Phosphorylation of Ribonucleotide Reductase R2 Protein: *In Vivo* and *in Vitro* Evidence of a Role for p34^{cdc2} and CDK2 Protein Kinases†

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ABSTRACT: Ribonucleotide reductase is responsible for supplying the deoxyribonucleotides required for DNA synthesis and repair. The active enzyme consists of two dissimilar protein components called R1 and R2. Immunoprecipitation of R1 and R2 proteins from [^{32}P]orthophosphate-labeled exponentially growing mouse L cells showed that the R2 protein but not the R1 protein of ribonucleotide reductase could be phosphorylated *in vivo*. Two-dimensional phosphopeptide mapping experiments of trypsin-digested R2 protein showed a major spot containing more than 90% of the total radioactivity and a minor spot with the remaining radioactivity. Phosphoamino acid analysis of R2 phosphorylated protein indicated that phosphorylation occurred exclusively on serine. Protein kinase C, cAMP-dependent protein kinase, p34^{cdc2}, and CDK2 were capable of phosphorylating the R2 protein *in vitro*, whereas casein kinase II was not. To determine whether any of these enzymes could phosphorylate peptides observed to be phosphorylated in actively growing cells, tryptic phosphopeptide maps of R2 that had been phosphorylated *in vitro* were compared with maps of R2 that had been isolated from [^{32}P]-labeled cells. Only the phosphopeptide maps obtained with p34^{cdc2} and CDK2 matched the pattern found in [^{32}P]-labeled cells. Experiments in which tryptic digests from different samples were mixed prior to two-dimensional separation demonstrated comigration of phosphopeptides obtained by *in vivo* phosphorylation with phosphopeptides derived from p34^{cdc2} or CDK2 obtained by *in vitro* phosphorylations. These studies indicate that protein R2 phosphorylation may play an important role in the regulation of ribonucleotide reduction, DNA synthesis, and cell cycle progression, and suggest a potentially important p34^{cdc2} and/or CDK2 regulation point in DNA replication.

Mammalian ribonucleotide reductase consists of two dissimilar protein components often called R1 and R2 (Reichard, 1988; Wright *et al.*, 1990; Hurta & Wright, 1992a). Protein R1 is a dimer with a molecular weight of 170 000 and contains substrate and effector binding sites (Thelander *et al.*, 1980). Protein R2 is also a dimer, has a molecular weight of 88 000, and contains a non-heme iron which stabilizes a tryosyl free radical required for the reduction process (McClarty *et al.*, 1990). The activity of ribonucleotide reductase correlates closely with DNA synthesis (Lewis *et al.*, 1978; Weber, 1983), and in actively proliferating cells, S phase appears to be dependent upon synthesis of the R2 component, which is rate-limiting for ribonucleotide reduction (Wright *et al.*, 1990; Bjorklund *et al.*, 1990). Alterations in ribonucleotide reductase are associated with major changes in the biological properties of cells (Wright, 1989). For example, altered ribonucleotide reductase has been reported as a mutator locus in mammalian cells (Weinberg *et al.*, 1981). There is evidence that the enzyme may be involved in certain immunodeficiency diseases in man (Ullman *et al.*, 1979). It is important in DNA repair, and in critical early events in mechanisms of tumor promotion (Hurta & Wright, 1992a,b; Chen *et al.*, 1993), and the levels of enzyme activity have been correlated with tumor progression (Weber, 1983). Alterations in ribonucleotide reductase gene expression are also observed in malignant cells in response to growth factor treatment (Hurta *et al.*, 1991; Amara *et al.*, 1993; Wright *et al.*, 1993).

In addition, protein phosphorylation mechanisms appear to play a potentially important role in the regulation of ribonucleotide reductase. For example, rapid elevation of enzyme activity, R1 and R2 messages, and R2 protein was observed when mouse fibroblasts were treated with the protein phosphatase inhibitors okadaic acid and calyculin A (Hurta & Wright, 1992b). Also, there is evidence that the enzyme is phosphorylated in S49 T lymphoblasts and that phosphorylation *in vitro* can be carried out by cyclic AMP-dependent protein kinase, in keeping with a proposed regulatory relationship between cyclic AMP and ribonucleotide reductase in some cells (Albert & Nodzenski, 1989; Hurta & Wright, 1993). In the present study, we investigated the phosphorylation of ribonucleotide reductase in actively growing mouse L cells, and provide evidence for the direct phosphorylation of the R2 protein by p34^{cdc2} and CDK2 protein kinases, mediators of G_2/M- and G_1/S-phase transition events (Maller, 1990; Nurse, 1990; Elledge *et al.*, 1992; Gu *et al.*, 1992).

EXPERIMENTAL PROCEDURES

Materials

[^{32}P]Orthophosphate and [γ-^{32}P]ATP were obtained from Amersham Ltd. (Oakville, Ontario), and [^{35}S]methionine (Tran [^{35}S]-label) was obtained from ICN Biomedical (St. Laurent, Quebec). Fetal bovine serum (Fetalclone II) was supplied by Hyclone (Logan, UT). The catalytic subunit of cAMP-dependent protein kinase was from Sigma Chemical Co. (St. Louis, MO). Protein kinase C and heat-killed, formalin-fixed *Staphylococcus aureus* cells (Pansorbin) were

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obtained from Calbiochem (La Jolla, CA). Rabbit polyclonal antibody specific to CDK2 was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Synthetic peptide corresponding to an epitope within the C-terminal domain (residues 287–298 of human CDK2) was used as antigen in preparation of the antibody. This antibody specifically recognizes human and mouse CDK2 ($p33^{cdk2}$) protein kinases but does not recognize the related human and mouse $p34^{cdc2}$ protein kinases, as determined in Western and immunoprecipitation experiments. Furthermore, $p34^{cdc2}$-specific antibody prepared against a C-terminal peptide (residues 290–297 of $p34^{cdc2}$) as described previously (Litchfield *et al.*, 1991, 1992) was unable to recognize protein immunoprecipitated by the CDK2-specific antibody. Casein kinase II was purified as previously described (Litchfield *et al.*, 1990) and had a specific activity exceeding 1 μmol min^{-1} mg^{-1} when assayed at 30 °C using a synthetic peptide substrate (Arg-Arg-Arg-Asp-Asp-Asp-Ser-Asp-Asp-Asp). The $p34^{cdc2}$ protein kinase was highly purified from nocodazole-arrested human Jurkat T cells using 15–40% ammonium sulfate fractionation followed by gel filtration on Sephacryl S-300HR (Pharmacia, Baie d'Urfe, Quebec), and then ion exchange on DEAE-Sephacel (Pharmacia), Econo Q (Bio-Rad Laboratories, Mississauga, Ontario), and finally Econo S (Bio-Rad Laboratories).[1] $p34^{cdc2}$ was monitored by assaying kinase activity with a synthetic peptide substrate (Gln-Leu-Gln-Leu-Gln-Ala-Ala-Ser-Asn-Phe-Lys-Ser-Pro-Val-Lys-Thr-Ile-Arg) as previously described (Litchfield *et al.*, 1991). The presence of $p34^{cdc2}$ in active fractions was confirmed on immunoblots developed with antibodies against a C-terminal peptide of $p34^{cdc2}$ (Litchfield *et al.*, 1991). The purified material had a specific activity of 0.33 μmol min^{-1} mg^{-1} when assayed at 30 °C using histone H1 (Boehringer Mannheim, Laval, Quebec) as substrate. This preparation exhibited no activity toward a synthetic peptide substrate of casein kinase II (Arg-Arg-Arg-Asp-Asp-Asp-Ser-Asp-Asp-Asp) (Litchfield *et al.*, 1990) or a synthetic peptide (Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala) that is based on phosphorylation sites on ribosomal protein S6 that can be phosphorylated *in vitro* by a number of protein kinases (including cAMP-dependent protein kinase, protein kinase C, and also growth factor-activated protein kinases) (Ahn *et al.*, 1990; Kemp & Pearson, 1991). Trypsin (TPCK-treated) was obtained from Worthington (Freehold, NJ). Thin-layer chromatography (TLC) plates for phosphoamino acid analysis and phosphopeptide mapping were from Merck (Darmstadt, Germany). Okadaic acid was from Gibco/BRL (Burlington, Ontario). Anti-R2 antiserum was obtained from New Zealand rabbits immunized with recombinant mouse R2 protein (Mann *et al.*, 1991), and anti-R1 monoclonal antibody (AD203) was obtained from InRo Biomedtek (Umea, Sweden).

Methods

Cell Culture and Labeling. Mouse L cells were routinely cultured in α-modified minimal essential medium (α-MEM) supplemented with 8% fetal bovine serum (FBS) (Fetalclone II, Hyclone) incubated at 37 °C in a 5% CO_2 atmosphere. For metabolic labeling with [^{35}S]methionine, methionine-free Dulbecco's modified Eagles's medium (D-MEM) (Gibco) containing 50 μCi/mL [^{35}S]methionine and 10% FBS was added to exponentially growing cells and then incubated at 37 °C for 3 h. For labeling with [^{32}P]orthophosphate, phosphate-free D-MEM containing 100 μCi/mL [^{32}P]orthophosphate and 10% dialyzed FBS was added to cells, which were then incubated at 37 °C for 3 h.

Expression and Purification of Recombinant R2 Protein. Mouse recombinant R2 protein was prepared from bacteria containing the plasmid pETM2 according to the method of Mann *et al.* (1991). No endogenous R2 kinase activities were detected when the partially purified R2 preparation was treated with kinase assay buffer.

Immunoprecipitation. Immunoprecipitations of the R2 protein and CDK2 were performed according to the method of Firestone *et al.* (1982) with some modifications. Labeled cells were lysed with solubilizing buffer (SB150) [25 mM Tris-HCl, pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA),[2] 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 1 mM PMSF]. The lysates were preabsorbed with *S. aureus* cells (Pansorbin) for 10 min at room temperature. For immunoprecipitation of R2 protein, 5 μL of anti-R2 rabbit antiserum was added to the sample and incubated at room temperature for 15 min, followed by the addition of 25 μL of a 10% suspension of Pansorbin. For the immunoprecipitation of CDK2 from mouse cell lysate, CDK2-specific rabbit antiserum which does not recognize $p34^{cdc2}$ protein kinase was used.

Phosphorylation of R2 Protein in Vitro by Purified Kinases. *In vitro* phosphorylation of R2 protein was carried out according to Haystead *et al.* (1990). R2 protein was immunoprecipitated and analyzed by electrophoresis on a 10% SDS–polyacrylamide gel (Laemmli, 1970). One unit of enzyme activity is the amount of protein that will transfer 1 nmol of phosphate from [γ-^{32}P]ATP to substrate per minute. Protein kinase C and $p34^{cdc2}$ activities were determined using histone H1 as substrate. Partially dephosphorylated casein was used as a substrate to measure the activities of CDK2 and cAMP-dependent protein kinase, and a synthetic peptide (Arg-Arg-Arg-Asp-Asp-Ser-Asp-Asp-Asp) was used to measure casein kinase II activity. When R2 was used as a substrate for each of these kinases, 0.72, 0.9, 0.25, 0.04, and 0.8 units of protein kinase C, casein kinase II, $p34^{cdc2}$, CDK2, and cAMP-dependent protein kinase, respectively, were added to kinase reactions.

Proteolytic and Chemical Digestion of R2 Protein. Proteins were extracted from dried polyacrylamide gels into buffer containing 50 mM NH_4HCO_3, pH 7.8, 0.1% SDS, and 1% mercaptoethanol as previously described (Beemon & Hunter, 1978; Kazlauskas & Cooper, 1989). The performic acid treated protein samples were digested with 10 μg of trypsin (TPCK-treated) at 37 °C for 18 h. An additional 5 μg of TPCK-trypsin was added, and digestion was allowed to continue for another 6 h before two-dimensional phosphopeptide analysis was performed. For phosphoamino acid analysis, the immunoprecipitated R2 protein was recovered from dried polyacrylamide gels as described (Cooper *et al.*, 1983), or from poly(vinylidene difluoride) membranes (Kamps & Sefton, 1989) and treated with 5.7 N constant-boiling HCl at 110 °C for 1 h. Samples were dried on a Speedvac concentrator, and partial hydrolysis products were analyzed by two-dimensional electrophoresis as described below.

Two-Dimensional Phosphopeptide Mapping and Phosphoamino Acid Analysis. Peptides resulting from tryptic digestion were spotted onto 10 × 10 cm cellulose TLC plates and electrophoresed at 1000 V for approximately 20 min in pH 1.9 buffer (15% acetic acid/5% formic acid) followed by ascending chromatography in solvent that contained isobutyric acid, pyridine, acetic acid, butanol, and water in a ratio of

[1] D. W. Litchfield, unpublished results.

[2] Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride.

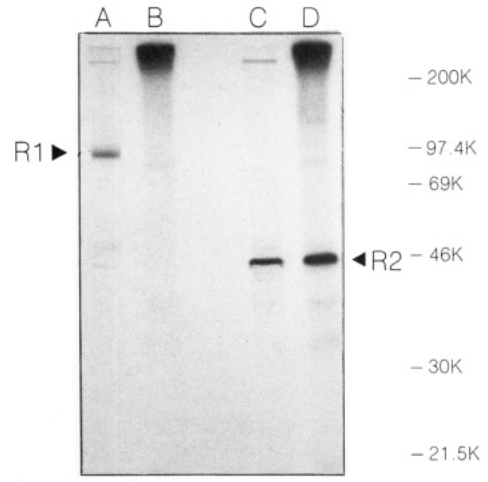

FIGURE 1: Immunoprecipitation of R1 and R2 proteins from [^{35}S]-methionine-labeled and [^{32}P]-labeled cell lysates using specific antibodies. The R1 (lanes A and B) and R2 (lanes C and D) subunits of ribonucleotide reductase were isolated by immunoprecipitation from lysates of [^{35}S]-labeled cells (lanes A and C) or from ^{32}P-labeled cells (lanes B and D). Immunoprecipitation of the R1 subunit was performed with R1-specific monoclonal antibody (AD203), and immunoprecipitation of the R2 subunit was performed using R2-specific polyclonal rabbit antibodies. Immunoprecipitates were subjected to 10% SDS–polyacrylamide gel electrophoresis. Radiolabeled proteins were detected by autoradiography. The positions of R1 (M_r 88 000) and R2 (M_r 44 000) are indicated. Molecular weight markers, from top to bottom, are as follows: myosin heavy chain, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

65:5:3:2:29, respectively (Litchfield *et al.*, 1991, 1992; Scheidtmann *et al.*, 1982). Phosphopeptides were visualized by autoradiography, and the relative intensities of the signals were determined with the aid of a densitometer (Bio-Rad, Model 620). For phosphoamino acid analysis, the partial hydrolysis products were mixed with unlabeled phosphoamino acid standards, spotted onto cellulose TLC plates, and subjected to two-dimensional electrophoresis (Cooper *et al.*, 1983). Electrophoresis in the first dimension was carried out at 1000 V for 45 min in pH 1.9 buffer (7.5% acetic acid and 2.5% formic acid) and in the second dimension at 1000 V for 20 min in pH 3.5 buffer (0.5% pyridine and 5.0% acetic acid). Phosphoamino acid standards were visualized by staining with ninhydrin.

RESULTS

Phosphorylation of Ribonucleotide Reductase R2 Protein. Ribonucleotide reductase R1 and R2 proteins were isolated by immunoprecipitation from exponentially growing cells that had been labeled with [^{35}S]methionine or with [^{32}P]orthophosphate. The immunoprecipitates were analyzed by 10% SDS–polyacrylamide gel electrophoresis and visualized by autoradiography (Figure 1). As observed in other studies, R2-specific antibody immunoprecipitated a [^{35}S]methionine-labeled protein band with a molecular weight of 44 000, corresponding to the molecular weight of the R2 component of ribonucleotide reductase (McClarty *et al.*, 1990). Interestingly, a protein band with the same molecular weight was also immunoprecipitated from cells labeled with [^{32}P]orthophosphate, providing evidence for *in vivo* phosphorylation of the R2 protein in mouse L cells. The R1-specific antibody immunoprecipitated the R1 protein with a molecular weight of 88 000 from [^{35}S]methonine-labeled cells as previously reported (Choy *et al.*, 1988). However, a band with a molecular weight corresponding to the R1 protein was not detected with [^{32}P]orthophosphate-labeled cell lysate, suggesting that the R1 protein of ribonucleotide reductase is not phosphorylated *in vivo* in mouse L cells.

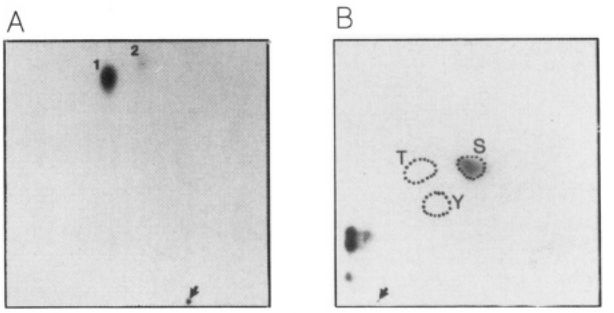

FIGURE 2: Phosphopeptide mapping and phosphoamino acid analysis of the R2 subunit. (A) *In vivo* phosphorylated R2 protein was proteolytically digested with TPCK-trypsin and analyzed by electrophoresis (horizontal dimension with –ve electrode at left) and ascending chromatography (vertical dimension). Phosphopeptides were visualized by autoradiography. Individual phosphopeptides are numbered, and the arrow indicates the origin. (B) Two-dimensional electrophoresis of partially acid-hydrolyzed R2 protein (first dimension is horizontal with –ve electrode at left). Positions of phosphoamino acid standards are marked, and the radioactive signal detected by autoradiography is associated with only phosphoserine.

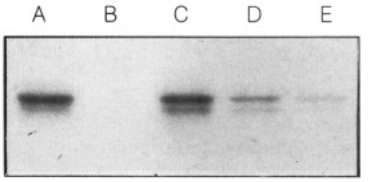

FIGURE 3: Phosphorylation of R2 protein by various purified protein kinases. Recombinant R2 protein was used as an *in vitro* substrate for purified protein kinases as follows: lane A, p34^{cdc2}; lane B, casein kinase II; lane C, cAMP-dependent protein kinase; lane D, protein kinase C; lane E, p33^{cdk2}. The products of the kinase reactions were subjected to immunoprecipitation using R2-specific antiserum. Immunoprecipitates were separated by 10% SDS–polyacrylamide gel electrophoresis. The phosphorylated R2 protein was detected by autoradiography.

Phosphopeptide and Phosphoamino Acid Analysis of in Vivo [^{32}P]Orthophosphate-Labeled R2 Protein. To further examine the *in vivo* phosphorylation of the R2 protein, two-dimensional phosphopeptide mapping was used. Phosphorylated R2 protein was recovered from 10% SDS–polyacrylamide gels and exhaustively digested with trypsin. Following electrophoresis and chromatography, tryptic phosphopeptides were visualized by autoradiography (Figure 2A), which showed one major spot containing more than 90% of the radioactivity as determined by densitometry, and one minor radioactive spot. Phosphoamino acid analysis of the phosphorylated R2 demonstrated that phosphorylation occurred exclusively on serine (Figure 2B).

Comparison of Phosphopeptide Maps of R2 Phosphorylated in Vitro by Purified Protein Kinases, with in Vivo Phosphorylated R2. In an attempt to identify the protein kinases that are responsible for R2 phosphorylation *in vivo*, recombinant R2 protein was tested as an *in vitro* substrate for the following purified protein kinases: protein kinase C, cAMP-dependent protein kinase, p34^{cdc2}, CDK2, and casein kinase II. Phosphorylated R2 was isolated from phosphorylation reactions by immunoprecipitation. Figure 3 shows that all of these enzymes, with the exception of casein kinase II, can phosphorylate R2 *in vitro*.

To determine whether any of these enzymes can phosphorylate peptides that are phosphorylated in cells, comparative phosphopeptide mapping studies were performed. Tryptic phosphopeptide maps of R2 that had been phosphorylated *in*

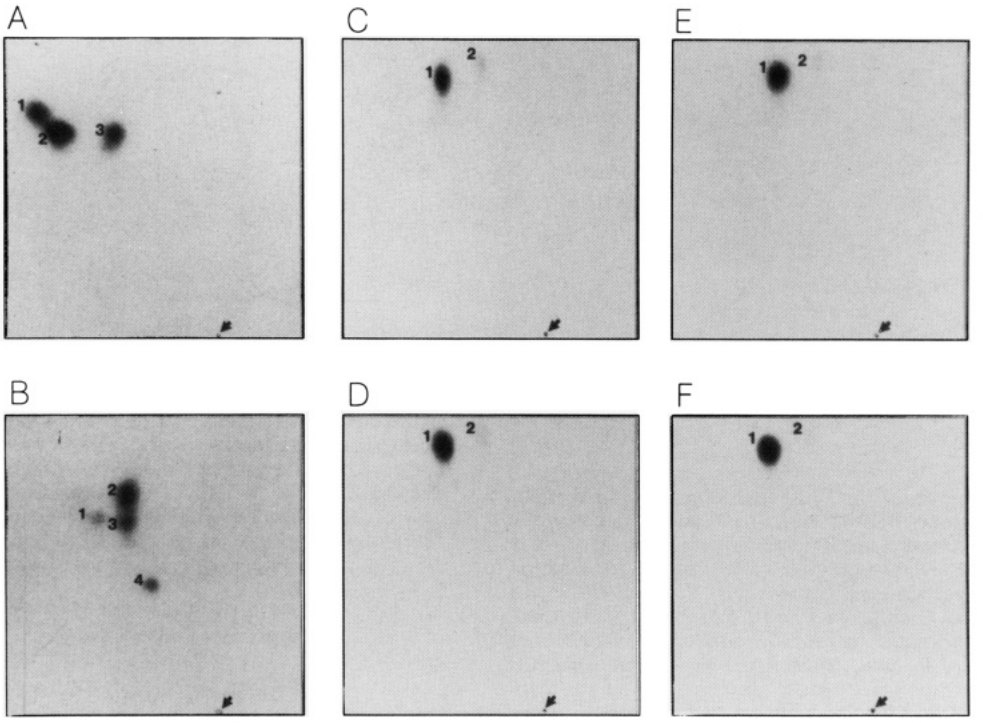

FIGURE 4: Phosphopeptide maps of phosphorylated R2 protein from *in vitro* and *in vivo* sources. R2 proteins that were phosphorylated *in vitro* by purified protein kinases or *in vivo* by the cell labeling method were exhaustively digested with trypsin. Tryptic phosphopeptides were subjected to electrophoresis (horizontal dimension) and ascending chromatography (vertical dimension). Tryptic phosphopeptides were prepared from the following samples: (A) R2 phosphorylated by purified cAMP-dependent protein kinase; (B) R2 phosphorylated by purified protein kinase C; (C) R2 phosphorylated by purified $p34^{cdc2}$; (D) R2 phosphorylated by immunopurified CDK2; (E) mix of phosphopeptides from *in vitro* phosphorylation of R2 by $p34^{cdc2}$ with phosphopeptides derived from *in vivo* phosphorylated R2 (equal cpm of each); (F) mix of phosphopeptides from *in vitro* phosphorylation of R2 by CDK2 with phosphopeptides derived from *in vivo* phosphorylated R2 (equal cpm of each). Phosphopeptides were detected by autoradiography. The origins are marked with arrows.

vitro were compared with maps of R2 that had been isolated from ^{32}P-labeled cells (Figure 1). These studies indicate that phosphorylation of R2 by cAMP-dependent protein kinase resulted in the appearance of three distinct spots of similar intensity on phosphopeptide maps (Figure 4A). Phosphorylation of R2 by protein kinase C resulted in the appearance of four spots of varying intensities with spots 2 and 3 being predominant (Figure 4B). The phosphopeptide map of $p34^{cdc2}$-phosphorylated R2 protein exhibited two spots (Figure 4C), with the intensities of the two spots unevenly distributed. The intensity of spot 1 was approximately 10-fold greater than that of spot 2 as determined by densitometry. The phosphopeptide map generated from CDK2-phosphorylated R2 (Figure 4D) was very similar to the one obtained with $p34^{cdc2}$, again showing one major spot that was approximately 10 times more intense than a minor spot. Interestingly, the phosphopeptide patterns obtained following phosphorylation of R2 with $p34^{cdc2}$ or CDK2 appeared to be identical to the pattern obtained with R2 that had been isolated from ^{32}P-labeled cells. By comparison, the phosphopeptide maps of R2 that had been phosphorylated by cAMP-dependent protein kinase or protein kinase C did not bear any resemblance to the maps obtained from R2 that had been phosphorylated *in vivo*.

To determine whether or not the phosphopeptide maps of R2 phosphorylated by $p34^{cdc2}$, or CDK2, and by *in vivo* kinases are indeed identical, mixing experiments were conducted in which tryptic digests from different samples (equal cpm) were mixed prior to two-dimensional separation. Figure 4E shows that the phosphopeptide map of the mixed sample containing peptides from *in vivo* phosphorylated R2 and $p34^{cdc2}$-phosphorylated R2 consisted of only two spots which were positioned identically to the two spots observed for the maps of the individual samples. Similarly, the mixed sample containing phosphopeptides from *in vivo* phosphorylated R2 and CDK2-phosphorylated R2 protein also demonstrated comigration of phosphopeptides derived from the two samples (Figure 4F). These results suggest that *in vitro* $p34^{cdc2}$ and CDK2 can phosphorylate the same peptides on R2 that are phosphorylated in actively growing cells.

DISCUSSION

This investigation indicates the potential importance of direct phosphorylation of the R2 protein in the regulation of ribonucleotide reduction and DNA synthesis. Although several different enzymes are capable of carrying out this phosphorylation process *in vitro*, comparative phosphopeptide mapping experiments suggest that R2 phosphorylation *in vivo* in actively growing cells is mediated by the cell cycle regulatory $p34^{cdc2}$ and CDK2 protein kinases. According to the predicted (Ser/Thr-Pro) consensus of $p34^{cdc2}$ (Maller, 1990; Nurse, 1990; Kemp & Pearson, 1991), there are 3 potential $p34^{cdc2}$ phosphorylation sites (Thr-6, Ser-20, Thr-33) within the N-terminal 34 amino acids of R2. Since phosphoamino acid analysis of the major site phosphorylated in ^{32}P-labeled cells and by $p34^{cdc2}$ and CDK2 *in vitro* indicated phosphorylation of a serine, we suggest that the major site of phosphorylation on R2 is at Ser-20. This residue is located within the sequence Ser-Pro-Leu-Lys-Arg-Leu, which also has a basic residue in the third position on the C-terminal side of the phosphorylation site. The presence of a basic residue in this position has been noted for many $p34^{cdc2}$ phosphorylation sites (Maller, 1990; Nurse, 1990; Kemp & Pearson, 1991). If Ser-20 was the major site of phosphorylation on R2, the minor spot observed on phosphopeptide maps could rise through the inability of trypsin to quantitatively cleave the protein at Lys-23 and Arg-24. The inability of trypsin to function efficiently as an

exopeptidase could result in the production of phosphopeptides terminating with Lys-23 or with Arg-24, thus producing related phosphopeptides that differed by the presence of Arg-24. The resultant peptides would differ in electrophoretic mobility and to a lesser extent in chromatographic mobility. While we have no formal proof that trypsin does indeed produce a mixture of peptides terminating with Lys-23 or with Arg-24, the appearance of two phosphopeptides with similar electrophoretic and chromatographic properties (spots 1 and 2) on two-dimensional maps is consistent with this possibility (Figure 4C–4F). Alternatively, the minor phosphopeptide could result from the phosphorylation of a different residue on R2. At present, we have not obtained any information to resolve this issue.

The phosphopeptide maps for the *in vitro* phosphorylation of R2 by cAMP-dependent protein kinase and protein kinase C did not match the *in vivo* phosphorylation pattern, indicating that they were unlikely to be important in directly regulating the enzyme during logarithmic growth. However, it is still possible that kinase activities other than $p34^{cdc2}$ or CDK2, including cAMP-dependent protein kinase or protein kinase C, may have some other role in ribonucleotide reductase control, perhaps in more specialized situations like DNA repair or during cellular differentiation. Indeed, there is evidence that cAMP-dependent protein kinase activity may play a role in ribonucleotide reductase regulation in S49 T lymphoblasts (Albert & Nodzenski, 1989), and in altered mechanisms of malignant cell proliferation (Hurta & Wright, 1993).

The demonstration that $p34^{cdc2}$ or CDK2 can phosphorylate a site that is phosphorylated in cells does not prove that these enzymes carry out this function *in vivo*. Given the recent identification of a number of proline-directed protein kinases and $p34^{cdc2}$-related kinases, it is possible that R2 could also be phosphorylated by some of these enzymes (Kemp & Pearson, 1991; Meyerson *et al.*, 1992). Nevertheless, the observation that $p34^{cdc2}$ and CDK2 can phosphorylate a physiological site on R2 suggests that the ribonucleotide reductase could be regulated by phosphorylation during different stages of the cell cycle. The mammalian $p34^{cdc2}$-like kinases are closely related to the yeast $p34^{cdc2}$/cdc28 proteins (about 65% identical), and are capable of complementing $p34^{cdc2}$ *Saccharomyces pombe* or cdc28 *Saccharomyces cerevisiae* mutants (Lee & Nurse, 1987; Norbury & Nurse, 1992). The first mammalian homolog to be characterized, $p34^{cdc2}$, is most active during mitosis and has been directly linked to the control of G_2/M transitions and perhaps but less likely to G_1/S transitions (Draetta & Beach, 1988; Th'ng *et al.*, 1990). The structurally related CDK2 has a number of characteristics that closely tie it to the $p34^{cdc2}$ family. For example, the human CDK2 protein sequence is identical to $p34^{cdc2}$ in a 16 amino acid motif (PSTAIRE sequence), that exhibits conservation among other $p34^{cdc2}$-like proteins (Meyerson *et al.*, 1992). There is evidence that CDK2 is an indispensable regulator of G_1/S transitions in higher eukaryotes. For example, depletion of CDK2 from *Xenopus* extract blocks DNA synthesis, without affecting entry into mitosis (Fang & Newport, 1991). These observations suggest that DNA replication (G_1/S) and mitosis (G_2/M) in higher eukaryotes are controlled by different but related $p34^{cdc2}$-like proteins (Meyerson *et al.*, 1992; Fang & Newport, 1991). The finding that $p34^{cdc2}$ and CDK2 kinases are capable of phosphorylating the cell cycle regulated ribonucleotide reductase R2 protein is in keeping with conceptual roles of these kinases as mediators of key transition points during cell cycle progression. Studies are underway to directly determine the effects of protein phosphorylation on ribonucleotide reduction, and to examine the R2 phosphorylation patterns during the various phases of the cell cycle, during cell differentiation, and in the presence of agents that modify ribonucleotide reductase in an S-phase uncoupled fashion. These studies should provide insight into a potentially important $p34^{cdc2}$/CDK2 regulation point in DNA replication.

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