Mitosis-specific Phosphorylation and Subcellular Redistribution of the RII\(\alpha\) Regulatory Subunit of cAMP-dependent Protein Kinase*

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Phosphorylation of the RII regulatory subunits of cyclic AMP-dependent protein kinases (PKAs) was examined during the HeLa cell cycle. Three RII\(\alpha\) isoforms of 51, 54, and 57 kDa were identified by RII\(\alpha\) immunodetection and labeling with 8-azido[\(\text{\textsuperscript{32}P}\)]cAMP in different cell cycle phases. These isoforms were characterized as different phosphorylation states by the use of selective PKA and cyclin-directed kinase inhibitors. Whereas RII\(\alpha\) autophosphorylation by PKA caused RII\(\alpha\) to shift from 51 to 54 kDa, phosphorylation of RII\(\alpha\) by one or a combination of several kinases activated during mitosis caused RII\(\alpha\) to shift from 51 to 57 kDa. In vitro incorporation of [\(\text{\textsuperscript{32}P}\)]orthophosphate into mitotic cells and RII\(\alpha\) immunoprecipitation demonstrated that RII\(\alpha\) was hyperphosphorylated on a different site than the one phosphorylated by PKA. Deletion and mutation analysis demonstrated that the cyclin B-p34\(\text{cdk2}\) kinase (CDK1) phosphorylated human recombinant RII\(\alpha\) in vitro on Thr\(54^\prime\). Whereas RII\(\alpha\) was associated with the Golgi-centrosomal region during interphase, it was dissociated from its centrosomal localization at metaphase-anaphase transition. Furthermore, particulate RII\(\alpha\) from HeLa cell extracts was solubilized following incubation with CDK1 in vitro. Our results suggest that at the onset of mitosis, CDK1 phosphorylates RII\(\alpha\), and this may alter its subcellular localization.

Cyclic AMP-dependent protein kinases (PKAs)\(^1\) are present in mammalian tissues as two major isozymes, type I and type II (for reviews, see Refs 1 and 2). The inactive holoenzyme is composed of a regulatory subunit dimer that binds two catalytic subunits. Binding of cAMP to the regulatory subunits in situ demonstrates different phosphorylation patterns during the cell cycle. In mitotic-arrested cells, RII\(\alpha\) labeling with 8-azido[\(\text{\textsuperscript{32}P}\)]cAMP, immunostaining with a specific antibody, and in situ incorporation of [\(\text{\textsuperscript{32}P}\)]orthophosphate in HeLa cells revealed different sites of RII\(\alpha\) phosphorylation compared with that observed during interphase. Inhibition of PKA activity with a selective inhibitor, H89, or inhibition of mitotic kinase activity with olomoucine showed that RII\(\alpha\) is mainly phosphorylated by the catalytic subunit during interphase and by a mitotic kinase on another phosphorylation site(s) during mitosis. Moreover in vivo incorporation of [\(\text{\textsuperscript{32}P}\)]orthophosphate into mitotic and interphase HeLa cells followed by RII\(\alpha\) immunoprecipitation demonstrated that in mitosis RII\(\alpha\) was hyperphosphorylated on at least two sites. Recombinant native human RII\(\alpha\) was phosphorylated in vitro by purified CDK1 on a site located in the N-terminal domain of this protein. Site-specific mutagenesis of the putative Thr\(54^\prime\) phosphorylation site demonstrated that this site was the target of CDK1. During mitosis, RII\(\alpha\) was dissociated from centrosome at the met-
aphase-anaphase transition concomitantly with its phosphorylation by PKA and CDK1.

MATERIALS AND METHODS

Cell Culture, Synchronization, and 32P Incorporation—HeLa cells were grown as monolayer cultures at 37°C in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. They were arrested or slowed down in their cell cycle by different techniques previously described (15). Briefly, cells enriched in late G2 were obtained by a 2.5 mM thymidine block for 17 h, followed by 7 h of release from this block and a subsequent block with 400 mM thymidine for 16 h. For early S phase arrest, subconfluent cultures were arrested in G0 by serum deprivation for 48 h followed by 5 μg/ml of aphidicolin for 24 h. A double-thymidine block followed by 100 μM olomoucine treatment for 6 h gave a mixed population of cells arrested in G0 and in G1 (16). HeLa cells were synchronized in mitosis by a double thymidine block followed by 1 μM nocodazole treatment for 18 h. The cell cycle stage was analyzed by flow cytometry. Human osteosarcoma (SaOS-2) and human skin fibroblast (HS27) cell lines were grown as monolayer cultures on glass slides for immunocytochemistry in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

For analysis of RIIa phosphorylation, kinase inhibitors were incorporated into certain experiments. Cells in interphase (essentially in G0/G1 and G2/M phases) were treated for 6 h with 20 μM protein kinase A inhibitor olomoucine (H89) (17). Cells entering mitosis were accumulated by treatment with 1 μM nolocazole for 18 h with additional 20 μM H89 during the last 6 h of this period. Olomoucine (100 μM), a cyclin-directed kinase inhibitor, was added 2 h before the end of mitotic synchronization to avoid inhibition of entry into mitosis.

For 32P-labeling, HeLa cells arrested either in interphase at the G0/G1 border or in mitosis were incubated in 1 h in phosphate-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal calf serum, then 1 μCi/ml carrier-free [32P]orthophosphate (Amersham Pharmacia Biotech) was added for an additional 2 h at 37°C.

Cell Fractionation and Cdk2 Kinase Assay—Both interphase (G0/G1 and S) and mitotic HeLa cells were washed twice in 80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM MgCl2, 100 mM sucrose, 1 mM diithiothreitol, pH 7.2 (EBS buffer). They were centrifuged at 800 g for 10 min to discard unbroken cells and nuclei. The post-nuclear fractions were then centrifuged at 400,000 g for 30 min in a TL100 Beckman centrifuge to obtain cytosol and particulate fractions.

Cell lysates were obtained as described above in the presence of 1% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride. After homogenization, lysates were centrifuged at 100,000 g for 30 min, and the supernatant was used for determination of cyclin-dependent kinase activity as described below.

Protein content of each fraction was quantified according to Bradford (18).

The activity of mitotic cyclin-dependent kinase CDK1 was measured in cell lysates with [γ-32P]ATP and the BIOTRAK Cdk2 kinase enzyme assay system (Amersham Pharmacia Biotech). This system is based on a highly specific substrate (SV40 large T antigen) for the Cdk2 kinase (19).

Phosphoaffinity-labeling of the Regulatory Subunits with 8-Azido-[32P]cAMP—Phosphoaffinity incorporation of 8-azido[32P]cAMP was performed as described (20). Subsequently proteins were resolved by SDS-PAGE on 6–15% polyacrylamide gradient gels. For all cytosols prepared from synchronized cells, the incorporation of 8-azido[32P]cAMP was linear with regard to the amount of protein used up to 150 μg.

Antibodies—A monoclonal antibody (clone 4D7) raised against purified human testis RIIa (21) was used as purified IgG1, mouse ascites at 1/1000 dilution for Western blotting. Polyclonal antibody against human RIIa was raised against a specific peptide from the N-terminus of human RIIa kinase inhibitory acidic sequence (21), affinity-purified, and used at 1 μg/ml dilution for Western blots and 10 μg/ml for immunoprecipitation.

Antiseras raised against rat heart RIIa (7) was used at 1/30 dilution for immunoprecipitation. A monoclonal antibody CTR 453 (IgG3), obtained from a library of monoclonal antibodies raised against centrosomes isolated from human lymphoblasts was previously characterized as specific for the centrosome (22) and used for immunocytochemistry as purified immunoglobulins at 140 ng/ml dilution.

Electrophoresis and Immunoblotting—The cytosolic proteins solubilized in sample buffer were separated on 6–15% polyacrylamide gradient gels and electrophoretically transferred to nitrocellulose filters as described previously (23). The blots were incubated with the appropriate primary antibodies and then with anti-rabbit immunoglobulins coupled to alkaline phosphatase (for RIIa, HRP and PKA) or alkaline phosphatase-conjugated antiserum raised against a specific peptide from the C-terminus of yeast p13 species. Subsequently, the blots were developed with NBT/BCIP (for RIIa) or NBT/BCIP containing 0.1 M D-glucose (for PKA HRP). Immunoprecipitation—Metabolically labeled HeLa cells in interphase and in mitosis were washed twice with PBS and lysed in 1 ml of modified radioimmune precipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet-P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin; 100 mM sodium orthovanadate, 1 mM okadaic acid, and 50 mM sodium β-glycerophosphate). Immunoprecipitation of RIIa from metabolically labeled cells was performed as described previously (24). The radioactive polypeptide bands were cut out from the gel, and limited digestion of RIIa was carried out using trypsin-EDTA (Boehringer Mannheim) as described by Takio et al. (24).

The reaction was stopped after 5 min at room temperature by the addition of phenylmethylsulfonyl fluoride, and the peptides were separated by SDS-PAGE on a 1-mm-thick slab gel consisting of a 4% stacking gel and a 15% separating gel. Labeled proteolytic products were visualized by autoradiography of the dried gel using Kodak BIOMAX films.

Heterologous Expression of Recombinant Human RIIa and Site-specific Phosphorylation—Recombinant His-tagged human RIIa in E.coli were expressed in E.coli strains BL21 (DE3), and purified from bacterial lysates by adsorption to glutathione agarose beads as described elsewhere (25). Subsequently, purified fusion protein was digested with thrombin, and GST was absorbed on glutathione agarose beads to obtain soluble RIIa protein that contained an extra two- amino acid N-terminal extension from the linker segment/thrombin cleavage site of GST.

Mutants RIIa-Thr24-Ala and RIIa-Thr24-Glu were made by polymerase chain reaction of two overlapping amplification products, both with the Thr24-Ala or Thr24-Glu mutation introduced. This was accomplished using RIIa wild type cDNA (26) as a template and primers covering nucleotides 189–208 and 335–350 for the 5' amplification product and primers covering nucleotides 336–351 and 1523–1542 for the 3' amplification product. Subsequently, the overlapping products were mixed, denatured, and allowed to refold. The overexpressed protein was purified by chromatofocusing, filled with Klenow, and subjected to a second round of polymerase chain reaction as described above using only the outside primers. Polymerase chain reaction products of the full-length open reading frame were subcloned to pCRII vector (Invitrogen, Leek, The Netherlands) and sequenced to identify mutations. RIIa-mutated genes were then subcloned to pGEX-KG, and RIIa mutants were expressed in E.coli and purified as described above.

Phosphorylation in Vivo of Human Recombinant Wild Type and Mutated RIIa by Different Kinases—The catalytic subunit C of PKA type II (stock solution 0.21 mg/ml) was purified from bovine heart as described by Lohmann et al. (27). The starfish cAMP-dependent kinase was purified from starfish oocytes by a protocol including affinity chromatography on p13-Sepharose and consisted of two major polypeptides identified as p34cdc2/cyclin B (28). The specific activity of starfish CDK1 preparations varied from 150 to 1000 pmols of phosphate/min/μg incorporated into histone H1 as a substrate. HeLa CDK1 was enriched by affinity chromatography on p13-Sepharose beads and consisted of two major polypeptides identified as p34cdc2/cyclin B (29). The specific activity of starfish CDK1 preparations varied from 150 to 1000 pmols of phosphate/min/μg incorporated into histone H1 as a substrate. HeLa CDK1 was enriched by affinity chromatography on p13-Sepharose beads and consisted of two major polypeptides identified as p34cdc2/cyclin B (29).

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Recombinant human RII subunits were phosphorylated in vitro at 20 °C either in PKA phosphorylation buffer (50 mM Tris, pH 7.4, 1 mM MgCl₂, 10 mM β-mercaptoethanol, 20 μM cAMP, 100 μM ATP, and 1 μCi of [γ-32P]ATP (specific activity up to 5000 Ci/mmol) (1 Ci = 37 GBq; American Radiolabeled Chemicals) or for CDK1 and microtubule-associated protein kinase in EBS phosphorylation buffer without sucrose containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin), 100 μM ATP, and 1 μCi of [γ-32P]ATP. The reaction was stopped by the addition of Laemmli buffer. For subsequent 8-azido[32P]cAMP-labeling of the RII subunits, phosphorylation was performed in the absence of radioactive ATP, and the reaction was stopped at 4 °C with the addition of 10 mM EDTA. After affinity-labeling, the samples were boiled in Laemmli buffer. 

Phosphorylation of the Triton X-100-insoluble Fraction of HeLa Cells with CDK1 Isolated on p13 src1-Sepharose Beads—Cells were arrested in G1-S border with a double thymidine block. The cells were also arrested in a pseudometaphase state with nocodazole, which produced 49 ± 6% mitotic cells. The CDK1

activity was quite high (2264 ± 4% pmol/mg/min). The 8-azido[32P]cAMP-labeling of the PKA regulatory subunits (Fig. 1B) was performed both in the absence and in the presence of an excess of unlabeled cAMP, the latter used as a check

**RESULTS**

**Photoaffinity-labeling with 8-Azido[32P]cAMP and RIIα Immunodetection Identify Different RII Forms in Mitotic and Interphase HeLa Cytosols—**

8-Azido[32P]cAMP affinity-labeling of R subunits was used to follow the different regulatory subunit isoforms present in cytosols from HeLa cells arrested during the cell cycle. The position of the cells in the cell cycle was monitored by flow cytometry after DNA staining. The mitotic p34cdc2 kinase activity was measured with a specific substrate for this kinase as described under "Materials and Methods." The photoaffinity labeling with 8-azido[32P]cAMP was used to affinity-label the regulatory subunits of cAMP-dependent protein kinases present in cytosols obtained from HeLa cells synchronized at different phases of the cell cycle. A, HeLa cells were synchronized in late G1 (left panel) with a simple thymidine block (2.5 mM) followed by 18 h treatment with 400 μM mimosine; in S phase (second panel) by serum deprivation followed by treatment for 24 h with 100 μM nocodazole; as a mixed population of G1 and G2 cells (third panel) by a double thymidine block followed by treatment for 6 h with 100 μM olomoucine; and finally in mitosis (right panel) by a double thymidine block followed by treatment for 24 h with 1 μM nocodazole. Cell synchrony was analyzed by flow cytometry after DNA staining. The mitotic p34cdc2 kinase activity was measured with a specific substrate for this kinase as described under "Materials and Methods." The autoradiograph showing the affinity-labeling of 8-azido[32P]cAMP of R subunits in cytosols of the synchronized cells. Cytosols were prepared as described under "Materials and Methods." The photoactivated incorporation of 8-N1[32P]cAMP was performed under standard conditions in the absence (lanes 2-5) or in the presence of 10 μM unlabeled cAMP (lanes 1 and 6). The molecular mass of the treated regulatory subunits are indicated at the right side. Shown are samples of cell cytosol from G1, with (lane 1) and without (lane 2) 10 μM cAMP, S phase (lane 3); mixed G1 and G2 (lane 4) and mitosis, without (lane 5) and with (lane 6) 10 μM cAMP. Each lane contained 100 μg of protein. Use of unlabeled cAMP, which blocks 8-azido[32P]cAMP-labeling in lanes 1 and 6 demonstrated the specificity of labeling of R subunits. C, immunodetection of RIα and RIIα in HeLa cytosols. Proteins of HeLa cells arrested in G1, S, or M phases were separated by SDS-PAGE on 8% minigel and transferred to nitrocellulose. RIα was immunodetected with anti-human RIα monoclonal antibody diluted 1:1,000, with an affinity-purified anti-human RIIα antibody diluted to 1:1,000, both followed by a secondary antibody coupled to alkaline phosphatase.
of the specificity of the labeling. In the cytosol of G$_1$, S- or G$_2$-enriched cells, or in the mixed population of G$_1$ and G$_2$ cells, three polypeptides of 49, 51, and 54 kDa specifically bound the 8-azido$^{32}$P-cAMP (Fig. 1A, lanes 2–4). In the cytosol of mitotic cells, four polypeptides of 49, 51, 54, and 57 kDa were labeled (Fig. 1B, lane 5). No 8-azido$^{32}$P-cAMP-labeling was observed in the presence of excess unlabeled cAMP (Fig. 1B, lane 4) or treated with 100 µM olomoucine 2 h before the end of mitotic synchronization (lane 3). Interphase cells were untreated (lane 4) or treated for 6 h with 20 µM H89 (lane 5). Each lane contained 100 µg of protein. The molecular mass of the labeled regulatory subunits are indicated at the left side.

In HeLa cells only two regulatory subunit isoforms have been identified, RI$_{\alpha}$ and RI$_{\beta}$. This was observed with antibodies directed against nonhuman R isoforms by Weber et al. (32) and Nigg et al. (6) and more recently with specific antibodies directed against the known human isoforms of R subunits, RI$_{\alpha}$, RI$_{\beta}$, RI$_{\gamma}$, and RI$_{\beta\gamma}$ (data not shown). To identify the 8-azido$^{32}$P-cAMP-labeled R isoforms in synchronized cell cytosols, RI$_{\alpha}$ and RI$_{\beta}$ were immunodetected in different HeLa cytosols with an affinity-purified antibody directed specifically against the N-terminal domain of human RI$_{\alpha}$ and a monoclonal antibody directed against human RI$_{\beta}$. RI$_{\alpha}$ was always immunocytochemically detected in interphase HeLa cells as a polypeptide doublet of 51 and 54 kDa (Fig. 1C, right panel), corresponding to the well known unphosphorylated and autophosphorylated forms of this regulatory subunit (6, 32). However, in cells arrested in mitosis, an additional slower migrating polypeptide 57 kDa), which cross-reacted with anti-hRI$_{\alpha}$ antibody, was always detected. Western blot analysis revealed no difference in the total amount of RI$_{\alpha}$ detected during the cell cycle. In HeLa cytosols, the human RI$_{\alpha}$ monoclonal antibody detected only one polypeptide at 49 kDa, which also did not vary in amount during the cell cycle (Fig. 1C, left panel).

The 54- and 57-kDa 8-azido$^{32}$P-cAMP-labeled RI$_{\alpha}$ Subunits Are Different Phosphorylation States of RI$_{\alpha}$—In interphase cells, H89, a cell permeant inhibitor of cAMP-dependent protein kinase, led to the disappearance of the 54-kDa R-subunit (Fig. 2, lanes 1 versus 5). In mitotic cells treated with H89 (PKA activity in these cells was completely inhibited as verified using Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) as a substrate), we detected no 57-kDa RI$_{\alpha}$ isoform. Although PKA activity was abolished, we still observed a faint 8-azido$^{32}$P-cAMP-labeled R-subunit at 53–54 kDa (Fig. 2, lane 2). To distinguish between noncomplete H89 inhibition of RI$_{\alpha}$ autophosphorylation and another nonautophobicphorylation event on RI$_{\alpha}$ in mitosis, we made use of olomoucine. Treatment of mitotic cells with 100 µM olomoucine led to an inhibition of CDK1 (as previously measured in synchronized cell lysates) and to a disappearance of the 57-kDa R-subunit in mitotic cytosol (Fig. 2, lane 3). In contrast, olomoucine treatment of G$_2$-arrested cells did not modify the 8-azido$^{32}$P-cAMP binding pattern (not shown, see also Fig. 1B, lane 4 for a mixed population of G$_1$- and G$_2$-arrested cells with olomoucine). Unfortunately the addition of both H89 and olomoucine on nocodazole-arrested cells led to cell death. Whereas phosphatase treatment of cytosols isolated from either the mitotic or the interphase cells abolished the 54- and 57-kDa RI$_{\alpha}$ phosphorylated isoforms, only the 57-kDa RI$_{\alpha}$ isoform disappeared when the homogenization of mitotic cells was performed in the absence of okadaic acid (data not shown).

In Vivo $^{32}$P Incorporation Reveals at Least Two Different Phosphorylation Sites on RI$_{\alpha}$ in Mitotic Cells—To confirm the phosphorylation states of RI$_{\alpha}$ during mitosis, HeLa cells in interphase or arrested in mitosis were metabolically labeled with $^{32}$P-orthophosphate, and cell lysates were prepared as described under “Materials and Methods.” RI$_{\alpha}$ was immunoprecipitated with either anti-rat RI$_{\alpha}$ (lanes 1 and 2), preimmune sera from the rabbit later immunized with rat heart RI$_{\alpha}$ (lanes 3 and 4), or with affinity-purified anti-human RI$_{\alpha}$ (lanes 5 and 6). The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Both antibodies showed that RI$_{\alpha}$ was hyperphosphorylated during mitosis. A autoradiogram of tryptic digests. The 57-kDa phosphorylated polypeptide band from the mitotic immunoprecipitate (M) and the 51–54-kDa area from the interphase immunoprecipitate (I) were excised from the gel and digested with trypsin-ENDTA for 5 min at 4 °C, and the trypptic digest was submitted to SDS-PAGE on a 20% polyacrylamide gel. Two proteolytic products of RI$_{\alpha}$ (36 and 17 kDa) were observed to be phosphorylated.
weight phosphorylated polypeptides also coimmunoprecipitated with RIIα. Two of them were reproducibly observed at 350 and 150 kDa, even after immunoprecipitation with affinity-purified anti-hRIIα Ab.

In 2 experiments, we next excised from wet gels the weakly labeled polypeptides at 51–54 kDa and the 57-kDa labeled polypeptide from interphase and mitotic cell immunoprecipitates, respectively. Excised polypeptides were then digested with trypsin-EDTA for 5 min. The trypsin digests were submitted to electrophoresis on a 20% polyacrylamide gel, and the gel was autoradiographed (Fig. 3B). Whereas no phosphorylation was observed in trypsin digest from interphase cells (lane 1), two major heavily 32P-labeled proteolytic products of 36 and 17 kDa were observed in immunoprecipitates from mitotic cells (lane 2). When HeLa cells were arrested in G1 and then stimulated with 10 μm forskolin (to increase the cAMP concentration in these cells (34)), incorporation of 32P was increased only in the 54-kDa RIIα isoform (not shown).

Human Recombinant RIIα Is Phosphorylated by CDK1 at Thr54 in the N-terminal Domain—As observed on Coomassie Blue-stained SDS-PAGE gels (Fig. 4, left), a preparation of recombinant RIIα showed the presence of four polypeptides corresponding to the GST-RIIα complex (75 kDa), the cleaved human RIIα (51 kDa), a major (46 kDa) proteolytic product of RIIα, and some contamination of cleaved GST (26 kDa). The 46-kDa protein corresponded to the 51-kDa human RIIα cleaved by thrombin at amino acid Glu57 as demonstrated by peptide sequencing of this polypeptide. Both the full-length recombinant protein and the 46-kDa cleavage product were phosphorylated by the catalytic subunit of PKA (see below Fig. 5). The human RIIα preparation was phosphorylated in vitro at 20 °C by the starfish CDK1 kinase for 2.5–30 min. Fig. 4 shows that native RIIα was phosphorylated in vitro by CDK1 with a modification in the electrophoretic mobility from 51 to 54 kDa. The GST-RIIα complex was slightly phosphorylated. The 46-kDa cleaved RIIα was not phosphorylated, suggesting that phosphorylation occurs in the N-terminal domain of RIIα. The phosphorylated polypeptide at 49 kDa observed in the presence of CDK1 alone corresponded to cyclin B. Cyclin B has been reported to be phosphorylated by the catalytic subunit of CDK1, the p34cdc2 kinase, in Xenopus oocytes and eggs (35) and in cultured human cells (36). We also noted a shift in the electrophoretic mobility of cyclin B when phosphorylated for 30 min.

8-Azido[32P]cAMP-labeling was performed after in vitro phosphorylation of RIIα by either PKA or CDK1 (Fig. 5). Incubation of recombinant RIIα without or with cold ATP did not modify the 8-azido[32P]cAMP-labeling of RIIα (Fig. 5, compare lanes 1 and 2). The phosphorylation of RIIα with PKA induced a shift of RIIα from 51 to 54 kDa (Fig. 5, lane 3), which was observed both on Coomassie-stained gels (Fig. 5, left) and after 8-azido[32P]cAMP-labeling (Fig. 5, right). CAMP (100 μM) competed the 8-azido[32P]cAMP-labeling of all RIIα subunits present in the preparation, independent of their phosphorylation (competition is shown after PKA phosphorylation (Fig. 5, lane 4)). Phosphorylation with starfish CDK1 also induced an electrophoretic mobility shift that was slightly smaller than that induced by PKA (Fig. 5, compare lanes 5 and 6). The 46-kDa RIIα proteolytic product bound 8-azido[32P]cAMP, but coinubation with CDK1 did not induce an electrophoretic mobility shift of the cleavage product.

Because the 46-kDa RIIα proteolytic product corresponding to the C-terminal part of RIIα starting at amino acid Glu57 was not phosphorylated by CDK1, we mutated a putative phosphorylation site at Thr54 of the N-terminal sequence Ala52-Ala53-Thr-Pro-Arg-Gln57 to either a glutamic acid or an alanine residue.

The native RIIα and mutated RIIα Thr54-Glu recombinant proteins were incubated for 10 min in vitro with [γ-32P]ATP and 3 different purified kinases, i.e, the catalytic subunit of bovine heart PKA type II, purified starfish CDK1, and human recombinant extracellular signal-regulated kinase 1. The phosphorylation of both native and mutated RIIα Thr54-Glu by PKA induced an electrophoretic mobility shift from 51–54 kDa both on Coomassie Blue-stained gels (not shown) and on autoradiograms (Fig. 6A, lanes 1 and 3). Whereas a distinct mobility shift and [32P] incorporation were observed when native RIIα was incubated with starfish CDK1 (Fig. 6A, lane 2), only the phosphorylation of cyclin B associated by starfish CDK1 was observed after incubation of mutated RIIα with CDK1 (Fig. 6A, lane 3).
Human recombinant extracellular signal-regulated kinase 1, shown to be active with myelcid basic protein, did not phosphorylate native or mutated RIIα (not shown).

8-Azido[32P]cAMP binding to different recombinant RIIα subunits was also used to demonstrate that mutated RIIα Thr54-Glu was not phosphorylatable by mitotic kinase (Fig. 6B). The RIIα Thr54-Glu preparation contained mainly 2 polypeptides (51 and 46 kDa) that bound 8-azido[32P]cAMP (Fig. 6B, lane 2). These polypeptides corresponded to the full-
beads. HeLa cells arrested at the G1-S border of the cell cycle were stained and solubilized with 0.5% Triton X-100 in stabilizing buffer (PHEM, see “Materials and Methods”). Then the extracted CDK1 bound to p13 could be observed. The CDK1-bound RII activity could be detected by Western blot for the presence of RII in situ with the polyclonal antibody hRII Ab. Whereas the total amount of supernatant was loaded on the gel, only 1/5 of the amount of pellet was loaded. Lanes 1 and 2, proteins from the supernatant (S) obtained after incubation with p13-Sepharose beads alone (lane 1) or with CDK1 bound to beads (lane 3). Lanes 2 and 4, protein pellets (P) after incubation with p13-Sepharose beads alone (lane 2) or with CDK1 bound to beads (lane 4). The upper band observed in the pellet did not correspond to RII as this staining was not competed by the hRII peptide used for immunization. The major 54-kDa RIIa form was observed both in supernatant and pellet. C, exponentially growing HeLa cells were fixed with methanol and then double-labeled with human RII Ab (hRII Ab (A)) and a specific centrosomal marker (monoclonal Mab 453 (C)) and were counterstained with 4',6-diamino-2-phenylindole dihydrochloride (Dapi (B)). The white arrowhead shows the staining of centrosome at mitotic poles in metaphase cell with monoclonal Ab 453 but no staining with hRII Ab was observed. The thin white arrow indicates that a weak staining with hRII Ab was sometimes observed at centrosome as a tiny dot. Large white arrows show the staining of centrosome in interphase cells both with hRII Ab (A) and monoclonal Ab 453 (C). Bar, 20 μm.

**Fig. 7.** A, immunodetection of RIIa in HeLa subcellular fractions. HeLa cells arrested at the G1/S border (G1) or in mitosis (M) were extracted in situ with 0.5% Triton X-100 for 1 min and fractionated into Triton X-100-insoluble (I) and -soluble (S) fractions. Proteins were separated by SDS-PAGE on 8% minigels and transferred to nitrocellulose. RIIa was immunodetected with an affinity-purified anti-human RIIa Ab. Whereas no or very little 54-kDa RIIa was released to the supernatant by incubation with p13-Sepharose beads alone (lane 1), a large amount of RIIa was released when CDK1 bound to the same beads were used (lane 3). RIIa was not present in the mitotic kinase-enriched preparation of p13-Sepharose beads as verified by Western blot. The RIIa solubilization was observed in three separate experiments with variable specific activity of the human mitotic kinase bound to Sepharose beads. Immunofluorescence studies showed that whereas RIIa was associated with centrosomes during interphase, RIIa was either no longer observed at mitotic poles (Fig. 7C). In mitotic HeLa cells, RIIa was either not observed in the centrosome region or detected as a large amount of supernatant was loaded on the gel, only 1/5 of the amount of pellet was loaded. Lanes 1 and 2, proteins from the supernatant (S) obtained after incubation with p13-Sepharose beads alone (lane 1) or with CDK1 bound to beads (lane 3). Lanes 2 and 4, protein pellets (P) after incubation with p13-Sepharose beads alone (lane 2) or with CDK1 bound to beads (lane 4). The upper band observed in the pellet did not correspond to RII as this staining was not competed by the hRII peptide used for immunization. The major 54-kDa RIIa form was observed both in supernatant and pellet. C, exponentially growing HeLa cells were fixed with methanol and then double-labeled with human RII Ab (hRII Ab (A)) and a specific centrosomal marker (monoclonal Mab 453 (C)) and were counterstained with 4',6-diamino-2-phenylindole dihydrochloride (Dapi (B)). The white arrowhead shows the staining of centrosome at mitotic poles in metaphase cell with monoclonal Ab 453 but no staining with hRII Ab was observed. The thin white arrow indicates that a weak staining with hRII Ab was sometimes observed at centrosome as a tiny dot. Large white arrows show the staining of centrosome in interphase cells both with hRII Ab (A) and monoclonal Ab 453 (C). Bar, 20 μm.
very low levels at mitotic poles compared with the staining of RIIα at centrosome in interphase (Fig. 7C, thin white arrow). In HeLa cells arrested in mitosis with nocodazole, no RIIα was detected at unsplit centrosomes (not shown). This was observed for several cells such as SaOS2 osteosarcoma cells, neuroblastoma cells, and human skin fibroblasts in primary cultures.

**DISCUSSION**

cAMP-dependent protein kinase has been directly implicated in cell cycle regulation, and its down-regulation may possibly play a role in the induction of mitosis and nuclear envelope breakdown in mammalian cells (37). Subsequently, during the metaphase-anaphase transition, concomitant with a drop in mitotic CDK1 activity, PKA is up-regulated to stimulate exit from mitosis (38, 39). Although most of the regulation of cAMP-dependent protein kinases derives from modifications in cAMP concentration, the phosphorylation states of the catalytic and regulatory subunits may also modulate the properties of these proteins. Because an increasing number of kinases are the targets of the mitotic kinase CDK1 (40–43), we questioned whether PKA might be also a target for CDK1 or a kinase activated only during mitosis. We previously reported that the regulatory subunit RIIβ of PKA type IIβ is phosphorylated in vitro by CDK1 (14). However PKA type IIβ is poorly expressed in dividing cells, whereas PKA type IIα is ubiquitously expressed. Moreover the amino acid sequences of RIIβ and RIIα differ in the N-terminal domain (2).

Both 8-azid[32P]cAMP-labeling and RIα or RIIα immunodetection with specific antibodies were performed to follow the regulatory subunits of PKA in HeLa cytosols during the cell cycle. First, care was taken to prevent changes in the phosphorylation state of RII during cell homogenization (44). Okadaic acid and orthovanadate were added to prevent the action of serine/threonine type-1 protein phosphatase, type-2 protein phosphatase class A (45), and tyrosine phosphatases (46), respectively. Phosphorylated RII has been shown to be a substate for type I protein phosphatase and type-2 protein phosphatase class A (47). Calcineurin (calcium-dependent type-2 protein phosphatase class B) has been shown to associate with RII-anchoring protein, AKAP 79 (48). The catalytic subunit of type-2 protein phosphatase class B has been shown to dephosphorylate RII on the site phosphorylated by the catalytic subunit of PKA (49). EGTA was present in the homogenization buffer to prevent the activation of calcineurin. Homogenization carried out in the presence of phosphatase inhibitors, ATP, and dithiothreitol generates mitotic cytosols and extracts with a more stable CDK1 activity (50). Both 8-azido[32P]cAMP-labeling and RIIα immunodetection showed that the number of RIIα forms was dependent on the cell cycle phase in which cells were arrested. In cells arrested in late G1 or S phases or in a mixed population of G1 + G2-arrested cells, a major RII isoform at 51 kDa was observed in HeLa cytosols. This was not the case in the cytosol of mitotic-arrested cells in which the amount of the 54-kDa RII form was more abundant than the 51-kDa isoform, and moreover, a third RII isoform appeared at 57 kDa. Immunodetection with specific antibodies identified only one RIIα isoform at 49 kDa.

Further treatment of synchronized cells with kinase inhibitors showed that the slowly migrating forms of regulatory subunits were phosphorylated forms. In interphase cells treated with the PKA inhibitor H89, the auto phosphorylated RII isoform (54 kDa) was not observed. In mitotic cells treated with H89 (PKA activity in these cells was completely inhibited as verified using Kemptide as a substrate) we still detected a minor amount of the 53–54-kDa RII isoform but no 57-kDa RII isoform. This can be explained either by a noncomplete H89 inhibition of RII autophosphorylation by PKA or another modification occurring on RII during mitosis. Olomoucine, which has been shown to inhibit mainly the cyclin-directed kinases in several cell types (16), suppressed the 57-kDa RII isoform. In this case, only autophosphorylated RII was observed at 54 kDa. Both 57- and 54-kDa 8-azido[32P]cAMP-labeled RII subunits were absent after alkaline phosphatase treatment of the mitotic cytosols, also suggesting that these R subunits are phos-
centrosomes during interphase is removed at metaphase just before anaphase. Bailly et al. (22) show that CDK1 associates with centrosome at the onset of mitosis. The removal of centrosomal RIIα cannot be explained by only a redistribution of the pericentriolar material at metaphase-anaphase transition, as several pericentriolar antigens are still present at the end of metaphase. Furthermore, the RIIβ subunit, which is expressed in differentiated normal cells and in neoplastic dividing cells, was not subject to cell redistribution from the centrosome at mitosis (data not shown). This serves as a control for redistribution of centrosomal RIIα and indicates that RII redistribution is not linked to a modification in the amount of anchoring protein or a kinase-anchoring protein in the pericentriolar material during the cell cycle. Future immunofluorescence studies on in situ expressed mutated RIIα in cells will address the question of whether the phosphorylation of Thr54 in human RIIα alters the subcellular localization of PKA type II at the onset of mitosis.

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Mitosis-specific Phosphorylation and Subcellular Redistribution of the RIIα Regulatory Subunit of cAMP-dependent Protein Kinase

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