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IN VITRO PHOSPHORYLATION OF THE TUMOR SUPPRESSOR GENE RB PROTEIN BY MITOSIS-SPECIFIC HISTONE H1 KINASE

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SUMMARY: The major components of the mitosis-specific histone H1 kinase are CDC2 kinase and cyclin and the consensus amino acid sequence for phosphorylation by this enzyme has been proposed. We have noted the presence of such sequences in six sites of the tumor suppressor gene RB protein and determined whether or not RB protein is in fact phosphorylated by this kinase. Highly purified enzyme was used for this purpose. HeLa cell extracts immunoprecipitated with anti-RB antiserum as well as RB proteins expressed in *E. coli* cells were shown to be phosphorylated by this kinase *in vitro*. Synthetic peptides for the six expected sites were also phosphorylated. These results suggest the possibility that the function of RB protein is regulated by CDC2 kinase.

Eukaryotic cells contain a so-called "mitosis-specific" or "growth-associated" histone H1 kinase (H1 kinase) that is most active at metaphase. This kinase has recently been shown to be identical to the M-phase promoting factor (MPF) (1,2) and shown to contain $p34^{cdc2}$ and cyclin as major components (1-8). In the case of histone H1, four to five phosphorylation site sequences for this kinase, which are of the type Lys-Ser/Thr-Pro-Lys or Lys-Ser/Thr-Pro-X-Lys (9), have been identified in calf thymus histone H1. In trout histone H1, all of the identified phosphorylation sites have the sequence Lys-Ser-Pro-Lys (10). Phosphorylation sites similar to those in histone H1 have also been identified in chicken erythrocyte histone H5 although lysine residues are sometimes replaced by arginine residues (11). We also found that H1

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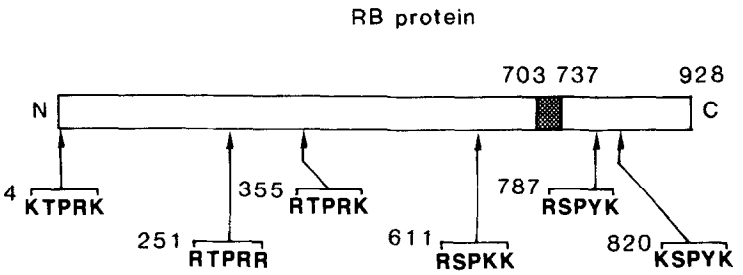

Fig. 1. Schematic presentation of the location of consensus sequences for phosphorylation by H1 kinase in the RB protein. Numbers represent positions from the N-terminus. The shaded area (703-737) is a region involved in E1A binding (21).

kinase purified from mouse FM3A cells recognize the sequence Lys-Ser/Thr-Pro (Yasuda, H., Kamijo, M., Bradbury, E.M. and Ohba, Y., manuscript in preparation).

When we searched for similar amino acid sequences in cancer-related proteins functioning in the cellular nucleus, we noted the presence of such sequences in six regions of the tumor suppressor gene RB protein (Fig. 1). Thus, we investigated whether or not RB protein can be phosphorylated by H1 kinase.

The RB gene was first cloned by Friend *et al.* (12) and its nucleotide sequence has been determined (13,14). Tumor suppressor activity of this gene was also demonstrated by Su Huang *et al.* (15). The RB protein was shown to be a nuclear phosphoprotein which has molecular weight of 110-114 Kd, and which is directly or indirectly bound to DNA (13). It was also found that the RB protein forms a specific complex with the adenovirus E1A protein (16) or SV40 large T antigen (17) or the human papilloma virus-16 E7 protein (18).

MATERIALS AND METHODS

H1 kinase: This enzyme was highly purified from mouse FM3A cells to a degree of about 700 fold using a synthetic peptide AKAKKTPKAKK as a substrate. The detail of purification and characterization will be reported elsewhere (Yasuda, H., Kamijo, M., Peter, P.M., Bradbury, E.M. and Ohba, Y., manuscript in preparation). The purified kinase showed major protein bands of 62 Kd, 45 Kd and 33 Kd in SDS-polyacrylamide gel electrophoresis. It also showed high substrate specificity to histone H1 and, in contrast, casein, angiotensin II, phosvitin, core histones and S6 peptide were not phosphorylated.

Immunoprecipitation of RB protein from HeLa cells: HeLa cells of subconfluent culture (2×10^6 cells) were lyzed with RIPA buffer and immunoprecipitated with anti-RB antisera and 20 µl of protein A-

Sepharose as described previously (16,19). Rabbit polyclonal anti-RB antisera used here were raised against a synthetic peptide of the carboxy-terminal 15 residues of the RB protein (gift from Drs. M. Ikeda, N. Tsuchida and K. Oda).

Expression of a protein fragment of RB protein in E. coli: A 1.95 Kb *Bsp*H1-*Hin*dIII fragment which contained the C-terminal coding region and 3'-noncoding region of the RB gene was excised from a RB-gene cDNA clone p4.95 BT (12) and inserted into the *Nco*I-*Hin*dIII site of pKK233-2. An *E. coli* clone which contains this construct pRB-BSP234 was cultured to mid-log phase and induced by 0.5 mM IPTG for 2 hrs at 37°C.

Synthetic peptides: Synthesis was performed with a model 431A peptide synthesizer (Applied Biosystems).

RESULTS

Phosphorylation of HeLa cell RB proteins.

RB proteins were immunoprecipitated from HeLa cells and tested for phosphorylation by purified H1 kinase (Fig. 2). A phosphorylated band of approximately 110 Kd which corresponds to the size of RB protein was detected in the presence of H1 kinase, but not detected without H1 kinase. Thus, RB proteins of HeLa cells were presumably phosphorylated *in vitro* by H1 kinase.

Phosphorylation of RB proteins expressed in E. coli.

The construct pRB-BSP234 is expected to produce a protein of 234 residues which corresponds to the C-terminal portion of the RB protein. Expression of such a protein was confirmed by immunoblotting as shown in Fig. 3A. The molecular weight of the detected band (30 Kd) is somewhat larger than that expected from the number of amino acid residues. However, this is reasonable because this region of RB protein contains many proline residues and proline-rich proteins usually have higher apparent molecular weight. This region also contains two consensus sequences for H1 kinase (Table 1, RB peptide E and F).

Phosphorylation of crude extracts from such expressed *E. coli* cells by H1 kinase was carried out as shown in Fig. 3B. A strongly phosphorylated band of 32 Kd was detected in the case of extracts induced by IPTG but a very faint band was detected by the extract of *E. coli* without induction, indicating that RB proteins expressed in *E. coli* is phosphorylated by H1 kinase. The phosphorylated band has significantly slower mobility than the band observed in immunoblotting. However, it is expected that the phosphorylated form has a slower mobility.

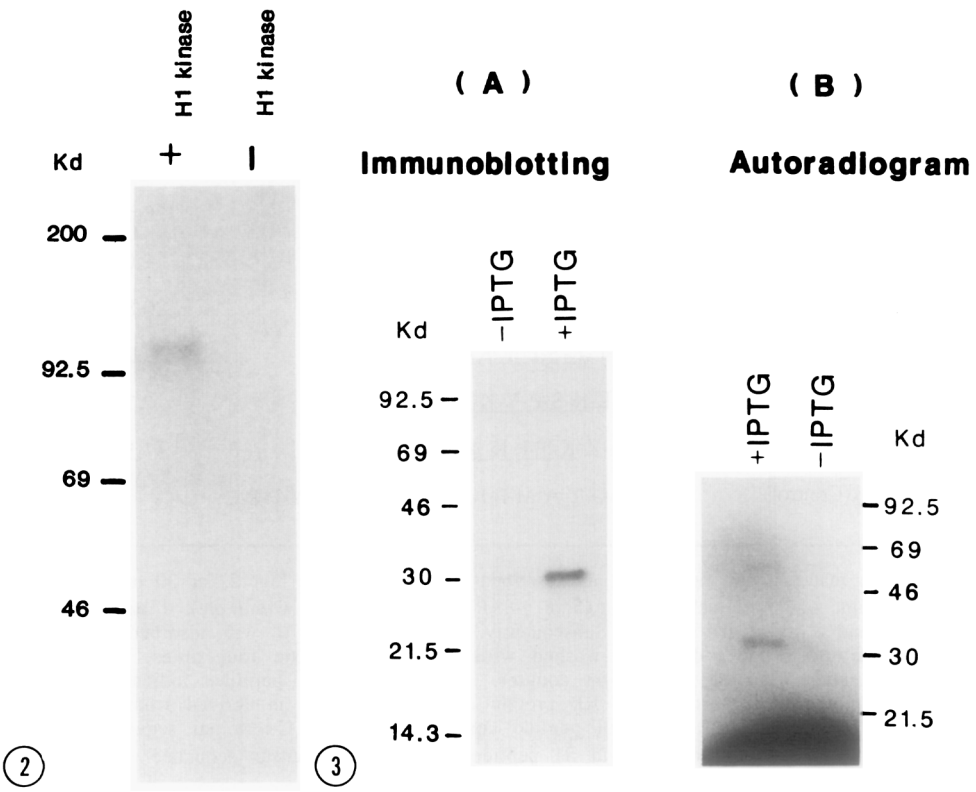

Fig. 2. Phosphorylation of the RB protein of HeLa cells by H1 kinase. Reaction mixture of 40 µl contained 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$,0.1 mM EGTA, 10 mM β-mercaptoethanol, 10 µCi γ-^{32}P-ATP (Amersham; specific activity 3,000 Ci/mmole), 8 µl H1 kinase and 20 µl of immunoprecipitates described in MATERIALS AND METHODS. The reaction was performed for 2 hrs at 30°C and washed with RIPA buffer three times, boiled for 5 min. in 70 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1 M dithiothreitol and 0.004% bromphenol blue, and then analyzed by 8% SDS-PAGE and autoradiography for 14 hrs with a Kodak XAR-5 film.

Fig.3. Phosphorylation of the RB protein expressed in *E. coli*. Cells were collected from 1.5 ml of culture induced as described in MATERIALS AND METHODS and suspended in 0.5 ml of a solution containing, 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM $MgCl_2$ and 5 µg/ml each of leupeptine, antipain, chymostatin and pepstatin, sonicated and then centrifuged for 10 min. at 15,000 rpm by a microcentrifuge. The resultant supernatant was used for (A) immunoblotting or (B) phosphorylation. (A) 10 µl of supernatant was electrophoresed by 14% SDS-PAGE and immunoblotted using Vectastain ABC kit according to the method of the supplier. (B) 4.5 µl of supernatant was incubated in a total volume of 15 µl with 8 µl of H1 kinase, 10µCi γ-^{32}P-ATP (ICN; specific activity 7,000 Ci/mmole) in addition to a buffer and salts described in the legend to Fig. 2. After incubating for 20 min. at 30°C, reaction mixture was directly analyzed by 14% SDS-PAGE and autoradiography for 10 min. with a Kodak XAR-5 film. Strong radioactivity in the bottom of the gel was caused by non-reacted ATP.

Table 1. Assay for phosphorylation of synthetic peptides using phosphocellulose filters

		cpm
RB-A	^{1}M-P-P-K-T-P-R-K-T-A-A-T-A-A^{14}	20,450
RB-B	^{246}I-N-G-S-P-R-T-P-R-R-G-Q-N-R^{259}	28,130
RB-C	^{351}F-E-T-Q-R-T-P-R-K-S-N-L-D-E^{364}	2,010
RB-D	^{607}L-S-P-V-R-S-P-K-K-K-G-S-T^{619}	20,800
RB-E	^{783}P-H-I-P-R-S-P-Y-K-F-P-S-S^{795}	7,240
RB-F	^{816}I-S-P-L-K-S-P-Y-K-I-S-E-G^{828}	2,700
S1(H1)	A-K-A-K-K-T-P-K-A-K-K	37,570
C1(Control)	C-L-V-G-T-A-M-F-I-A-S-K-Y-E-E-M-Y-P-P-E	0

The reaction was carried out as described in the legend for Fig. 2 for 30 min. in a total volume of 20 µl including 1 µCi of γ-^{32}P-ATP (ICN, 7000 Ci/mmole), 1 µg of peptide and 2 µl of H1 kinase. Subsequently, an aliquot of 10 µl was adsorbed to a phosphocellulose paper disc and washed with 75 mM phosphate four times, and then counted using a liquid scintillation counter. Numbers in RB peptides indicate positions from the N-terminus of RB protein (13). S1 peptide is derived from the sequence of histone H1. Control peptide corresponds to the C-terminal sequence of p34^{cdc2}. Underlining of RB and S1 peptides show the consensus sequence.

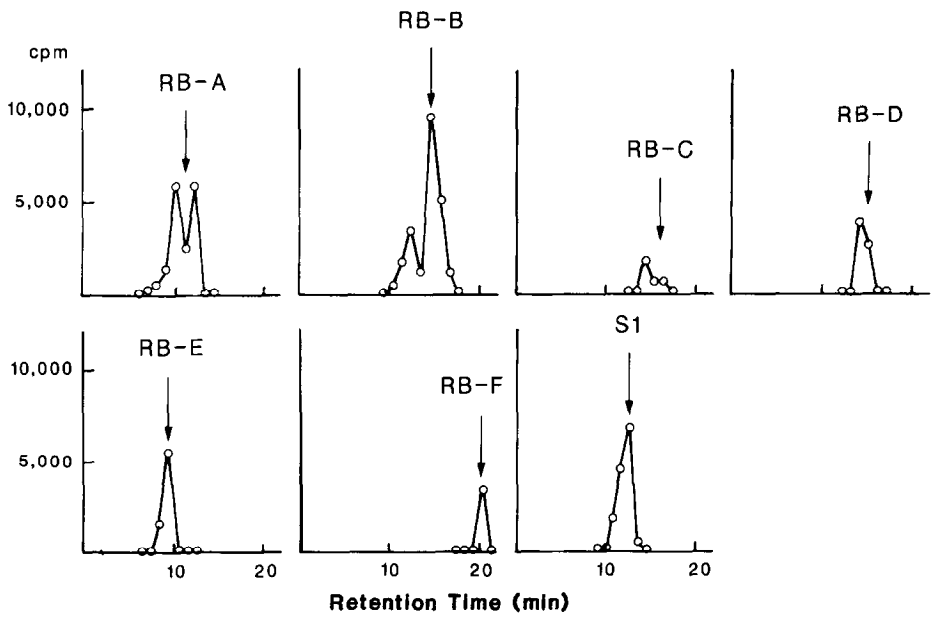

Fig. 4. Analysis of phosphorylated peptides by HPLC. 5 µl of reaction mixtures described in Table 1 were mixed with 5 µg each of non-reacted peptide and subjected to HPLC using a Cosmosil packed column 5C18-300 (Nakarai Tesk Co. Ltd). After washing with 0.1 % trifluoroacetic acid peptides were eluted with a gradient of acetonitrile (0-50%). Fractions of 1 ml were collected and counted using a liquid scientillation counter. Arrows indicate the position of non-reacted peptides.

Phosphorylation of synthetic peptides.

Six peptides of the RB protein which contain the consensus sequences for H1 kinase substrates were synthesized and tested for phosphorylation. First, a filter assay using phosphocellulose paper was performed. As shown in Table 1, all the RB peptides were phosphorylated, although phosphorylation was weak with two peptides (C and F). In contrast, no phosphorylation was detected using control peptide C1. As it is possible that peptides with a high content of acidic residues did not adsorb to the phosphocellulose paper, peptides were also analyzed by HPLC after phsphorylation . As shown in Fig. 4, the phosphorylation of two peptides, C and F, were also weak compared to other peptides.

DISCUSSION

We have shown that the RB protein is phosphorylated by H1 kinase *in vitro*. Putative phosphorylation sites are present in six places. Experiments to determine the precise phosphorylation sites *in vitro* and to determine whether or not identical sites are phosphorylated *in vivo* in M-phase are in progress.

Ludlow *et al.* (20) observed preferential binding of SV40 large T antigen to underphosphorylated RB protein, suggesting that phosphorylated form of the RB protein is inactive and nonphosphorylated form is active in the growth suppression function. It will be interesting to determine the relationship between the phosphorylation they detected and that which we have shown here. If they are identical, phosphorylation of the RB protein by H1 kinase could be involved in release of the cells from growth suppression by nonphosphorylated RB protein. It is also interesting to speculate that the RB protein is involved in regulation of cell division because H1 kinase seems to have identical role to MPF which is the major regulatory factor of cell division.

The sequence of the RB peptide A is located in the N-terminus of the RB protein and unusual clusters of alanine and proline are present adjacent to this sequence (13,14). On the other hand, sequences of peptide D, E and F are located near the region (position 703-737) involved in E1A binding (21). It will also be interesting to study their correlation.

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