IIa promotor. Reh cells (which are RIIα deficient) were electroporated (320 V, 960 μF) with 15 μg of linearized constructs. Stably transfected cells were selected with hygromycin B (250 μg ml^{-1}). RIIα and RIIα(T54E) with *Sac*II/*Xho*I ends were amplified using the above constructs as templates and subcloned into pEGFP-N1 to yield constructs directing expression of RIIα with green fluorescent protein (GFP) fused to the C terminus. Additional mutants, RIIα(T54L)-GFP and RIIα(T54V)-GFP were made by the mutation of Thr54 to Leu or Val, respectively. Reh cells (20×10^6) were transfected with 20 μg of DNA by electroporation (320 V, 960 μF), incubated for 24-48 hours and analysed for GFP fluorescence.

Antibodies and recombinant proteins

A mouse monoclonal antibody against human RIIα (developed by K. Taskén in collaboration with Transduction Laboratories) was used at 1 μg μl^{-1} for western blotting and 2.5 μg μl^{-1} for immunoprecipitation and immunofluorescence. In some western blotting experiments, a polyclonal antiserum against human RIIα (Keryer et al., 1999) was used at 1:500 dilution. An affinity-purified polyclonal antibody raised against AKAP 450 (a gift from W. A. Kemmner (Max Planck Institute for Development Biology, Tuebingen, Germany)) was used at 25 μg μl^{-1} for immunofluorescence and 0.1 μg μl^{-1} for immunoprecipitation. In the microtubule repolymerization experiments, we used an affinity-purified anti-RIIα polyclonal antibody (4 μg μl^{-1}) and a monoclonal anti-α-tubulin at 0.1 μg μl^{-1} (Sigma, T-9026). A mouse monoclonal antibody against human RIIβ (Transduction Laboratories) was used at a 1 μg μl^{-1} dilution for western blotting and immunofluorescence. HRP-conjugated anti-mouse IgGs (1:5000 dilution, Transduction Laboratories) and anti-mouse or anti-rabbit IgGs (1:10,000 dilution, Jackson Immunoresearch) were used as secondary antibodies. Recombinant human RIIα wild-type was expressed as a glutathione-*S*-transferase (GST) fusion protein in the *Escherichia coli* strain BL21, purified and cleaved as described (Keryer et al., 1998). Two putative RII-binding domains in AKAP450 were expressed as fusion proteins referred to as GST-AKAP450 (amino acids 1390-1595) and GST-AKAP450 (amino acids 2327-2602). GST-AKAP79 (amino acids 178-427) and GST-AKAP149 (amino acids 285-387) were expressed as previously described (Herberg et al., 2000). For the surface plasmon resonance (SPR) experiments, wild-type and mutated RIIα (Thr54 to Ala (T54A), Asp (T54D), Leu (T54L) or Val (T54V)) were cloned into pRSET, expressed in the *E. coli* strain BL21 and purified by using cAMP coated beads as described (Herberg et al., 2000).

Immunofluorescence

Immunofluorescence analysis of cells or nucleus-centrosome complexes was done as previously described (Collas et al., 1996). TRITC- or FITC-conjugated secondary antibodies were used at 1:100 dilution and DNA was stained with 0.1 μg ml^{-1} Hoechst 33342. Observations were made and photographs were taken as previously described (Collas et al., 1999).

Immunoprecipitation and phosphorylation

Whole cells were sonicated and extracted in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) and the lysate centrifuged at 13,000*g*. The supernatant was precleared with protein A/G agarose (1:25 dilution) and RIIα was immunoprecipitated using anti-RIIα mAb (2.5 μg μl^{-1}). To phosphorylate RIIα, the beads containing the immune complexes were prewashed in EBS phosphorylation buffer (80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 100 mM sucrose, 1 mM dithiothreitol, pH 7.2) before incubation with 100 μM ATP and purified starfish oocyte CDK1 (mitotic kinase) (Labbé et al., 1989) (9 pmol $minute^{-1}$ μl^{-1}) for 45 minutes at 22°C.

Electrophoresis and immunoblotting

Proteins were separated by 7.5% or 10% SDS-PAGE containing 16% glycerol in the separating gel or 4.5% PAGE containing 2 M urea and transferred by electroblotting to PVDF membranes. The filters were blocked in 5% non-fat dry milk in PBS for 1 hour, incubated overnight at 4°C with primary antibodies, washed for 1 hour in PBS with 0.1% Tween-20 and incubated with a horseradish-peroxidase-conjugated secondary antibody. Blots were developed by enhanced chemiluminescence (Amersham). For RII overlay, filters were

blocked, incubated with [^{32}P]-RIIα and washed in blotto/BSA (0.1% BSA, 0.02% Na-azide, 0.05% Tween-20, 5% nonfat dried milk in PBS) solution as described previously (Bregman et al., 1989).

CDK1 phosphorylation of RIIα in Triton-X-100-insoluble fractions and purified nucleus-centrosome complexes

Interphase cells (20×10^6) were washed in PHEM buffer (45 mM Pipes, 45 mM Hepes, 10 mM EGTA, 5 mM $MgCl_2$, pH 6.9) containing anti-proteases (10 μg ml^{-1} each of antipain, chymostatin, leupeptin and pepstatin A) and phosphatase inhibitors (1 μM okadaic acid and 0.1 mM sodium orthovanadate). The cells were pelleted (400*g*) and then extracted in the same buffer containing 0.5% Triton X-100; Triton-X-100-soluble and -insoluble fractions were separated by centrifugation. The Triton-X-100-insoluble fraction was sonicated twice for 10 seconds each, washed twice in EBS phosphorylation buffer (described above) before incubation for 30 minutes at 22°C with 100 μM ATP and CDK1 (30 pmol minute^{-1} μl^{-1}). The pellet and supernatant were subsequently separated by centrifugation and both fractions were boiled in Laemmli buffer and analyzed for the amount of RII by western blotting. Nucleus-centrosome complexes were purified according to Maro and Bornens (Maro and Bornens, 1980) from cells by resuspension in cold buffer containing 0.25 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 mM PMSF, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.4, with protease inhibitors. NP-40 was added to a final concentration of 0.5% and the cells were disrupted by vortexing for 15 seconds. Nucleus-centrosome complexes were pelleted at 200*g* for 10 minutes and washed in PHEM buffer containing 0.5% Triton X-100. After pelleting, the complexes were resuspended in EBS phosphorylation buffer and incubated in presence or absence of CDK1 and 100 μM ATP.

RII overlays

Purified recombinant human RIIα and purified bovine RIIα were radiolabeled by purified CDK1 or catalytic subunit (C) of PKA and [γ-^{32}P] ATP. RIIα (1.5 μg) was incubated with CDK1 (24 pmol minute^{-1} μl^{-1}) in EBS buffer (described above) containing 0.7 μCi μl^{-1} of [γ-^{32}P] ATP for 1 hour at 22°C. Phosphorylation by PKA was done using 0.7 μCi μl^{-1} of [γ-^{32}P] ATP and 24 pmol minute^{-1} μl^{-1} of active C for 1 hour at 0°C. PKA- and CDK1-labeled RIIα (6 nM final concentration each; 4.9×10^5 cpm μg^{-1} and 2.4×10^4 cpm μg^{-1}, respectively) was purified by gel filtration (G-25 sepharose) and used in a modified western blot protocol as previously described (Bregman et al., 1989).

GST precipitation

R subunit (50 ng) was incubated in 20 μl EBS phosphorylation buffer (described above) containing 100 μM ATP for 1 hour at 22°C in presence or absence of purified CDK1 (10 pmol minute^{-1} μl^{-1}). The R subunit was then diluted to 20 nM, mixed with 20 nM of different GST-AKAP fragments in a buffer containing 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF, 0.1% Triton X-100, 20 mM EDTA (to inactivate CDK1), 5 mM benzamidine, protease inhibitors and incubated at room temperature for 30 minutes with rotation. Subsequently, 25 μl of glutathione-agarose beads were added and incubation continued further for 2 hours at 4°C with rotation, after which beads were pelleted by centrifugation at 1000*g* for 5 minutes and washed three times in 300 μl of the same buffer. Precipitates were eluted by boiling in SDS-sample buffer, subjected to SDS-PAGE and immunodetection of RIIα.

Surface plasmon resonance (SPR)

Recombinant RIIα was purified over a 8-amino-hexyl-amino-cAMP resin (Biolog, Bremen) and quality tested as described before (Herberg et al., 2000). To obtain cAMP-free RIIα, RIIα was unfolded with 6 M urea and refolded in buffer A (150 mM NaCl, 20 mM MOPS, pH 7.0, 0.005% surfactant P20 (Biacore AB)) (Buechler et al., 1993). Studies on the interaction between the RIIα of PKA and AKAP protein were performed by SPR spectroscopy using a Biacore 2000 instrument (Biacore AB, Sweden) and a CM5 chip coated with 8-AHA cAMP as described before (Herberg et al., 2000). For review of the SPR technique, see Szabo et al. (Szabo et al., 1995). The surface concentration of wild-type and mutant RIIα proteins was adjusted to 120 or 500 RU for analysis of interaction with GST-AKAP450 (1390-1595) and GST-AKAP450 (2327-2602) (fusions of GST with the two putative RII-binding domains or AKAP450 (Schmidt et al., 1999, Witczak et al., 1999)). A response (i.e. a change in the resonance signal) of 1000 Relative Units (RU) corresponds to a change in surface concentration on the sensor chip of about 1 (ng protein) mm^{-2} (Stenberg and Nygren, 1991).

Microtubule repolymerization assay

Cells were settled onto freshly L-lysin-coated coverslips for 30 minutes at 25°C. Microtubules were depolymerized at 4°C for 1 hour in medium. The polymerization was initiated by incubating the cells in RPMI medium at 37°C. Microtubule regrowth was stopped after 0-15 minutes by fixing with ice-cold 100% methanol. The cells were prepared for immunofluorescence as described above, except that a cytoskeleton stabilization buffer (PHEM buffer) was used instead of PBS.

Statistical analysis

For comparison of two groups, the Mann-Whitney *U* test was used. Statistical analysis was performed using Statistica (Statsoft, Tulsa, OK). *P* values are two-sided and are considered significant when <0.05.

RESULTS

Subcellular localization of RIIα in Reh-RIIα and Reh-RIIα(T54E) cells

Stably transfected cell lines expressing wild-type RIIα or RIIα(T54E) were generated. RIIα was detected in the cell lines transfected with wild-type RIIα (Reh-RIIα) (Fig. 1A, lane 3) and mutated RIIα (Reh-RIIα (T54E), lane 4). Both cell lines expressed similar levels of RIIα (51 kDa). RIIα was not detected in the untransfected Reh cells (lane 1) or in Reh transfected with vector (Reh-pMEP4) (lane 2). Next, RIIα immunoprecipitates from different cell lines were subjected to CDK1 phosphorylation in the presence of ATP. Only RIIα immunoprecipitated from Reh-RIIα cells was phosphorylated and migrated with apparent mobility of 53 kDa (Fig. 1B, lower panel, lane 2), although both Reh-RIIα and Reh-RIIα(T54E) cells contained RIIα (upper panel). Consistent with our earlier observations (Keryer et al., 1998), this indicates that the human RIIα sequence (AAT^{54}PRQSL) contains a CDK1 phosphorylation site at T54.

The subcellular localization of RIIα in the different cell lines was determined by double immunofluorescence using anti-RIIα monoclonal antibody (mAb) and an affinity-purified polyclonal antibody raised against AKAP450 to detect centrosomes (Fig. 1C-E). RIIα was detected in the Golgi/centrosomal region in interphase Reh-RIIα and Reh-RIIα(T54E) cells but was absent from untransfected Reh cells. In mitotic Reh-RIIα cells, RIIα was dissociated from centrosomes and redistributed to the cytoplasm and chromatin. Variable amount of RIIα was colocalized with chromatin in mitotic RIIα-transfected Reh cells, whereas association was observed to be stronger in other cell types (Landsverk et al., 2001; Collas et al., 1999). By contrast, mutant RIIα remained associated within centrosomes in mitotic Reh-RIIα(T54E)

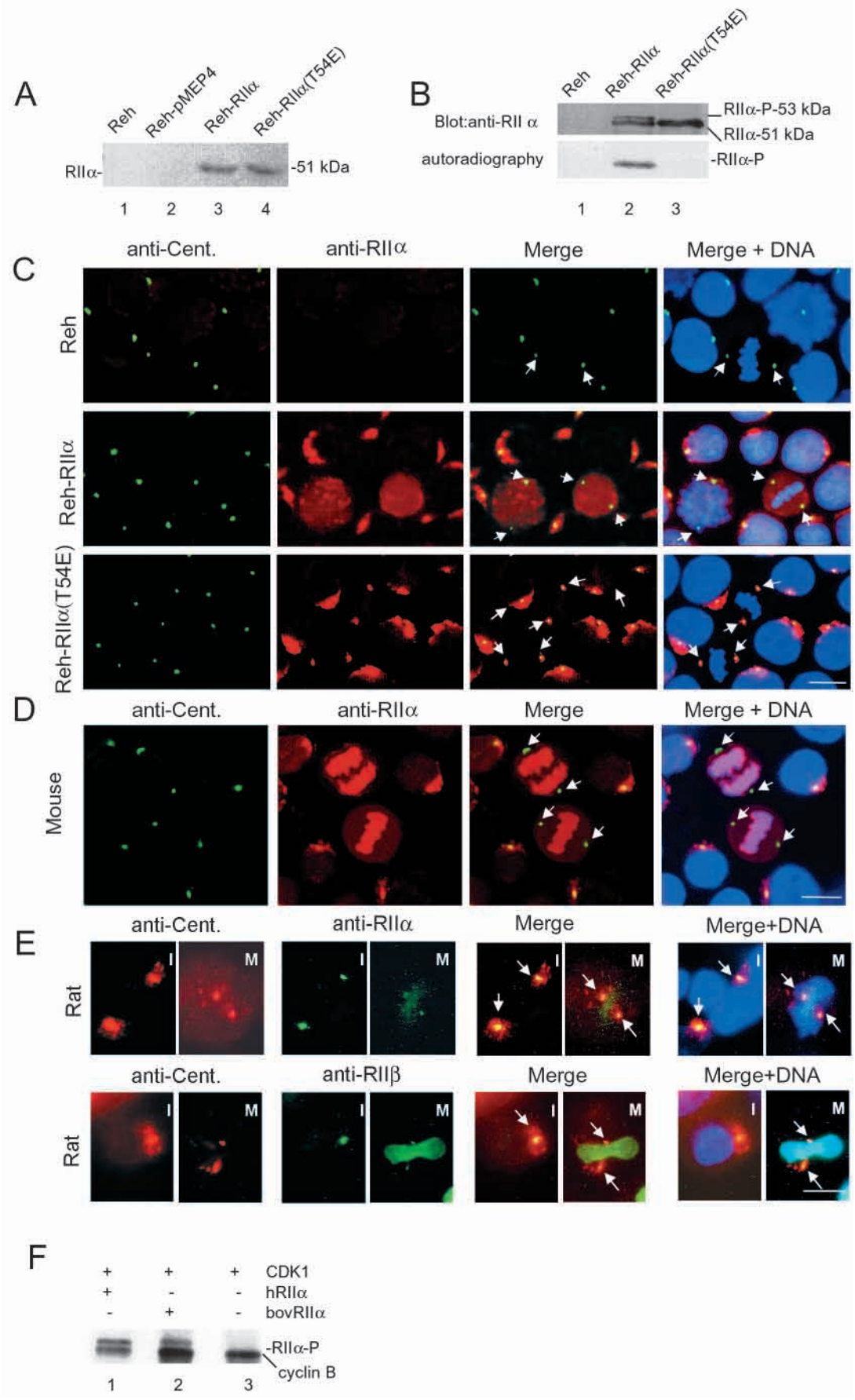

Fig. 1. Subcellular distribution of RIIα in cell lines expressing wild-type or mutant (T54E) RIIα. (A) Analysis of RIIα expression in stably transfected cell lines. Interphase cells (2×10^6 cells per lane) were subjected to SDS-PAGE and immunoblotting with a polyclonal antibody against human RIIα. Lanes: 1, wild-type Reh (RIIα-deficient); 2, Reh-pMEP4 (vector transfected); 3, Reh-RIIα; 4, Reh-RIIα(T54E). (B) RIIα was immunoprecipitated from interphase lysates (4×10^7 cells per lane) of Reh-RIIα (lane 2) and Reh-RIIα(T54E) (lane 3) cells. Precipitation from Reh was performed as control (lane 1). Half of the immune precipitates were immunoblotted using a anti-RIIα polyclonal antibody (upper panel). The other half was incubated for 45 minutes at 22°C in EBS phosphorylation buffer with [γ-^{32}P]ATP in the presence of CDK1 (9 pmol minute^{-1} μl^{-1}), separated by SDS-PAGE, dried and subjected to autoradiography (lower panel). The positions of non-phosphorylated (51 kDa) and phosphorylated (53 kDa) RIIα are indicated. (C-E) Interphase (I) and mitotic (M) Reh cell lines (C), mouse A9 fibroblasts (D) and primary cultures of peritubular cells prepared from rat testes (fibroblast-like) (E) were analyzed by immunofluorescence using anti-RIIα (upper panel; red in C and D, green in E) or anti-RIIβ mAbs (lower panel; green in E) and an affinity-purified polyclonal antibody against AKAP450 (green in C and D, red in E). DNA was stained with Hoechst 33342 (blue). Arrows indicate mitotic centrosomes. Bar: 10 μm. (F) CDK1 phosphorylation of purified recombinant human (lane 1) and bovine (lane 2) RIIα (150 ng of each). The positions of phosphorylated RIIα (lanes 1 and 2) and autophosphorylated cyclin B (49 kDa, lane 3) are indicated.

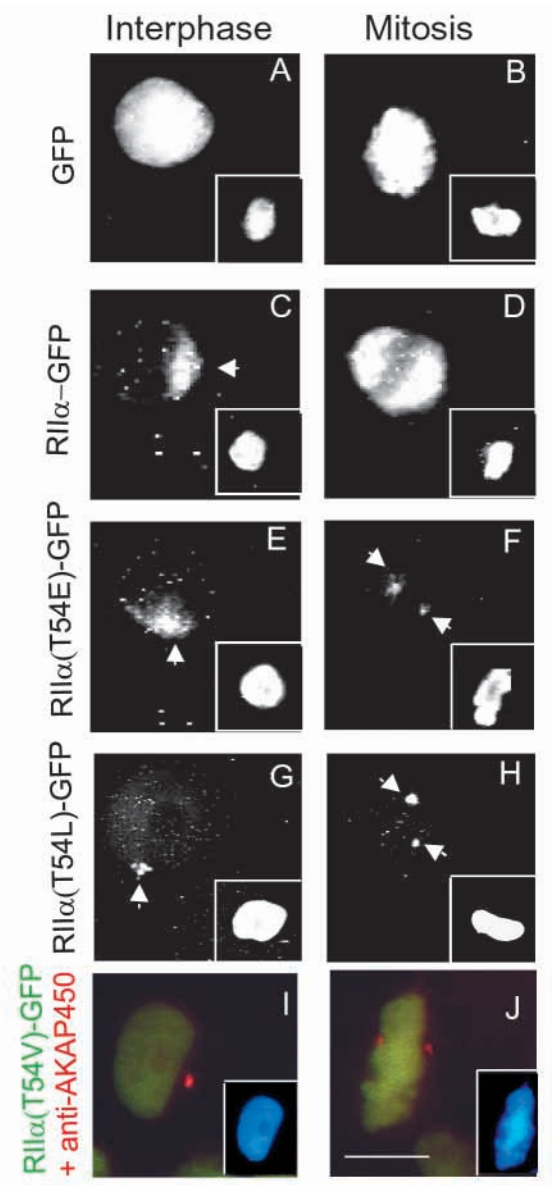

Fig. 2. Different locations of wild-type and mutated RIIα-GFP in mitotic cells. Reh cells were transiently transfected with GFP (A,B), RIIα-GFP (C,D), RIIα(T54E)-GFP (E,F), RIIα(T54L)-GFP (G,H) or RIIα(T54V)-GFP (I,J), fixed and stained with affinity-purified anti-AKAP450 polyclonal antibodies to stain centrosomes (I,J) and with Hoechst 33342 to stain DNA (inserts). GFP fluorescence was examined in interphase (A,C,E,G,I) and mitotic (B,D,F,H,J) cells. Arrows indicate the centrosomal association of RIIα.

cells. Thus, the two RIIα proteins expressed in Reh-RIIα and Reh-RIIα(T54E) cells were differently localized in mitotic cells. Because RIIα(T54E) could not be phosphorylated by CDK1, this suggests that cell cycle redistribution of RIIα at mitosis correlates with CDK1 phosphorylation. Cell-cycle-dependent localization of RIIα was also observed in mouse fibroblasts (Fig. 1D), indicating that this redistribution also occurs in cell lines from other species. By contrast, RIIα was not redistributed at mitosis in fibroblast-like primary cultures of peritubular cells from the rat testis, but were found to be associated with the centrosomal region (Fig. 1E). These observations are consistent with the presence of an N-terminal CDK1 phosphorylation site in mouse (IVS^{49}PTTFH) and the lack of a corresponding site in rat RIIα peptide sequence (IAPPTTFH). In the rat, however, the RIIβ subunit seemed to associate more strongly with mitotic chromatin (Fig. 1E, bottom row). Last, we tested whether bovine RIIα was accessible for CDK1 phosphorylation. Bovine RIIα was incubated with CDK1 and ATP, and detected by immunoblotting. Fig. 1F shows that purified bovine RIIα (lane 2), like human RIIα (lane 1), was phosphorylated in vitro by CDK1 with a change in electrophoretic mobility from 56 kDa to 58 kDa. The phosphorylated polypeptide at 49 kDa observed in the presence of CDK1 alone corresponds to autophosphorylated cyclin B (lane 3).

Subcellular location of RIIα-GFP and RIIα(T54E, L or V)-GFP in Reh cells

We next examined the distribution of wild-type and mutant RIIα by GFP tagging in transiently transfected Reh cells. To avoid disruption of the dimerization and AKAP-binding domains of RIIα, the GFP coding sequence was fused to the C terminus of the open reading frame of wild-type or mutated RIIα in the vector pEGFP-N1 and transfected into Reh cells. As a negative control, we transfected Reh cells with vector expressing GFP only. Examination of transfected cells by fluorescence microscopy revealed that GFP alone was localized both in the cytoplasm and nucleus-chromatin of interphase and mitotic cells (Fig. 2A,B). RIIα-GFP, RIIα(T54E)-GFP and RIIα(T54L)-GFP were localized to the Golgi/centrosome region in interphase cells (Fig. 2C,E,G). In mitotic cells, most of the RIIα-GFP was localized to the cytoplasm but some weaker fluorescence also overlapped with chromatin (Fig. 2D). By contrast, RIIα(T54E)-GFP and RIIα(T54L)-GFP were still attached to the mitotic centrosomes (Fig. 2F,H). We conclude that the GFP fusion proteins are localized similarly to the proteins expressed in the stable transfectants. Cells transfected with RIIα(T54V)-GFP did not display any centrosomal RIIα-GFP staining (Fig. 2I,J, centrosomes evident from double staining with anti-AKAP450 antibody), although centrosomes were visualized by staining with anti-AKAP450 antibody (red). These observations are consistent with the low affinity of AKAP450 for this mutant protein as measured by SPR (Table 1).

RIIα is solubilized by CDK1-phosphorylation in Reh-RIIα, but not in RIIα(T54E) cells

In contrast to RIIα, RIIα(T54E) was neither phosphorylated by CDK1 nor redistributed at mitosis and remained associated with the centrosomes. Because of this, we wanted to analyze the solubility of RIIα in the transfected cell lines. Triton-X-100-insoluble fractions of Reh-RIIα and Reh-RIIα(T54E) interphase cells were incubated with or without CDK1 and ATP. Supernatant and particular fractions were subsequently separated by centrifugation and the distribution of RIIα was analyzed by immunoblotting (Fig. 3A). The 51 kDa and 53 kDa RIIα were the major isoforms and were detected both in pellet (Fig. 3A, lanes 2-5) and supernatant (Fig. 3A, lanes 6-9) fractions from the two cell lines. The levels of the different RIIα isoforms in each lane (Fig. 3A) were quantified by densitometry (Fig. 3A, histogram). A decrease in the amount of particulate RIIα protein was seen for Reh-RIIα after incubation with CDK1 (lane 3), with a corresponding increase

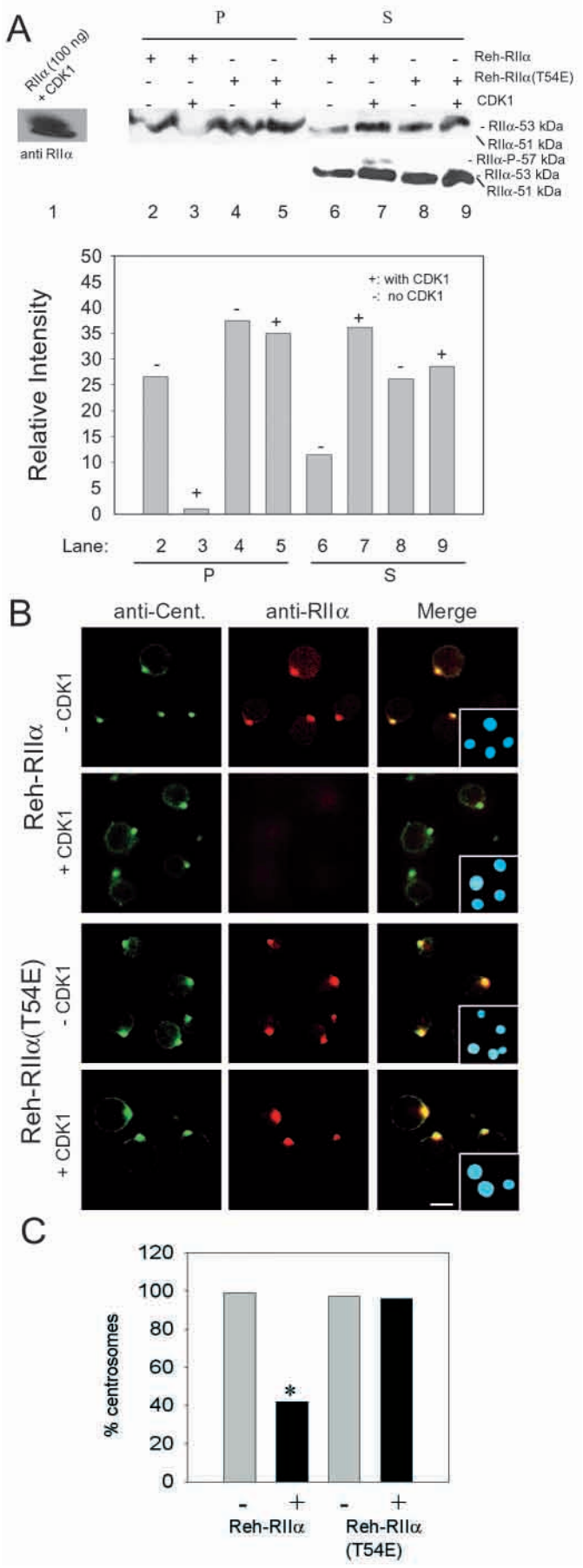

Fig. 3. Displacement of RIIα from Reh-RIIα but not Reh-RIIα(T54E) centrosomes after CDK1 phosphorylation. (A) Proteins from the Triton-X-100-insoluble fractions of Reh-RIIα and Reh-RIIα(T54E) interphase cells were incubated (for 45 minutes at 22°C in EBS phosphorylation buffer with 100 μM ATP) in the presence (lanes 3,5,7,9) or absence (lanes 2,4,6,8) of CDK1. After incubation, pellet (P; lanes 2-5) and supernatant (S; lanes 6-9; lower panel shows longer exposure) were separated by centrifugation and subjected to SDS-PAGE, and RIIα was immunodetected with anti-hRIIα mAb. The level of immunoreactive RIIα in each lane (in upper panel) was evaluated by densitometric scanning and relative intensities are given in the lower panel. As a positive control, 100 ng of human recombinant RIIα was incubated with CDK1 before the immunodetection (lane 1). (B) The localization of RIIα was analyzed by immunofluorescence in centrosome-nucleus complexes of interphase Reh-RIIα and Reh-RIIα(T54E) cells before and after incubation with CDK1. The location of RIIα and centrosomes was determined using anti-RIIα mAb (red) and anti-affinity purified anti-AKAP450 polyclonal antibodies (green). Inserts: DNA was stained with Hoechst 33342 (blue). Bars: 10 μm. (C) The percentage of centrosomes staining for RIIα before (–) and after (+) CDK1 incubation is given (n=200; ∗ means P<0.001). One representative experiment of two or more is shown for all panels.

of RIIα in the soluble fraction (lane 7). In addition, after longer exposure a phosphorylated 57-kDa RIIα isoform was also observed in the soluble fraction of Reh-RIIα after incubation with CDK1 (Fig. 3A, lane 7, lower blot), which most probably represents the PKA-RIIα double phosphorylated by PKA and CDK1 (Keryer et al., 1998). The levels in the pellet and supernatant fractions (lanes 5 and 9) of Reh-RIIα(T54E) were unchanged after CDK1 incubation. Thus, RIIα but not RIIα(T54E) was solubilized by CDK1 phosphorylation.

To examine the solubilization of RIIα further, nucleus-centrosome complexes from the two cell lines were isolated and incubated in presence or absence of CDK1 and ATP. Double immunofluorescence staining showed that RIIα was initially attached to centrosomes but dissociated after CDK1 incubation (Fig. 3B). By contrast, RIIα(T54E) remained attached to centrosomes regardless of the CDK1 treatment. Approximately 70% of the isolated nuclei were associated with centrosomes after purification. The number of centrosomes staining for RIIα or RIIα(T54E) was scored from 200 nucleus-centrosome complexes. After CDK1 treatment, only 42% of centrosomes from Reh-RIIα complexes stained for RIIα and, in addition, this staining was dim (P<0.001) (Fig. 3C). By contrast, 96% of the centrosomes from Reh-RII(T54E) complexes stained strongly for RIIα after CDK1 treatment. The results suggest that phosphorylation of T54 is a determining factor for dissociation of RIIα from centrosomes.

RIIα(T54E), but not RIIα, immunoprecipitates with AKAP450 in mitotic cells

RIIα has been shown to bind the A-kinase anchoring protein AKAP450 in purified centrosomal fractions from KE37 lymphoblast cells (Witczak et al., 1999). We analyzed the association of the RIIα with AKAP450 in Reh-RIIα and Reh-RIIα(T54E) cells by immunoprecipitation. Mitotic and interphase cells were lysed and the RIIα-AKAP450 complex was immunoprecipitated using anti-RIIα antibody. Precipitates

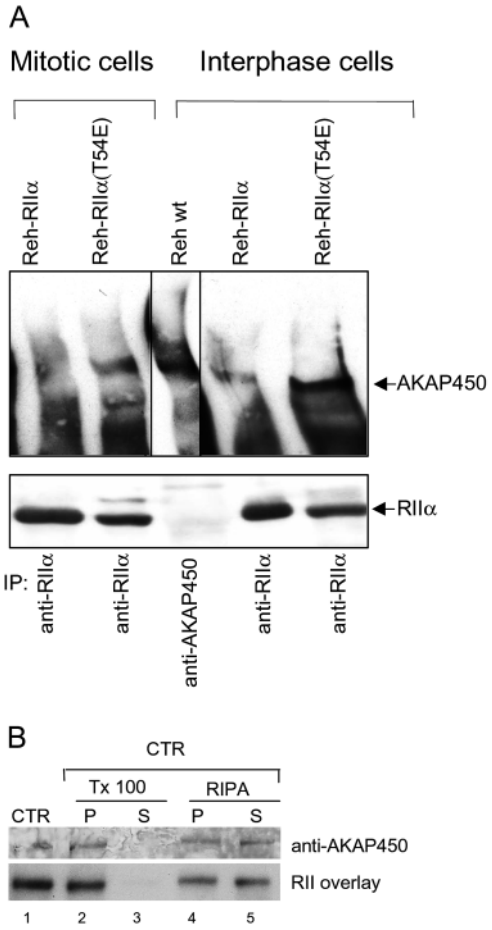

Fig. 4. (A) Co-immunoprecipitation of AKAP450 with anti-RIIα from Reh-RIIα and Reh-RIIα(T54E) cells in interphase and mitosis (obtained by double thymidine block). The upper panel shows immunoprecipitation of RIPA-buffer extracts of 200×10^6 cells using anti-RIIα antibody. As a control, Reh cells were immunoprecipitated with anti-AKAP450 antibody. Samples were separated by 4.5% PAGE containing 2 M urea, transferred to nitrocellulose and detected using anti-AKAP450 antibodies. The AKAP450 band is indicated by an arrow. The lower panel shows the RIIα content in the precipitated samples. (B) Immunospecificity of the affinity-purified polyclonal AKAP450 antibodies. Lane 1 contains purified centrosomes (untreated); lanes 2 and 3 contain Triton-X-100 insoluble and -soluble fractions from the centrosomal preparation in lane 1, respectively; lanes 4 and 5 contain RIPA-insoluble and -soluble centrosomal fractions, respectively. The blot was analyzed by RII overlay (lower panel) and western blotting using the affinity-purified polyclonal AKAP450 antibodies (upper panel).

were separated on a 4.5% acrylamide gel containing 2 M urea, and AKAP450 was detected by anti-AKAP450 antibody. As a control, we immunoprecipitated AKAP450 with anti-AKAP450 antibody from wild-type Reh cells. The presence of RIIα and RIIα(T54E) was analyzed by immunoblotting. AKAP450 immunoprecipitated with RIIα from interphase lysates of both Reh-RIIα and Reh-RIIα(T54E) (Fig. 4A). In the mitotic lysates, AKAP450 was detected in the RIIα-immunoprecipitates from Reh-RIIα(T54E) but not in precipitates from Reh-RIIα. This is consistent with the cell cycle redistribution of RIIα to chromatin at mitosis (Fig. 1C,D). We conclude that the redistribution of RIIα from centrosomes at mitosis occurs by dissociation from AKAP450. As a control, the immunospecificity of the affinity-purified polyclonal AKAP450 antibodies was assessed by immunoblot analysis of centrosomal preparations (Fig. 4B, upper panel, lane 1). This revealed a 450-kDa band that was resistant to detergent extraction (lanes 2 and 3) and partly solubilized by RIPA buffer extraction (lanes 4 and 5). RII overlay of the same blot to detect AKAPs (Fig. 4B, lower panel) demonstrated a band of the same mobility and distribution as that of the immunoreactive band (upper panel). We conclude that the immunoreactivity of the antibody is consistent with the characteristics of AKAP450.

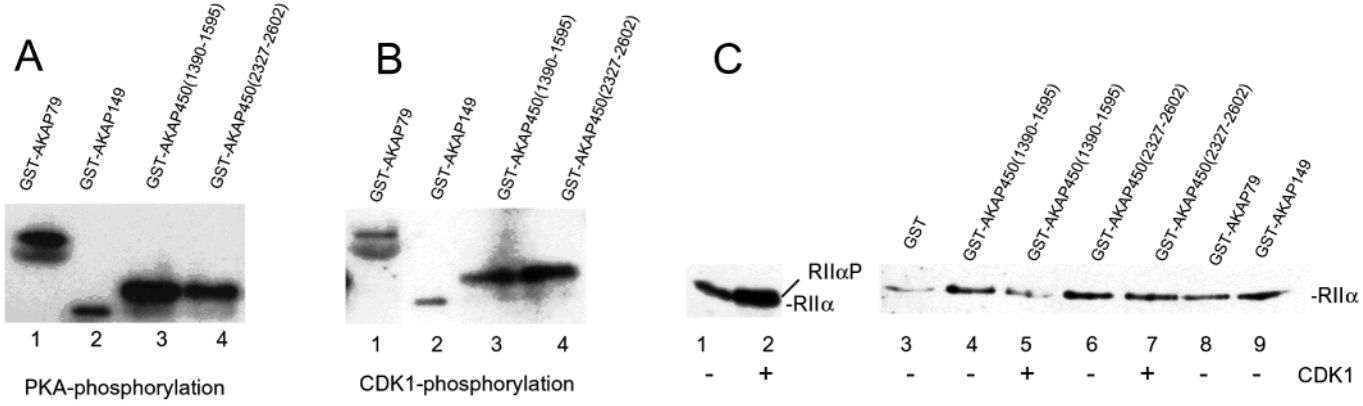

Fig. 5. Phosphorylated RIIα binds with different affinity to the RII-binding motifs in AKAP450. Purified recombinant human RIIα was radiolabeled using the catalytic subunit of either PKA (A) or CDK1 (B) and used for overlays on blots with different AKAPs (100 ng). Lanes: 1, GST-AKAP79 (178-427); 2, GST-AKAP149 (285-387); 3, GST-AKAP450 (1390-1595); 4, GST-AKAP450 (2327-2602) (numbers indicate the amino acid extent of the recombinant fragments used). (C) Precipitation of RIIα isoforms with different GST-AKAPs (20 nM of both R and AKAP). In some experiments, RIIα was preincubated with CDK1 before the GST precipitation. Lanes: 1, RIIα (50 ng); 2, RIIα after CDK1 preincubation; 3, GST + RIIα (negative control); 4, GST-AKAP450 (1390-1595) + RIIα; 5, GST-AKAP450 (1390-1595) + RIIα-P; 6, GST-AKAP450 (2327-2602) + RIIα; 7, GST-AKAP450 (2327-2602) + RIIα-P; 8, GST-AKAP79 (178-427) + RIIα; 9, GST-AKAP149 (285-387) + RIIα. Immunodetection was with anti-hRIIα mAb.

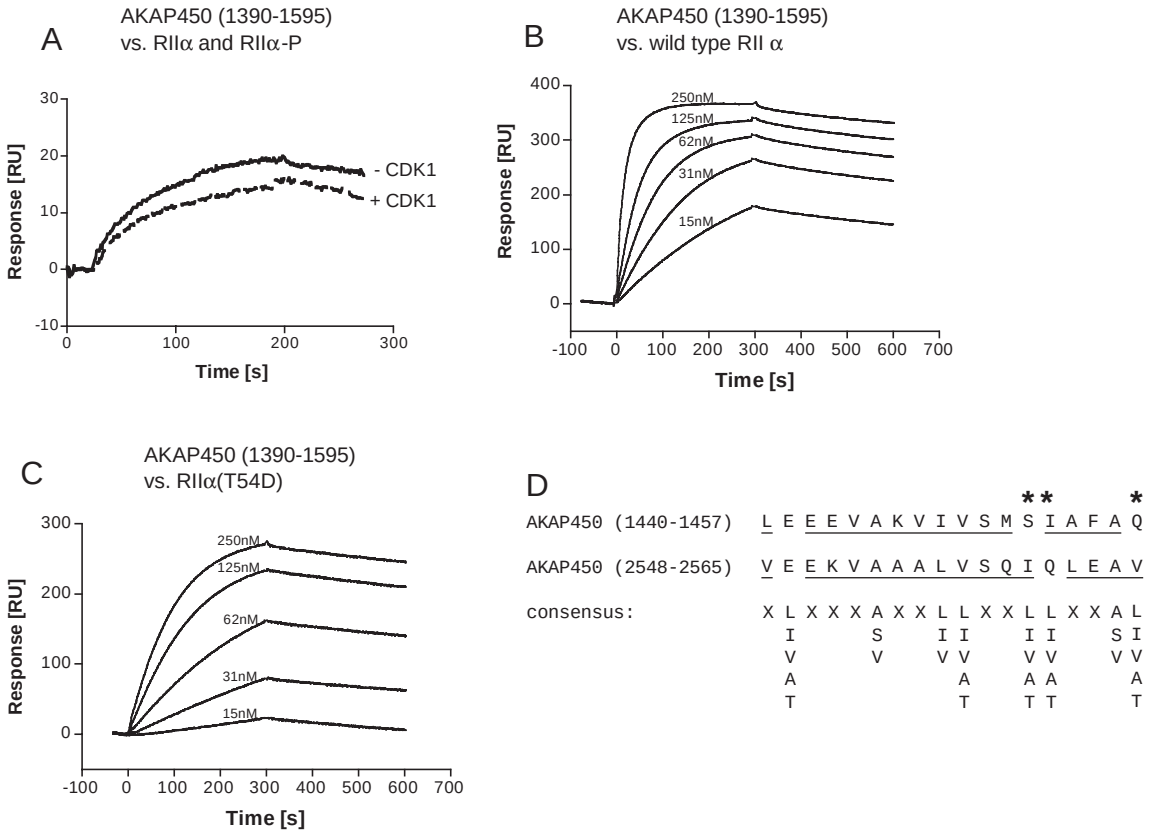

Fig. 6. Interaction of the AKAP450 N-terminal binding domain with wild-type hRIIα (A,B) and hRIIα(T54D) (C). A sensor chip with 60 RUs of 8-AHA-cAMP immobilized on each surface was used to capture (A) 120 RU and (B,C) 500 RU of the RIIα subunit to a separate flow cell. (A) AKAP450 (1390-1595) (100 nM) was run over immobilized CDK1-phosphorylated (dotted line) and unphosphorylated (solid line) RIIα for 180 seconds. The indicated concentrations of AKAP450 (1390-1595) were run over the immobilized wild-type hRIIα (B) and hRIIα(T54D) (C) subunits for 300 seconds and the association phases of AKAP450 (1390-1595) were monitored in 20 mM MOPS, pH 7.0, 150 mM NaCl, 1 mM DTT and 0.005% surfactant P20. The dissociation phase was monitored for another 300 seconds after omitting the AKAP from the running buffer. The immobilization of the R subunits and the regeneration are not shown. (D) Aligned sequences of the RII binding domains in AKAP450 along with the consensus binding sequence (Vijayaraghavan et al, 1999). * denotes residues that differ between the two AKAP450 domains and from the consensus.

CDK1 phosphorylation of RIIα affects its affinity to the two RII-binding domains of AKAP450

Human RIIα can be phosphorylated by both PKA and CDK1 (Keryer et al., 1998). We next compared the affinities of PKA- and CDK1-phosphorylated RIIα to AKAP450. Two putative RII-binding domains have been reported in AKAP450 (Schmidt et al., 1999; Witczak et al., 1999). Both domains were expressed as GST fusion proteins: GST-AKAP450 (1390-1595) and GST-AKAP450 (2327-2602). Binding to AKAP450 was first assessed by RII-overlay technique using RIIα phosphorylated by either PKA or CDK1 in the presence of [γ-^{32}P]ATP. PKA-phosphorylated RIIα appeared to bind more strongly to GST-AKAP450 (1390-1595) (Fig. 5A, lane 3) than to GST-AKAP450 (2327-2602) (lane 4). By contrast, CDK1-phosphorylated RIIα bound more weakly to GST-AKAP450 (1390-1595) (Fig. 5B, lane 3) than to GST-AKAP450 (2327-2602) (lane 4). However, binding is not limited by the affinity in the filter assay. To analyze the RIIα-AKAP450 interaction further in vitro, we did in-solution binding assays using GST precipitation to detect interactions. Purified recombinant human RIIα was incubated with CDK1 prior to the binding assay and subjected to GST precipitation to analyze the effect of phosphorylation of RIIα on binding to AKAP450. As a control for the phosphorylation reaction, 50 ng of purified recombinant human RIIα was incubated with CDK1 and ATP, and immunodetected with anti-RIIα antibody. This demonstrated similar levels of RIIα and phosphorylated RIIα

Table 1. Apparent association and dissociation rate constants for interaction of wild-type and mutated human RIIα with the two AKAP450 fragments*

	AKAP450 (1390-1595)			AKAP450 (2327-2602)		
Protein	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (nM)	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (nM)
RIIα wt	8.6×10^4	5.4×10^{-4}	5±1	1.2×10^6	4.3×10^{-4}	0.4±0.2
RIIα(T54A)	3.2×10^4	2.3×10^{-4}	7±1	1.7×10^6	4.5×10^{-4}	0.3±0.1
RIIα(T54L)	3.2×10^4	2.5×10^{-4}	7±2	1.7×10^6	2.3×10^{-4}	0.15±0.05
RIIα(T54V)	2.7×10^4	4.9×10^{-4}	18±5	1.5×10^6	2.8×10^{-4}	0.2±0.05
RIIα(T54D)	2.2×10^4	4.5×10^{-4}	20±3	1.6×10^6	3.9×10^{-4}	0.3±0.1

RII-AKAP interaction was analysed as described in legend to Fig. 6. Apparent association (K_a) and dissociation (K_d) rate constants were calculated from three different independent experiments using global fit analysis as described elsewhere (Herberg et al., 2000). $K_D=K_d/K_a$.

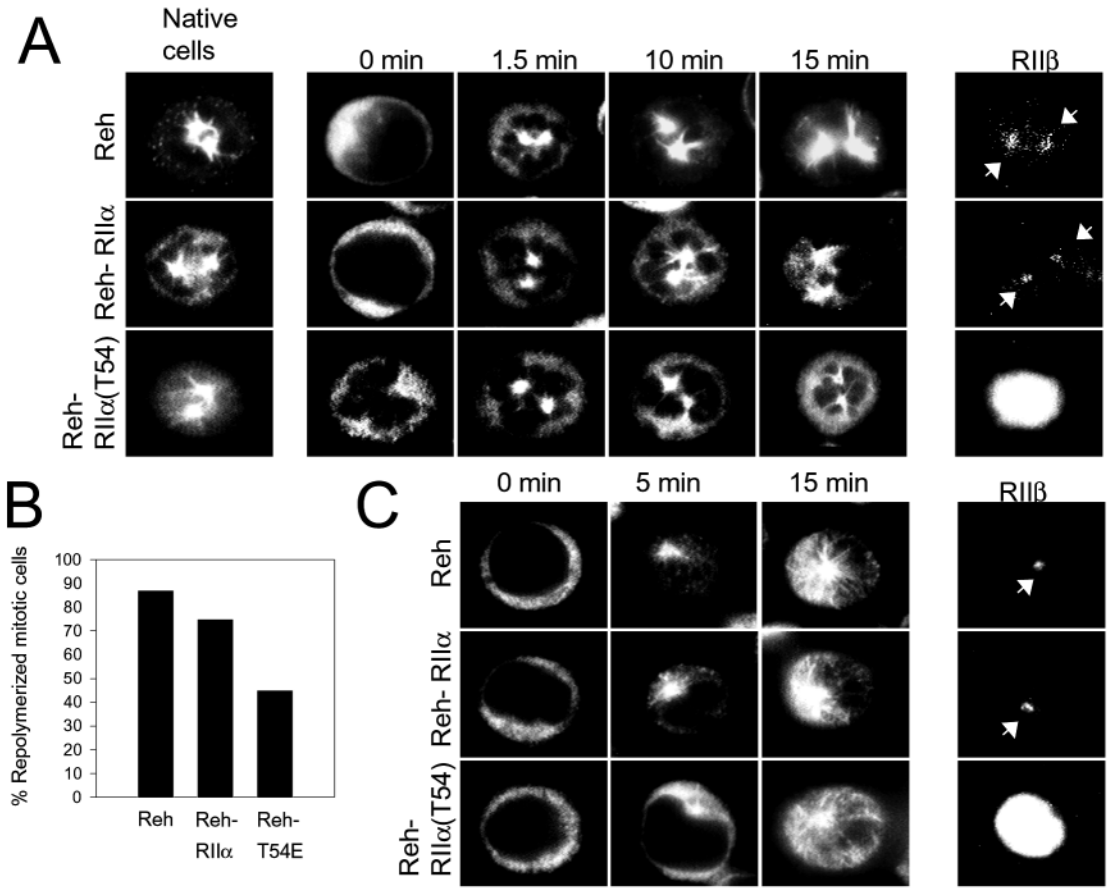

Fig. 7. Microtubuli repolymerization assays in mutant and wild-type RIIα-transfected cells. Reh, Reh-RIIα and Reh-RIIα(T54E) cells were incubated for 1 hour at 4°C to depolymerize microtubules. Repolymerization was started by heating to 37°C and samples were stopped at different time points (0-15 minutes) by methanol fixation. Aster formation was then analyzed by immunofluorescence using anti-α-tubulin mAb. Mitotic (A) and interphase (C) cells were examined. The right-hand columns show staining by anti-RIIβ antibody in cells prior to depolymerization. Arrows indicate the centrosomal association of RIIβ. (B) The number of mitotic cells that formed asters after depolymerization was scored. Reh vs RIIα, $P<0.005$; Reh vs RIIα(T54E), $P<0.001$.

(Fig. 5C, lane 1 and 2). RIIα (+/– CDK1 phosphorylation) was then diluted to 20 nM and mixed separately with 20 nM of different GST proteins. After CDK1 treatment, RIIα showed low-level binding to GST-AKAP450 (1390-1595) (Fig. 5C, lane 5) compared to the binding of non-phosphorylated RIIα to the AKAP (lane 4). Both phosphorylated and unphosphorylated RIIα bound well to GST-AKAP450 (2327-2602) (Fig. 5C, lanes 6 and 7). These observations were consistent with the RII overlay data. As positive controls, we used GST-AKAP79 (178-427) (Fig. 5C, lane 8) and GST-AKAP149 (285-387) (lane 9), which precipitated similar amounts of unphosphorylated RIIα to GST-AKAP450 (either binding domain; compare with lanes 4 and 6). Binding of RIIα(T54E) to GST-AKAP450 (1390-1595) and GST-AKAP450 (2327-2602) was not affected by CDK1 (data not shown).

Next, SPR was used to investigate the binding of GST-AKAP450 (1390-1595) to unphosphorylated and CDK1-phosphorylated RIIα immobilized to the same concentration on a cAMP surface. Before immobilization, RIIα was incubated in presence or absence of CDK1 to yield a 70% stoichiometry of CDK1 phosphorylation. The phosphorylated and dephosphorylated RIIα forms were then immobilized on the cAMP surface and AKAP450 was passed over them (Fig. 6A). From the time-dependent association-dissociation curves, it appeared that unphosphorylated RIIα bound better to AKAP450 (1390-1595) than CDK1-phosphorylated RIIα. In addition, four different RIIα mutants were analyzed for binding to AKAP450 (1390-1595) and AKAP450 (2327-2602) (Table 1). Wild-type RIIα, RIIα(T54A) and RIIα(T54L) bound equally well to AKAP450 (1390-1595) (K_D of 5-7 nM). The RIIα(T54D) is believed to mimic phosphorylation and bound with lower affinity to AKAP450 (1390-1595) (Fig. 6C, Table 1; K_D of 20 nM) than did wild-type RIIα (Fig. 6B, K_D of 5 nM). The lower affinity of RIIα(T54V) (K_D of 18 nM) might be explained by the insertion of a larger and more bulky residue than alanine or leucine. The four RIIα mutants and the wild-type all bound strongly to the C-terminal domain of AKAP450 (2327-2602) with K_D values of 0.16-0.4 nM (Table 1). This is consistent with the notion that binding of RIIα to AKAP450 (2327-2602) is not regulated by CDK1 phosphorylation. By comparison of the AKAP450 binding domains (Fig. 6D), the CDK1-sensitive N-terminal binding domain of AKAP450 differs from the C-terminal binding domain and from the consensus at positions 13 and 18, whereas the C-terminal binding domain differs from the consensus at position 14.

Stability of microtubules in wild-type and mutant-RIIα-transfected cell lines

PKA anchored at the centrosome and along microtubules has been implicated in regulation of microtubule stability (Lamb et al., 1991). We wanted to analyze the effect of the absence and presence of RIIα on microtubule dynamics using the characterized cell lines Reh, Reh-RIIα and Reh-RIIα(T54E). To examine microtubule formation, cells were settled onto poly-L-lysin-coated coverslips and incubated for 1 hour at 4°C to depolymerize microtubules. Repolymerization was induced by adding conditioned medium (37°C) and stopped by ice-cold methanol fixation. Aster formation was analyzed at different time points (0-30 minutes) by immunofluorescence with anti-α-tubulin. Spindles were also examined in mitotic cells before depolymerization and appeared to be similar for all three cell lines (Fig. 7A). After depolymerization and initiation of repolymerization from mitotic centrosomes, microtubule formation had started and appeared to be similar after 1.5 minutes in all three cell lines (Fig. 7A, mitotic cells). For Reh and Reh-RIIα, the asters extended with time and were after 10-15 minutes similar in size to spindles in native cells. Although aster formation also occurred in Reh-RIIα(T54E) cells, the asters were distinctly smaller and did not extend with time (Fig. 7A, representative cell shown). The repolymerization frequency in the mitotic cells (prometaphase) was 87% for Reh cells, 75% for Reh-RIIα and 45% for Reh-RIIα(T54E) (Fig. 7B). We conclude that the repolymerization in Reh-RIIα(T54E) mitotic cells was poor and occurred at a lower frequency. Microtubule repolymerization was also analyzed in interphase cells (Fig. 7C), where minor differences were observed that did not reach statistical significance. Furthermore, in native interphase cells, the microtubule network appeared to be similar in all the cell lines (not shown).

The centrosomal association of human RIIβ is not regulated by CDK1 and RIIβ remains associated with centrosomes during the whole cell cycle when present in cancer cell lines (Keryer et al., 1993). We examined the amount of RIIβ in the three cell lines by immunofluorescence. RIIβ was present in both interphase and mitotic centrosomes of Reh and Reh-RIIα (Fig. 7A,C). By contrast, RIIβ was not detected in the centrosomes of Reh-RIIα(T54E) but rather had a cytoplasmic redistribution. We speculate that RIIβ is depleted from the centrosomes in Reh-RIIα(T54E) cells because RIIα(T54E) has a higher affinity for the AKAP450 binding domains than RIIβ.

DISCUSSION

To address the mechanism of the previously described cell-cycle-dependent redistribution of RIIα (Keryer et al., 1998), we established stable transfectants of a neoplastic B-lymphoid cell line expressing wild-type RIIα or mutant RIIα(T54E) where the CDK1 phosphorylation site T54 is changed to glutamic acid (T54E). Our data indicate that CDK1 phosphorylation of T54 in RIIα might serve as a molecular switch that regulates the association and dissociation of PKA with the centrosome and AKAP450 based on the following observations. First, the location of RIIα is cell-cycle-dependent, it associates with the centrosomes in interphase and dissociates at mitosis. Second, RIIα is phosphorylated on T54 at mitosis and by the major mitotic kinase CDK1 in vitro (Keryer et al., 1998). Third, RIIα is solubilized from purified nucleus-centrosome complexes or particular fractions after CDK1 treatment. Fourth, in vitro, CDK1 phosphorylation of RIIα lowers the affinity of RIIα for AKAP450, consistent with the observation that AKAP450 co-immunoprecipitated with RIIα from interphase cells, but not mitotic cells. Fifth, the mutant RIIα(T54E) is not phosphorylated or solubilized by CDK1 and does not dissociate from mitotic centrosomes. Altogether, these data indicate that CDK1 phosphorylation of RIIα on T54 induces the dissociation of PKA from its centrosomal anchor at mitosis. Mutation of T54 to glutamic acid abolished this effect. The observation that mutation to glutamic acid mimicked the effect of dephosphorylated threonine and not phosphorylated threonine was somewhat surprising, and might be due to the fact that E or D mutations do not always have the ability to mimic a phospho-serine or phospho-threonine (Krek et al., 1992). However, our conclusions were confirmed by making additional T54 mutants. Threonine mutated to aspartic acid (T54D) or leucine (T54L) mimicked phosphorylated and dephosphorylated threonine, with respect to affinities for the N-terminal binding site in AKAP450.

The interaction of RIIα with the N-terminal PKA binding domain in AKAP450 (1390-1595) was sensitive to CDK1 phosphorylation in filter overlay and GST precipitations. Furthermore, detailed analysis by SPR of the interaction of RIIα with AKAP450 when RIIα was immobilized on a cAMP chip showed clearly that binding to AKAP450 (1390-1595) was reduced when RIIα was phosphorylated by CDK1 or when threonine was mutated to aspartic acid, mimicking a phospho-threonine. By contrast, binding to AKAP 450 (2327-2602) was not affected by any of the T54 mutations. The fact that mutagenesis did not affect binding to the C-terminal binding site could indicate that this site is not exposed on the surface of the native protein. Alternatively, this site might be occupied by RIIβ. Preliminary data on affinity of RIIβ to the N- and C-terminal binding sites of AKAP450 (not shown), indicate that the C-terminal site might be occupied by RIIβ. Comparison of the two binding domains of AKAP450 might give some indication of determinants for sensitivity to CDK1 phosphorylation in the AKAP, but such determinants might also reside outside the consensus binding domain. The observation that mutation of T54 to large residues such as V and D (loss of binding) and E (strong binding) distinctly affect interaction with AKAP450 as well as with AKAP95 (Landsverk et al., 2001), could indicate that T54, in addition to the well-characterized dimerization- and AKAP-binding motif in RIIα (Newlon et al, 1999), might be an important determinant for interaction with specific AKAPs. Although amino acids 45-75 in RIIα is the region of RIIα with the lowest conservation between species (Foss et al., 1997; Øyen et al., 1989), we found potential CDK1 phosphorylation sites in mouse (S49) and bovine (T53) RIIα by homology alignment. Thus, CDK1 phosphorylation of T54 is not a mechanism specific for human RIIα and cell-cycle-dependent redistribution of RIIα subunit also appears to operate in other species. In the rat, however, RIIα was not redistributed, which is consistent with the lack of an N-terminal CDK1 phosphorylation site in the rat sequence. Interestingly, RIIβ seemed to associate more with mitotic chromatin in the rat (this study; Landsverk et al., 2001). To our knowledge, distribution of RIIα and RIIβ during the cell cycle has not been examined

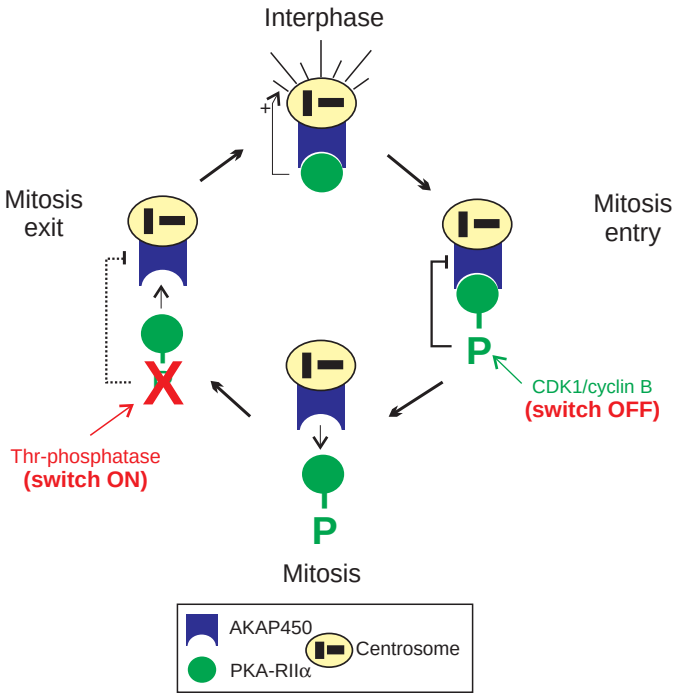

Fig. 8. CDK1 phosphorylation serves as a molecular switch that regulates the association of RIIα with centrosomal AKAP450. We propose this model for the regulation of RIIα association with AKAP450 by CDK1 phosphorylation. In interphase cells, unphosphorylated RIIα is tightly bound to the centrosomes through interaction with AKAP450. At mitosis entry, RIIα is phosphorylated at T54 by CDK1 and the centrosomal anchoring is disrupted. The binding is restored at mitosis exit by dephosphorylation of RIIα by a threonine phosphatase.

in vivo in the rat. The observations made here might suggest some redistribution of RIIβ instead of RIIα in rat, which could be pursued in future studies.

PKA has been shown to be involved in maintenance of the interphase microtubule network (Fernandez et al., 1995). PKA also switches off the effect of the destabilizing factor stathmin, which in turn promotes increased tubulin polymerization (Gradin et al., 1998). Overexpression of stathmin mutants that cannot be phosphorylated prevents the assembly of the mitotic spindle (Gradin et al., 1998). To address the function of the PKA-AKAP450 complex associated with the centrosome, we analyzed the microtubule nucleating activity of the transfected cell lines and showed that absence or presence of PKA-RIIα at mitosis had distinct effects on nucleation from mitotic centrosomes. This suggests that dissociation of PKA from the centrosome at mitosis is important for spindle formation. Based on our previous observations, centrosomal RIIβ is present in differentiated non-dividing cells and in all cancer cell lines examined, but not in normal cycling cells (Keryer et al., 1999). For this reason, we examined levels of RIIβ and found that Reh and Reh-RIIα cells both had centrosomal RIIβ. In the cell lines studied here, this might rescue effects that would come out more strongly in primary cycling cells. Also, requirements for accurate and timely progression through mitosis and segregation of chromosomes might be greater in the body than in cultured cancer cells and requirements for RIIα redistribution correspondingly stronger. Another possibility is that not all of PKA should be dissociated from centrosomes. This notion would be consistent with observations made with mutation of the PKA phosphorylation site in stathmin, which also produces aberrant spindle formation, indicating a PKA requirement either just before entry into mitosis or during mitosis.

In summary, we propose a model in which CDK1 phosphorylation of T54 in RIIα acts as a molecular switch that regulates the association and dissociation of PKA with centrosomal AKAP450 (Fig. 8); preliminary data indicate a similar distribution of C subunit (data not shown). During interphase, PKA is localized to the centrosome through interaction of RIIα with AKAP450. Upon mitosis entry, CDK1 is accumulated at the centrosome (Bailly et al., 1989) and RIIα is phosphorylated on T54 by CDK1. The switch is turned off and PKA dissociates from the centrosomal AKAP450. At mitosis exit, a yet-unknown threonine phosphatase dephosphorylates RIIα and turns the switch back. The affinity of RIIα for AKAP450 is now higher and the PKA-RIIα-AKAP450 complex reforms. In a parallel with this, we show an opposite situation with RIIα interaction with AKAP95. CDK1 phosphorylation of RIIα on T54 also seems to constitute a molecular switch controlling the association of PKA with chromosome-bound AKAP95 at mitosis (Landsverk et al., 2001; Collas et al., 1999). Anchoring of phosphorylated PKA to chromatin-associated AKAP95 is required to prevent premature decondensation of chromatin at mitosis. The two models fit together into a conceptual framework in which the association of RIIα to the centrosome or chromatin is determined by the phosphorylation state of RIIα, which is regulated by the mitotic kinase CDK1 and a threonine phosphatase. The threonine phosphatase, however, remains to be identified.

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