Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7900–7904, September 1992
Cell Biology

Phosphorylation of the retinoblastoma protein by cdk2

TETSU AKIYAMA*†, TOHRU OHUCHI*, SHUJI SUMIDA*, KUNIHIRO MATSUMOTO‡, AND KUMAO TOYOSHIMA*

*Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, Osaka 565, Japan; and ‡Department of Molecular Biology, Faculty of Science, Nagoya University, Nagoya 464, Japan

Communicated by Peter K. Vogt, June 4, 1992

ABSTRACT **The retinoblastoma gene product (the RB protein) is phosphorylated in a cell cycle-dependent manner and this modification is believed to be important for cells to progress through the cell cycle. We found that purified cdk2 (cyclin-dependent kinase/cell division kinase 2) can phosphorylate the RB protein *in vitro* at the sites phosphorylated in the cell. The timing of activation of cdk2 in the cell cycle was similar to that of the onset of phosphorylation of the RB protein. The kinase coprecipitated with the RB protein also exhibited a similar substrate specificity to cdk2 and a similar time course of activation during the cell cycle. We further showed that cdk2 formed a complex with the RB protein *in vitro* and that its formation was not competitively inhibited by the simian virus 40 large T antigen. These observations suggest that cdk2 or a cdk2-related protein is involved in the cell cycle-dependent phosphorylation of the RB protein.**

The retinoblastoma (RB) gene is an antioncogene whose inactivation is known to occur in retinoblastomas, in osteosarcomas, and in carcinomas of the breast, lung, and bladder (1). Reintroduction of RB cDNA into cells without the intact RB gene results in suppression of the neoplastic phenotype of these cells (2). The product of the RB gene (the RB protein) is a nuclear phosphoprotein of 110–115 kDa that forms complexes with the transforming proteins encoded by DNA tumor viruses, such as the adenovirus E1A protein, the simian virus 40 (SV40) large T antigen, and the papillomavirus E7 protein (3–5). Genetic studies have shown that the abilities of these oncoproteins to form complexes with the RB protein are closely linked with their transforming potentials (3–5). The RB protein also interacts specifically with several cellular proteins, including transcription factor E2F and the protooncogene product Myc (6–10). Thus, the normal function of the RB protein may involve formation of complexes with cellular proteins. Recently, microinjection of the RB protein into cells early in the G_1 phase of the cell cycle was found to inhibit progression to the S phase, suggesting that the RB protein regulates cell proliferation by restricting cell cycle progression at a specific point in the G_1 phase (11).

The function of the RB protein is thought to be regulated mainly by phosphorylation. The RB protein has been shown to be phosphorylated in a cell cycle-dependent manner, being underphosphorylated in G_0/G_1 and highly phosphorylated during the G_1 to S transition (12–16). Interestingly, only underphosphorylated RB protein can form a complex with large T antigen (17) and transcription factor E2F (7). The underphosphorylated RB protein is tightly associated with the nucleus, whereas the hyperphosphorylated form is easily extracted under the same conditions, suggesting that the underphosphorylated RB protein interacts tightly with nuclear proteins (18, 19). These findings suggest that underphosphorylated RB protein inhibits transition of cells from G_1 to S and that phosphorylation is a regulatory event leading to inactivation of the growth-suppressing activity of the RB protein.

The RB protein possesses a number of consensus sequences for phosphorylation by cdc2 kinase. In fact, cdc2 kinase phosphorylates the RB protein *in vitro* at the same sites as those phosphorylated in living cells, and the amino acid sequences of the five sites phosphorylated in the cells were recently shown to correspond closely to the consensus sequence for phosphorylation by cdc2 kinase (20–23). However, cdc2 kinase is known to be active in the late G_2 and M phases (24), whereas the RB protein becomes highly phosphorylated at about the G_1 to S transition. On the other hand, although in yeast a single cdc2 kinase regulates both the G_1 to S and G_2 to M transitions (24), in higher eukaryotes the G_1 to S transition is regulated by a kinase that is homologous with, but distinct from, cdc2 kinase (25–31). This kinase has been characterized independently by several groups and was originally named Eg1 (25), cdc2B (26), or cdk2 [cell division kinase 2 (27, 28)], but recently, following a new convention, it has been named cdk2, for cyclin-dependent kinase 2 (29). cdk2 does not associate with cyclin B but does associate with cyclin A (26, 30, 31) and a doublet of proteins of 54 kDa (26) and is expressed before cdc2 kinase in the cell cycle (30, 31). Most importantly, experiments using *Xenopus* egg cell-free extracts have clearly shown that depletion of cdk2 blocks DNA replication, whereas depletion of cdc2 kinase does not interfere with DNA replication but blocks mitosis (26). Thus, cdk2 may be one kinase responsible for phosphorylation of the RB protein at about the G_1 to S transition. Here we show that cdk2 phosphorylates the RB protein *in vitro* at the sites phosphorylated in living cells and that the timing of activation of cdk2 in the cell cycle corresponds to that of the beginning of phosphorylation of the RB protein. We further suggest the association of cdk2 with the RB protein.

MATERIALS AND METHODS

Cell Culture. A *Saccharomyces cerevisiae cdc28* deletion mutant carrying the human cdk2 gene was cultured as described (28). *Escherichia coli* BL21(DE3) transformed with PET3 vector (32) containing the full-length cdk2 coding region (nucleotides 84–1453; ref. 28) in the *Bam*HI site was cultured to an OD_{600} of 0.4 at 37°C in M9ZB medium containing ampicillin (50 μg/ml). Sf9, a clonal isolate of *Spodoptera frugiperda* IPLB-Sf21-AE, was grown as monolayers at 27°C in Grace's insect medium supplemented with 10% fetal bovine serum. Sf9 cells were infected with pAcYM1-RB [pAcYM1 (33) containing the entire coding sequence, nucleotides 78–3741, of an RB cDNA in its *Bam*HI site], pAcYM1-ΔRB (pAcYM1 containing the *Bss*HII–Dra III fragment, nucleotides 78–2134, of the RB coding sequence in its *Bam*HI site), or pAc373T2 (pAc373 carrying SV40 large T antigen cDNA; a gift from M. K. Bradley; ref. 34). Monkey

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Abbreviations: NP-40, Nonidet P-40; SV40, simian virus 40.

†To whom reprint requests should be addressed at: Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565, Japan.

kidney fibroblast CV-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. For cell cycle experiments, CV-1 cells were starved in methionine-free DMEM containing 2% dialyzed calf serum for 48 hr and then were fed with complete DMEM containing 10% calf serum. The cell cycle was monitored by flow cytometric analysis with a FACScan/CellIIFIT DNA System (Becton Dickinson). At the times indicated in Fig. 3, samples were taken for assays of cdk2- and RB-associated kinase activity. For labeling of the RB proteins, CV-1 cells were cultured in the presence of [^{32}P]orthophosphate for 4 hr.

Antibodies. Monoclonal anti-RB antibody C36 was kindly provided by E. Harlow (3). Anti-peptide antibodies against the 14 carboxyl-terminal amino acid residues of the RB protein were prepared as described (35). Anti-cdk2 antibodies were prepared by immunizing rabbits with a synthetic peptide corresponding to the 12 carboxyl-terminal amino acid residues of cdk2.

Preparations of RB Protein and cdk2. The RB protein was purified from pAcYM1-RB-infected Sf9 cells by immunoaffinity chromatography using anti-RB antibodies (36). *S. cerevisiae cdc28* deletion mutant cells carrying the human cdk2 gene were disrupted by vortex mixing with glass beads and cdk2 was purified from the supernatant by affinity chromatography on p13-agarose (Oncogene Science, Manhasset, NY) as described (37). Expression of the cdk2 gene in *E. coli* BL21(DE3) was induced by treatment of the bacteria with 0.4 mM isopropyl β-D-thiogalactopyranoside for 2 hr. The cells were then lysed in buffer A [50 mM Tris, pH 8.0/1.5 mM EDTA/0.4 mM NaCl/5 mM $MgCl_2$/5% (vol/vol) glycerol/0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride/0.1 mM dithiothreitol/0.1% (wt/vol) lysozyme/0.5% (vol/vol) Nonidet P-40 (NP-40)] by sonication (38). The lysate was centrifuged at 40,000 × *g* for 40 min and the supernatant obtained was subjected to affinity chromatography on p13-agarose. Then cdk2 was eluted with purified p13 protein (500 μg/ml) (39) obtained from Oncogene Science.

Immunoprecipitation. CV-1 cells were lysed in solubilizing buffer [50 mM Tris, pH 7.2/1% NP-40/0.15 M NaCl/50 mM β-glycerophosphate/5 mM dithiothreitol/1 mM Na_3VO_4/0.05 mM NaF/0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride with leupeptin at 5 μg/ml] (33). The lysates were incubated with antibodies for 1 hr at 4°C. The immunocomplexes were adsorbed to protein A-Sepharose 4B and washed extensively with solubilizing buffer containing 0.1% NP-40.

***In Vitro* Kinase Reaction.** Purified RB protein (1 μg) was incubated with 0.5 μg of cdk2, immunoprecipitated from unlabeled CV-1 cells or purified from *S. cerevisiae* carrying the human cdk2 gene, in 50 μl of kinase reaction buffer [50 mM Tris, pH 7.2/10 mM $MgCl_2$/1 mM dithiothreitol/20 μM [γ-^{32}P]ATP (5 μCi; 1 μCi = 37 kBq)] for 10 min at 25°C. The RB-associated kinase activity was measured by incubating the immunoprecipitate prepared from CV-1 cells with anti-RB antibody C36 in kinase reaction buffer for 10 min at 25°C. Samples were analyzed by SDS/PAGE followed by autoradiography.

Two-Dimensional Tryptic Phosphopeptide Mapping. The RB protein phosphorylated *in vitro* or in cultured cells was digested with trypsin and the resulting phosphopeptides were resolved by electrophoresis for 35 min at 1 kV in 88% formic acid/acetic acid/water, 78:25:897, (vol/vol) at pH 1.9 in the first dimension followed by chromatography in 1-butanol/pyridine/acetic acid/water, 15:10:3:12 (vol/vol).

Coprecipitation of the RB Protein with cdk2 Kinase *in Vitro*. The purified baculovirus-expressed RB protein (1 μg) was incubated with cdk2 purified from *E. coli* cells (1 μg) in a total volume of 150 μl for 1 hr at 4°C and then subjected to immunoprecipitation with anti-cdk2 antibodies. In another experiment, lysate prepared from pAcYM1-RB-infected Sf9 cells (25 μg of protein) was incubated with lysate prepared from *E. coli* cells expressing cdk2 (25 μg of protein) in a total volume of 300 μl for 1 hr at 4°C and then subjected to immunoprecipitation with anti-cdk2 antibodies or anti-RB antibody C36. The immunoprecipitates were subjected to SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-RB antibody PMG3-245 (Pharmingen, San Diego) or anti-cdk2 antibodies.

RESULTS

Purified cdk2 Phosphorylates the RB Protein *in Vitro*. Antibodies against the carboxyl-terminal portion of human cdk2 specifically recognized cdk2 in monkey kidney fibroblast CV-1 cells and human monocytic leukemia U937 cells; these antibodies detected a doublet of proteins of about 33 kDa and recognition of these proteins was prevented by preincubation of the antibodies with the antigenic peptide (Fig. 1*A*). The mobility differences between the two bands are known to be due to differences in the phosphorylation status of cdk2 (29–31). To further confirm the specificity of the antibodies, we analyzed lysates of *S. cerevisiae cdc28* mutant cells transformed with the human cdk2 or cdc2 gene. In these cells the endogenous CDC28 gene is mutated and the human cdk2 or cdc2 gene is expressed instead. As shown in Fig. 1*B*, anti-cdk2 antibodies recognized human cdk2 but not human cdc2 kinase or yeast CDC28 kinase.

On incubation of cdk2 immunoprecipitates prepared from CV-1 cells with the RB protein in the presence of [γ-^{32}P]ATP, ^{32}P was incorporated into the RB protein (Fig. 2*A*). The RB protein used as substrate in this reaction had been immunoaffinity-purified to near homogeneity from Sf9 cells infected with pAcYM1-RB (Fig. 1*C*). Phosphorylation of the RB protein was not detected when the immunoprecipitates prepared with anti-cdk2 antibodies that had been preadsorbed with the antigenic peptide were used for kinase assay (Fig. 2*A*, lane 3). In another experiment, we used affinity chromatography on p13-agarose to purify cdk2 from *S. cerevisiae cdc28* mutant cells expressing the human cdk2 gene and incubated the purified cdk2 with the baculovirus-expressed RB protein in the presence of [γ-^{32}P]ATP. The result also showed that the RB protein was a good substrate for cdk2 *in vitro* (Fig. 2*B*). Phosphorylation of the RB protein observed in this reaction was catalyzed by cdk2, not a contaminating kinase, because a sample depleted of cdk2

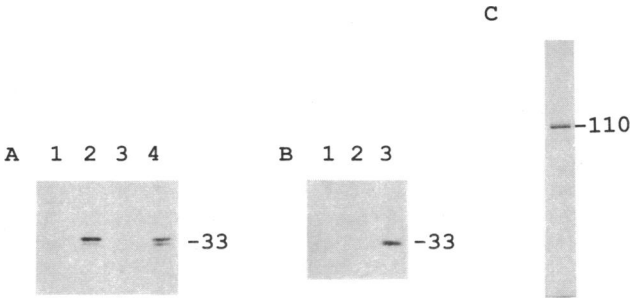

FIG. 1. Characterization of anti-cdk2 antibodies and the baculovirus-expressed RB protein. (*A*) cdk2 was immunoprecipitated from CV-1 (lanes 1 and 2) or U937 (lanes 3 and 4) cell lysate with affinity-purified anti-cdk2 antibodies (lanes 2 and 4) or anti-cdk2 antibodies that had been preabsorbed with an excess of peptide antigen (lanes 1 and 3). Western blot analysis was performed with anti-cdk2 antibodies. (*B*) Lysates prepared from wild-type *S. cerevisiae* (lane 1) or *S. cerevisiae cdc28* mutants expressing the human cdc2 gene (lane 2) or cdk2 gene (lane 3) were subjected to Western blot analysis with anti-cdk2 antibodies. (*C*) SDS/PAGE analysis of the RB protein purified by anti-RB immunoaffinity chromatography from Sf9 cells infected with pAcYM1-RB. The gel was stained with Coomassie brilliant blue. In *A–C*, the size of the major protein detected is given in kilodaltons.

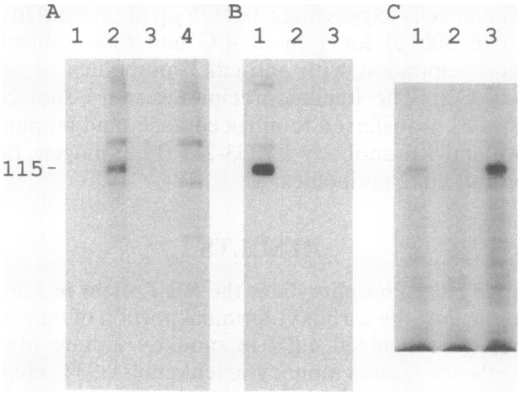

FIG. 2. Phosphorylation of the RB protein by cdk2 *in vitro*. (*A*) Phosphorylation of the RB protein by cdk2 immunoprecipitated from CV-1 cells. RB protein purified from Sf9 cells infected with pAcYM1-RB was incubated in the presence of [γ-^{32}P]ATP for 10 min at 25°C with (lanes 2–4) or without (lane 1) cdk2 immunoprecipitated from CV-1 cells. Lane 3, anti-cdk2 antibodies used for immunoprecipitation were preincubated with an excess of the peptides used for immunization; lane 4, the kinase reaction was performed in the absence of the RB protein. Samples were analyzed by SDS/PAGE followed by autoradiography. (*B*) Phosphorylation of the RB protein by cdk2 purified from *S. cerevisiae cdc28* mutant expressing the human cdk2 gene. Purified RB protein was incubated in the presence of [γ-^{32}P]ATP for 10 min at 25°C with (lanes 1 and 2) or without (lane 3) purified cdk2. Lane 2, the kinase reaction was carried out in the absence of the RB protein. (*C*) Phosphorylation of the RB protein by the coprecipitated kinase. The RB protein was immunoprecipitated from CV-1 cells with anti-peptide antibodies raised against the 14 carboxyl-terminal amino acid residues of the RB protein (lanes 1 and 2) or monoclonal anti-RB antibody C36 (lane 3). The immunoprecipitates were then incubated in the presence of [γ-^{32}P]ATP. Lane 2, anti-peptide antibodies were preincubated with the peptide used for immunization.

with anti-cdk2 antibodies showed no activity to phosphorylate the RB protein (data not shown).

Tryptic Phosphopeptide Map of the RB Protein. To compare the sites phosphorylated by cdk2 *in vitro* with those phosphorylated in living cells, we performed two-dimensional phosphopeptide mapping analysis. The RB protein immunoprecipitated from [^{32}P]orthophosphate-labeled CV-1 cells generated about 25 spots (Fig. 3*A*) with a pattern very similar to those reported by others (22). The baculovirus-expressed RB protein phosphorylated *in vitro* by cdk2 purified from *S. cerevisiae* expressing human cdk2 gave a peptide map remarkably similar to that of the RB protein labeled in CV-1 cells, although its pattern was slightly simpler, with about 17 spots (Fig. 3*B*). A quite similar pattern was also generated from the RB protein phosphorylated by cdk2 immunoprecipitated from CV-1 cells (data not shown). Mixing experiments confirmed that most of the *in vitro* labeled phosphopeptides comigrated with spots yielded by the RB protein labeled in CV-1 cells (Fig. 3*D*). These results revealed that purified cdk2 phosphorylated the RB protein at most of the sites phosphorylated in living cells.

Activation of cdk2 in the Cell Cycle. We next investigated the time course of activation of cdk2 in the cell cycle. CV-1 cells were arrested in the G_0 phase by incubation in medium that lacked methionine for 2 days and were then induced to proliferate by methionine refeeding. At the times indicated in Fig. 4, cdk2 was immunoprecipitated from CV-1 cells and its ability to phosphorylate the RB protein *in vitro* was measured. The activity of cdk2 was found to be enhanced 6–8 hr after addition of methionine to arrested CV-1 cells (Fig. 4*B*). In a parallel experiment, the phosphorylation state of the RB protein was examined by Western blot analysis with anti-RB antibody (Fig. 4*A*). Phosphorylated forms of the RB protein were also detected as bands migrating slower than the underphosphorylated form on SDS/PAGE 6–8 hr after addition of methionine to CV-1 cells. Thus the time course of activation of cdk2 in the cell cycle was similar to that of the initiation of phosphorylation of the RB protein.

Association of the RB Protein with Kinase Activity. When the RB protein was immunoprecipitated from CV-1 cells and subjected to protein kinase assay, phosphorylation of the RB protein was detected, suggesting that the protein kinase(s) coprecipitated with the RB protein (Fig. 2*C*). This kinase activity was precipitated with antibodies directed against different epitopes—namely, monoclonal antibody C36 and anti-peptide antibodies against the carboxyl-terminal portion of the RB protein. The kinase activity was not precipitated when antibodies were blocked with the peptide used for immunization. Two-dimensional tryptic phosphopeptide mapping showed that most spots generated from the RB

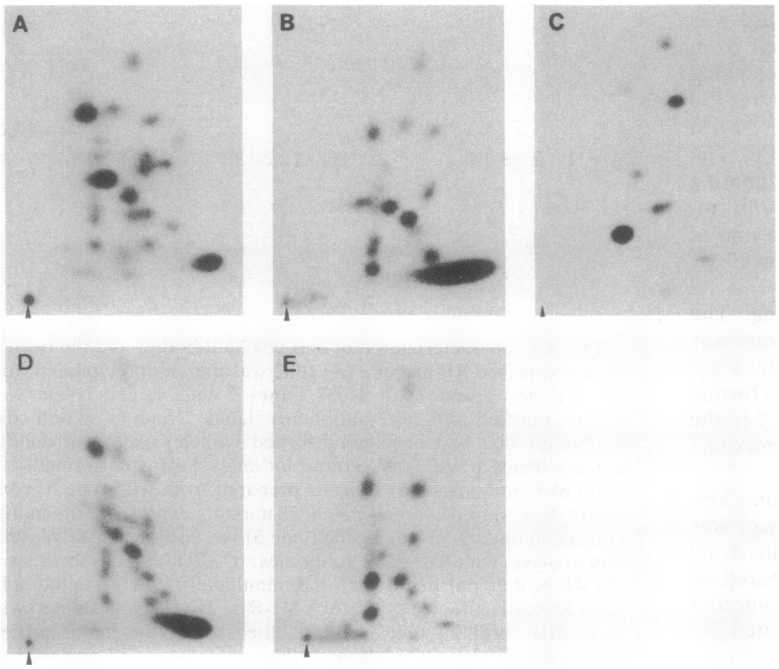

FIG. 3. Two-dimensional tryptic phosphopeptide mapping of the RB protein. (*A*) Map of the RB protein phosphorylated in CV-1 cells. The RB protein was immunoprecipitated from CV-1 cells labeled with [^{32}P]orthophosphate for 4 hr. (*B*) Map of the RB protein phosphorylated with purified cdk2 *in vitro* as described in Fig. 2*B*. (*C*) Map of the RB protein phosphorylated by the coprecipitated kinase *in vitro* as described for Fig. 2*C*, lane 3. (*D*) Mixture of the peptides in *A* and *B*. (*E*) Mixture of the peptides in *A* and *C*. Origin is indicated by arrowheads in the lower left corner of each autoradiogram.

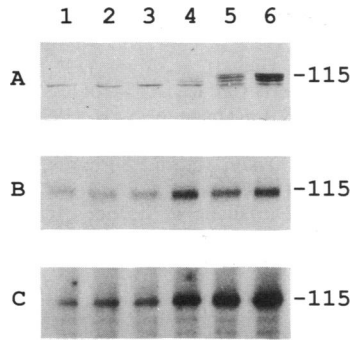

FIG. 4. Activation of cdk2. CV-1 cells were starved in methionine-free DMEM containing 2% dialyzed calf serum for 48 hr (lane 1). The cells were then fed with complete DMEM containing 10% calf serum for 2 hr (lane 2), 4 hr (lane 3), 6 hr (lane 4), 8 hr (lane 5), and 10 hr (lane 6) and samples were taken at the indicated times. (*A*) CV-1 cells were solubilized in solubilizing buffer containing 0.5% sodium deoxycholate and the lysates were subjected to SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-RB monoclonal antibody PMG3-245. (*B*) CV-1 cells were solubilized in solubilizing buffer, cdk2 was immunoprecipitated, and its ability to phosphorylate the RB protein was measured *in vitro* as described for Fig. 2*A*. (*C*) RB protein was precipitated and *in vitro* kinase reactions were carried out as described for Fig. 2*C*.

protein phosphorylated by the coprecipitated kinase coincided with those obtained from the RB protein phosphorylated in CV-1 cells and *in vitro* by cdk2, though the pattern obtained was simpler (Fig. 3*C* and *E*). This RB-associated kinase activity was also enhanced 6–8 hr after addition of methionine to CV-1 cells that had been arrested in the G_0 phase by depletion of methionine as described above (Fig. 4*C*), suggesting that cdk2 or a cdk2-related kinase may be associated with the RB protein.

Association of the RB Protein with cdk2 *in Vitro*. We next tested whether cdk2 could form a complex with the RB protein *in vitro*. For this purpose, cdk2 was expressed in *E. coli* and purified by affinity chromatography on p13-agarose. Purified cdk2 was incubated with the affinity-purified baculovirus-expressed RB protein and then immunoprecipitated with anti-cdk2 antibodies, and the immunoprecipitate was subjected to Western blot analysis using anti-RB antibody. The RB protein was detected in the immunoprecipitate (Fig. 5, lane 2), suggesting coprecipitation of the RB protein with cdk2. When a lysate of *E. coli* expressing cdk2 and a lysate of Sf9 cells expressing the RB protein were mixed and subjected to immunoprecipitation with anti-cdk2 antibodies, we also detected the RB protein in the immunoprecipitates by Western blot analysis (Fig. 5, lane 6). No coprecipitation was observed when the anti-cdk2 antibodies were preincubated with the peptides used for immunization (Fig. 5, lane 8). In addition, when, instead of the RB protein, bovine serum albumin was incubated with cdk2, no coprecipitation was observed (data not shown). On the contrary, when the RB protein was immunoprecipitated with anti-RB antibody, cdk2 was detected in the immunoprecipitates by Western blot analysis (Fig. 5, lane 11).

Since cellular proteins including transcription factor E2F have been reported to compete with DNA tumor virus oncoproteins for binding to the RB protein (refs. 6–8 and 10; unpublished observation), we tested the effect of SV40 large T antigen on formation of the complex between the RB protein and cdk2. For this test, we preincubated lysates of Sf9 cells expressing the RB protein with those expressing large T antigen. Interestingly, preincubation with T antigen did not inhibit coprecipitation of the RB protein with cdk2 (Fig. 4, lane 9). In addition, a mutant RB protein that lacks the carboxyl-terminal half of the T-antigen-binding domain (amino acid residues 666–928) was also coprecipitated with cdk2 (Fig. 5, lane 7). These results suggest that cdk2 binds to the RB protein at a site outside the T-antigen-binding domain.

DISCUSSION

The function of the RB protein is thought to be regulated by phosphorylation; its phosphorylation at the G_1 to S transition may inactivate its function and allow cells to enter S phase. Therefore, the kinase responsible for phosphorylation of the RB protein must be very important for regulating the G_1 to S transition. The G_1 to S transition in higher eukaryotes is regulated by cdk2, whereas the G_2 to M transition is controlled by cdc2 kinase (26). Thus, an interesting possibility is that one mechanism by which cdk2 regulates the G_1 to S transition is by phosphorylation of the RB protein. In this study, we showed that cdk2 phosphorylated the RB protein at the sites phosphorylated in living cells and that the time course of its activation in the cell cycle was parallel to that of the onset of phosphorylation of the RB protein in the cells. Further, we obtained evidence for association of cdk2 with the RB protein. These observations suggest that the kinase that phosphorylates the RB protein at about the time of the G_1 to S transition is likely to be cdk2 or a closely related kinase, although it may be difficult to identify this RB kinase unequivocally, since there are many cdc2 and cdk2-related kinases (29).

Very recently, Hu *et al.* (23) also showed that kinase activity was coprecipitated with the RB protein. They identified this kinase as cdc2 kinase or a closely related enzyme probably present as a complex with cyclin A. However, the initial phosphorylation of the RB protein occurs before the G_1 to S transition, several hours before cyclin A is expressed, suggesting that some other related kinase(s) contributes to phosphorylation of the RB protein in the late G_1 phase. The

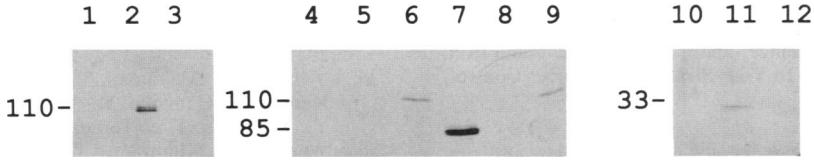

FIG. 5. Association of the RB protein with cdk2 *in vitro*. Lanes 1–9 show detection of the RB protein in cdk2 immunoprecipitates. The following samples were incubated for 1 hr at 4°C and immunoprecipitated with anti-cdk2 antibodies, and the immunoprecipitates were subjected to SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-RB antibody PMG3-245: Lane 1, purified RB protein; lane 2, a mixture of the purified RB protein and cdk2; lane 3, purified cdk2; lane 4, a mixture of lysates prepared from pAcYM1-RB-infected Sf9 cells and control *E. coli* cells; lanes 5–9, mixtures of lysates prepared from *E. coli* cells expressing cdk2 and from control Sf9 cells (lane 5), pAcYM1-RB-infected Sf9 cells (lanes 6 and 8), pAcYM1-ΔRB-infected Sf9 cells (lane 7), and pAcYM1-RB-infected Sf9 cells that had been preincubated with lysates prepared from pAc373T2-infected Sf9 cells (lane 9). For lane 8, anti-cdk2 antibodies preabsorbed with the immunizing peptide were used for immunoprecipitation. Lanes 10–12 show detection of cdk2 in RB immunoprecipitates. The following samples were subjected to immunoprecipitation with anti-RB antibody C36 and then to immunoblotting analysis with anti-cdk2 antibodies. Lanes 10 and 11, a mixture of lysates from pAcYM1-RB-infected Sf9 cells and control *E. coli* cells (lane 10) or *E. coli* cells expressing cdk2 (lane 11); lane 12, a mixture of lysates from control Sf9 cells and *E. coli* cells expressing cdk2.

antibody used by Hu *et al.* (23) did not crossreact with cdk2, since the amino acid sequence of the epitope, the carboxyl-terminal portion of the cdc2 kinase, is different from that of cdk2 (ref. 29; unpublished observation). On the other hand, the anti-cdk2 antibodies used in this study did not react with cdc2 kinase (Fig. 1*B*, lane 2). Taken together, these observations suggest that the RB protein is phosphorylated by cdk2 or a cdk2-related kinase in the late G_1 and S phases and is further phosphorylated by cdc2 kinase or a closely related kinase in the S, G_2, and M phases. However, several phosphorylation sites cannot be explained by these kinases, although most of the sites phosphorylated in the cell were phosphorylated by cdk2 and cdc2 kinases *in vitro*. Hu *et al.* (23) also detected sites phosphorylated in the cell that could not be phosphorylated by cdc2 kinase *in vitro*. These results suggest that some other kinase(s) besides cdk2- and cdc2-related kinases is also involved in phosphorylation of the RB protein.

We obtained the following evidence that cdk2 is associated with the RB protein. (*i*) The RB-associated kinase was shown to phosphorylate the RB protein at the sites phosphorylated by cdk2. The fewer spots detected in the peptide map may be explained by the fact that, in the assay of the RB-associated kinase, the RB protein is complexed with antibody C36, which may make some of the phosphorylation sites inaccessible to the kinase. (*ii*) The time course of activation of the RB-associated kinase was similar to that of cdk2. (*iii*) cdk2 was shown to associate with the RB protein *in vitro* and this interaction was not inhibited competitively by the large T antigen. However, we could not detect cdk2 in the immunoprecipitate from CV-1 cells with anti-RB antibodies. This may be because our anti-cdk2 antibodies were not suitable for detecting a small amount of RB-associated cdk2, although it is also possible that cdk2 is not associated with the RB protein in the cell. Thus, if cdk2 is associated with the RB protein, the amount associated must be much less than that associated with the cyclin A/E2F/107-kDa protein complex (40, 41). Hu *et al.* (23) also reported that the amount of either cdc2 kinase or cyclin A associated with the RB protein is very small.

There are several possible explanations for the significance of association of RB protein with kinases. This association might localize the kinase to the RB protein and induce specificity. Alternatively, the RB protein might act as an anchor targeting the kinase to other cellular proteins that associate with the RB protein. It is also possible that the RB protein regulates the activity of the kinase. These possibilities are not mutually exclusive. The complex composed of the RB protein and associated proteins might be important in cell cycle control, as in the case of association of tyrosine kinase with substrate proteins possessing an Src-homology 2 domain.

We thank Dr. E. Harlow and Dr. M. K. Bradley for providing anti-RB antibody C36 and pAc373T2, respectively. We thank Dr. K. Sugimoto for helpful discussion. We appreciate the technical assistance of A. Tokuoka. This work was supported by grants-in-aid from the Ministry of Education, Science, and Culture of Japan and from Welfare for a Comprehensive 10-Year Strategy for Cancer Control, Japan.

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