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Biochemistry

Human p53 is phosphorylated by p60–cdc2 and cyclin B–cdc2

(recessive oncogene/p53/cell cycle phosphorylation)

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ABSTRACT **The human anti-oncoprotein p53 is shown to be a substrate of cdc2. The primary site of phosphorylation is serine-315. Serine-315 is phosphorylated by both p60–cdc2 and cyclin B–cdc2 enzymes. The phosphorylation of p53 is cell cycle-dependent. The abundance of p53 also oscillates during the cell cycle. The protein is largely absent from cells that have just completed division but accumulates in cells during G_1 phase. Phosphorylation by cdc2 might regulate the antiproliferative activity of p53.**

The polypeptide known as p53 was first described as a cellular phosphoprotein that forms a complex with the large tumor antigen (TAg) in simian virus 40 (SV40)-infected cells (1). p53 also associates with the 58-kDa product of the E1B gene in cells infected with adenovirus (2). Initially, p53 was considered an oncoprotein since expression of p53 assisted in transformation of primary rodent cells (3). Subsequently, it was found that most if not all established cell lines carry mutant forms of p53, and mutant rather than wild-type genes were used in early studies. The wild-type gene is not oncogenic in typical assays and indeed can negate the effect of certain oncogenes (4). This observation coupled with the finding that mutations or full deletion of the p53 gene frequently occur in natural tumors (5) has led to the realization that p53 may normally act as an inhibitor of cell proliferation. The biochemical role of p53 in regulating cell proliferation has yet to be defined.

Another element directly involved in eukaryotic cell proliferation is the cdc2 protein kinase (6–11). cdc2 plays a broad role in cell cycle control. In the fission yeast, cdc2 gene function is required at two points during the cell cycle, before DNA synthesis and at the initiation of mitosis (12). In *Xenopus* oocytes cdc2 has been identified as a component of the M phase-promoting factor (13, 14) and cdc2 function is required for mitosis in mammalian tissue culture cells (15). It is uncertain whether cdc2 function is required for entry into S phase in vertebrate cells, but phosphorylation of SV40 TAg by cdc2 directly promotes the initiation of viral DNA replication *in vitro* (16).

To understand how cdc2 functions in the cell cycle, it is essential to identify substrates of the enzyme. cdc2 is predominantly a nuclear protein (15) and we have, therefore, investigated the ability of cdc2 to phosphorylate known nuclear phosphoproteins that might play a role in cell cycle regulation. We show here that p53 is a cdc2 substrate.

MATERIALS AND METHODS

Cell Culture, Labeling, and Elutriation. Human CEM cells were cultured in suspension at 37°C in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum. Cells were maintained at 2–4 × 10^6 cells per ml.

For ^{32}P labeling of proteins for tryptic peptide mapping, 1–2 × 10^8 exponentially growing CEM cells were incubated in 5 ml of phosphate-free Dulbecco's modified Eagle's medium (DMEM) containing 5 mCi (1 Ci = 37 GBq) of inorganic [^{32}P]phosphate (ICN) for 1.5 hr at 37°C.

Centrifugal elutriation and flow cytometric analysis of CEM cells were as described for HeLa cells (9), except 2 × 10^9 CEM cells were loaded at a pump speed of 100 ml/min and fractions were collected at pump speeds of 130, 150, 170, 190, 210, 230, 250, 270, 290, and 310 ml/min.

Purification of cdc2 Protein Kinase Complex and p53. Human cdc2 protein kinase complex was purified from HeLa cells as described (17) except that after lysis by Dounce homogenization the lysate was adjusted to 0.5 M NaCl and further homogenized to salt-extract the nuclei prior to centrifugation. This resulted in an approximately 5-fold increase in cdc2 kinase activity recovered (J.R.B., unpublished data).

To obtain quantities of human p53 sufficient for *in vitro* kinase experiments, a recombinant baculovirus (pEV55H-p53) was generated that contained human p53 coding sequences that had been cloned and characterized (18) (P.N.F. and C.P., unpublished data). pEV55Hp53 was constructed according to published procedures (19) using the transplacement plasmid pEV55 and DNA from wild type baculovirus *Autographa californica* (AcMPVL-1) (20). SF27 insect cells (2.5 × 10^7 cells per 150-mm dish) were infected with pEV55Hp53 (1–2 plaque-forming units per cell) and 48 hr after infection cells were lysed and p53 protein was purified using protein A-Sepharose beads to which the p53-specific monoclonal antibody PAb421 (21) had been cross-linked (22). Human p53 obtained by this approach displayed the correct electrophoretic mobility and antibody specificity as described (23). Similar quantities of human p53 (≈100 μg per 10^7 SF27 cells) were routinely purified, as were obtained from cells infected with pEV55p53 that expresses murine p53 (22).

Peptide Synthesis and Sequencing. Peptide CSH133 (RAALPNNTSSSPQPKKKPLDGEY—using the single-letter amino acid code) was synthesized on an Applied Biosystems model 430A instrument using small-scale (0.1 mmol) rapid cycles as *t*-Boc (*t*-butoxycarbonyl) symmetric anhydrides. These cycles were modified to allow double coupling of leucine, threonine, serine, lysine, and glutamic acid residues first in dimethylformamide and then in dichloromethane. Unreacted peptide was capped with acetic anhydride. Coupling yields exceed 99% on each cycle. The peptide was cleaved from the resin and deprotected in liquid HF containing anisole and dimethyl sulfide, 1:20 (vol/vol), for 2 hr at −10°C. After ether extraction the peptide was purified by high performance liquid chromatography and analyzed by plasma desorption mass spectrometry.

Sequence analysis was done on a Applied Biosystems model 473A protein sequencer using pulse-liquid delivery of trifluoroacetic acid and vapor-phase delivery of trimethylamine. After conversion, the product of each cycle was

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Abbreviations: TAg, tumor antigen; SV40, simian virus 40.

collected, mixed with 10 ml of Aquasol scintilation fluid, and measured for radioactivity.

Immunoprecipitation and Kinase Reactions. Immunoprecipitations of CEM extracts were as described (24).

In vitro phosphorylation of purified p53 by purified cdc2 protein kinase was at 30°C for 30 min in 50 mM Tris·HCl, pH 8/10 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM ATP/10–30 μCi of [γ-^{32}P]ATP (New England Nuclear, 3000 Ci/mM). The phosphorylation of purified p53 by anti-cyclin B and C160 immunoprecipitates utilized the same conditions described above. The phosphorylation of the p53 synthetic peptide (CSH133) used the same buffer conditions; the peptide concentration was 150 μM. The phosphorylation of the peptide was monitored by binding to P81 paper (Whatman) and counting in liquid scintilation fluid. The *in vitro* phosphorylation of the endogenous CEM p53 was as follows. Lysates were prepared as described (9), except the lysis buffer contained 125 mM NaCl. The CEM lysate was adjusted to 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM ATP/10–20 μCi of [γ-^{32}P]ATP and incubated for 30 min at 30°C. The reaction was then adjusted to 50 mM EDTA and immunoprecipitated with PAb421, a monoclonal antibody to p53 (21).

In vitro phosphorylation of histone H1 by G6 and C160 immunoprecipitates was as described (24).

Peptide Mapping and Phospho Amino Acid Analysis. Immunopurified p53 that had been ^{32}P-labeled *in vitro* or *in vivo* or phosphorylated peptide CSH133 was digested with trypsin as described by Beemon and Hunter (25). The resulting peptides were resolved in two dimensions on 100-μm cellulose thin-layer plates (EM Science) by electrophoresis at 800 V for 25–40 min at pH 1.9 in acetic acid/88% (vol/vol) formic acid/water, 156:50:1794 (vol/vol), followed by ascending chromatography in 1-butanol/pyridine/acetic acid/water, 75:50:15:60 (vol/vol). One-dimensional phospho amino acid analysis was according to Draetta *et al.* (26).

RESULTS

cdc2 Phosphorylates p53 on Serine-315. To test whether human p53 might be a cdc2 substrate, p53 was immunopurified from a baculovirus expression system. After incubation with [γ-^{32}P]ATP, no endogenous kinase activity was detected in the preparation of p53 (Fig. 1*A*). However, if p53 was mixed with cdc2 kinase purified from HeLa cells, phosphorylation of p53 was apparent in addition to the autophosphorylation of the cyclin B subunit of cdc2 (Fig. 1*A*).

To investigate the phosphorylation of p53, we used a T-lymphoblastoid cell line (CEM). Phosphorylation of endogenous p53 in crude lysates could be observed by the addition of [γ-^{32}P]ATP, followed by incubation at 30°C and immunoprecipitation with PAb421, a monoclonal antibody against p53 (21). An extract was prepared from CEM cells and was preincubated with either control Sepharose, or p13-Sepharose. p13 is a subunit of cdc2 (9, 27) and this polypeptide coupled to Sepharose is an effective reagent for clearing a cell lysate of cdc2 (10, 24). After addition of [γ-^{32}P]ATP to the cleared cell lysates, p53 was immunoprecipitated with PAb421. Preclearing the lysate with p13-Sepharose resulted in a significant reduction of p53 phosphorylation (Fig. 1*B*). On average, lysates precleared with p13-Sepharose had one-third the p53 kinase activity compared to lysates precleared with control Sepharose, but on no occasion was all activity removed. To be certain that the apparent clearing of the p53 kinase activity by p13-Sepharose was not due to binding of p53 to the beads, the amount of p53 protein in the precleared lysates was determined by immunoblot analysis. No significant difference in the amount of p53 was observed between extracts precleared with control Sepharose and p13-Sepharose (data not shown). Thus, p13-Sepharose, which has been shown to bind specifically to cdc2, removes a significant fraction of protein kinase activity that can phosphorylate p53 in a whole cell lysate. It should be noted that the p53 in CEM cells is probably not wild type.

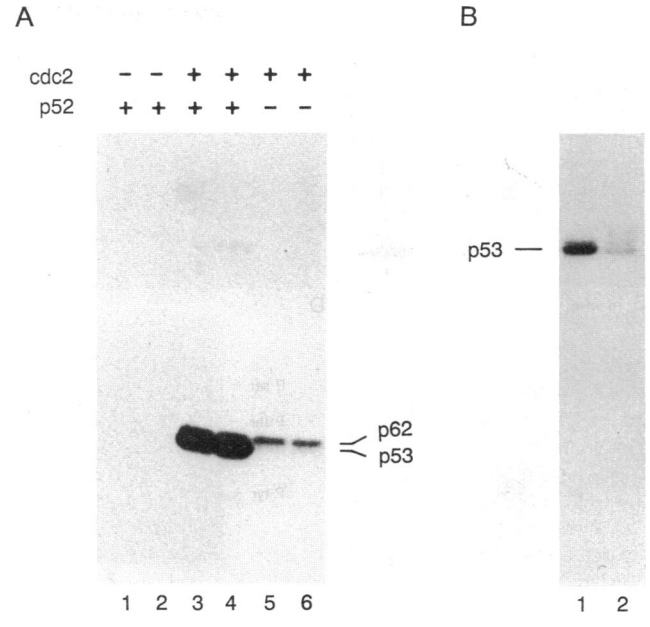

FIG. 1. *In vitro* phosphorylation of p53. (*A*) Lanes: 1, reaction mixture containing 160 ng of p53; 2, reaction mixture containing 320 ng of p53; 3, reaction mixture containing purified cdc2 protein kinase and 160 ng of p53; 4, same as lane 3, except containing 320 ng of p53; 5 and 6, reaction mixtures containing purified cdc2 protein kinase alone. Each reaction mixture was incubated with [γ-^{32}P]ATP and subjected to SDS/PAGE. (*B*) *In vitro* phosphorylation of p53 in crude lysates prepared from human CEM cells. Lanes: 1, 500 μg of CEM lysate precleared with control Sepharose was incubated with [γ-^{32}P]ATP and precipitated with anti-p53 (PAb421); 2, same as lane 1 except that the cell lysate was precleared with p13-Sepharose.

To examine whether cdc2 phosphorylates p53 at sites utilized *in vivo*, two-dimensional tryptic peptide mapping of p53 phosphorylated either *in vivo* or *in vitro* was performed. In the CEM cell line, no less than 11 tryptic phosphopeptides of p53 were resolved (Fig. 2*A*). A tryptic map of p53 phosphorylated *in vitro* by purified cdc2 protein kinase revealed only four phosphorylated peptides, of which one, referred to as peptide 3, was overwhelmingly the most heavily labeled (Fig. 2*B*). A mixture of the tryptic digests of p53 phosphorylated *in vivo* and *in vitro* was also resolved in two dimensions (Fig. 2*C*). It appears that the four sites phosphorylated *in vitro* by the cdc2 protein kinase were also phosphorylated *in vivo*. Most notably, the major fragment phosphorylated *in vitro* (Fig. 2 *B* and *C*, peptide 3) was also a major site of phosphorylation *in vivo*. Phospho amino acid analysis was performed on peptide 3 that had been phosphorylated either *in vivo* or *in vitro*. In both cases phosphoserine was detected (Fig. 2*D*). These observations show that cdc2 can phosphorylate p53 *in vitro* at sites that are phosphorylated *in vivo*. It also becomes apparent that cdc2 is not the only kinase that phosphorylates p53; this probably explains the inability of p13-Sepharose to clear all of the kinase activity that phosphorylates p53.

At least four sites of phosphorylation have been identified in murine p53 (28, 29). Among these is serine-312. Serine-312 is followed by a proline and, since cdc2 phosphorylation sites invariably contain the motif Thr/Ser-Pro (16, 30), we tested whether serine-315 [corresponding to murine serine 312 (18)] might be a site of cdc2 phosphorylation. Accordingly, we synthesized a peptide corresponding to amino acids 305–327 of human p53, which is expected to be cleavable at each end

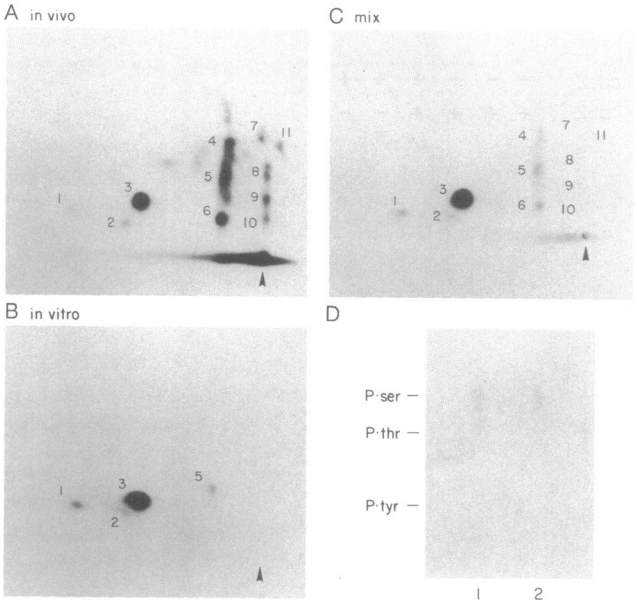

FIG. 2. Tryptic peptide mapping of p53. (*A*) Two-dimensional tryptic peptide map of ^{32}P-labeled p53 immunoprecipitated from CEM cells. (*B*) Similar peptide map of p53 labeled *in vitro* by purified cdc2 kinase. (*C*) Map of a mixture of p53 labeled *in vitro* and *in vivo*. (*A–C*) First dimension was electrophoresis from right (−) to left (+) and second dimension was ascending chromatography. Arrowheads mark the electrophoretic origins. (*D*) One-dimensional phospho amino acid analysis of peptide 3 labeled *in vivo* (lane 1) or *in vitro* (lane 2). Migration of marker phosphoserine (P·ser), phosphothreonine (P·thr), and phosphotyrosine (P·tyr) is indicated.

by trypsin, leaving a fragment containing serine-315. The peptide was readily phosphorylated by cdc2 and after trypsin digestion the phosphopeptide was resolved in two dimensions (Fig. 3*A*). The migration of the synthetic peptide was identical to that of tryptic peptide 3 from full-length human p53 phosphorylated *in vitro* by cdc2 (Fig. 3 *B* and *C*). Sequential degradation of the undigested phosphorylated peptide and radioactivity in each amino acid released was measured by scintillation counting. Although some ^{32}P was released during cycles 9 and 10 (serine-313 and -314, respectively), the peak of ^{32}P was released at cycle 11, which corresponded to serine-315 (data not shown). Thus, serine-315 is the major site of phosphorylation by cdc2 in human p53.

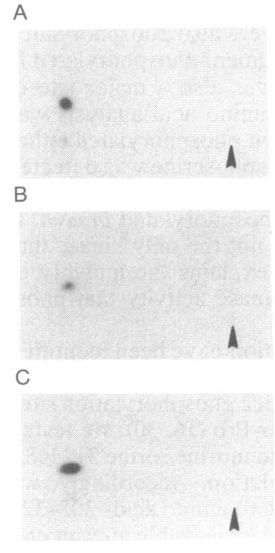

FIG. 3. Phosphorylation of peptide containing serine-315. (*A*) Peptide CSH133 (RAALPNNTSSSPQPKKKPLDGEY) corresponding to amino acids 305–327 of human p53 was phosphorylated *in vitro* by purified cdc2, digested with trypsin, and resolved in two dimensions. (*B*) p53 phosphorylated *in vitro* by cdc2 was digested with trypsin and resolved in two dimensions. (*C*) Map of a mixture of materials in *A* and *B*. Arrowheads mark the electrophoretic origins.

Phosphorylation by p60–cdc2 and Cyclin B–cdc2. The enzyme preparation used in the previous series of experiments (17) consisted primarily of cyclin B–cdc2. However, it also contained low levels of an interphase form of the enzyme, p60–cdc2 (D.B., unpublished observations). We thus asked which of these two enzymes might be responsible for phosphorylation of p53. Lysates from exponentially growing CEM cells were immunoprecipitated with either monoclonal antibody C160, which recognizes the p60 subunit of cdc2 (24), or anti-cyclin B antibody (31). p53 was added as substrate and was phosphorylated in each case (Fig. 4*A*). The C160 immunoprecipitates phosphorylated p53 to a greater extent at all concentrations of p53 examined. The lower level of phosphorylation in the anti-cyclin B precipitates does not necessarily reflect reduced affinity for p53, since the ability of these precipitates to phosphorylate histone H1 was also reduced compared to the C160 precipitates (data not shown).

Tryptic peptides of p53 phosphorylated *in vitro* by the p60- or cyclin B-associated cdc2 kinase were resolved in two dimensions. Both enzymes predominantly phosphorylated one major peptide (Fig. 4 *B* and *C*). When the two tryptic digests were mixed the same spectrum of spots was observed (Fig. 4*D*). The major site of phosphorylation with each enzyme corresponds to that of peptide 3 in Fig. 2. Thus, both cdc2 protein kinase complexes phosphorylate p53 at the same sites, predominantly at serine-315.

Cell Cycle Regulation. The cell cycle oscillation of the activity of cdc2-containing protein kinase complexes is well-established in mammalian cells (9, 24, 26, 31). In particular, the cyclin B-containing enzyme is most active during M phase, whereas the p60–cdc2 enzyme is most active in interphase.

To investigate the possible cell cycle regulation of p53 phosphorylation, CEM cells were fractionated by centrifugal elutriation to obtain populations enriched at various stages of

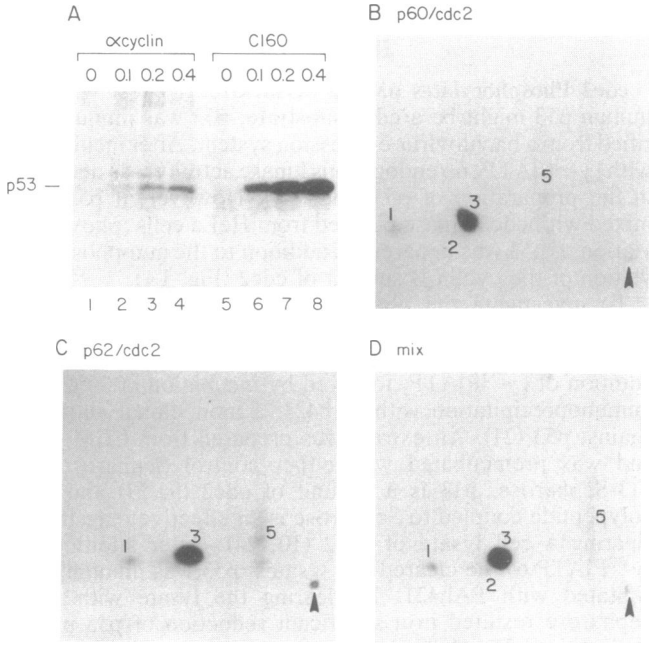

FIG. 4. Phosphorylation of p53 *in vitro* by p60–cdc2 and cyclin B–cdc2. (*A*) *In vitro* phosphorylation of 0, 0.1, 0.2, and 0.4 μg of p53 by anti-cyclin B immunoprecipitates of CEM cells (lanes 1–4) or by C160 immunoprecipitates (lanes 5–8). CEM lysate (250 μg) was immunoprecipitated for each phosphorylation reaction. (*B*) Two-dimensional tryptic peptide map of p53 phosphorylated by the C160 immunoprecipitate. (*C*) Two-dimensional tryptic peptide map of p53 phosphorylated by anti-cyclin B immunoprecipitate. (*D*) Two-dimensional tryptic peptide map of a mixture of material in *B* and *C*. Arrowheads mark the origins of electrophoresis.

the cell cycle. Crude lysates of the elutriated fractions were assayed for their ability to phosphorylate endogenous p53 or were immunoprecipitated with C160 or with G6, an anti-cdc2 antibody, for histone H1 kinase assay. For reasons that are not fully clear, antibody G6 can be used to preferentially measure the activity of cyclin B–cdc2 (24).

The phosphorylation of p53 in the whole-cell lysate of each elutriated fraction displayed clear cell cycle-dependence. The greatest incorporation of phosphate into p53 occurred in fraction 5 (Fig. 5*B*), in which a high percentage of cells were in S phase (Fig. 5*A*). The histone kinase activity of p60–cdc2, assayed with C160 immunoprecipitates, corresponded closely with the phosphorylation of p53 in whole-cell lysates (Fig. 5 *B* and *C*). The activity of cyclin B–cdc2 peaked one or two fractions later in the elutriation profile as compared to p60–cdc2 (Fig. 5*D*).

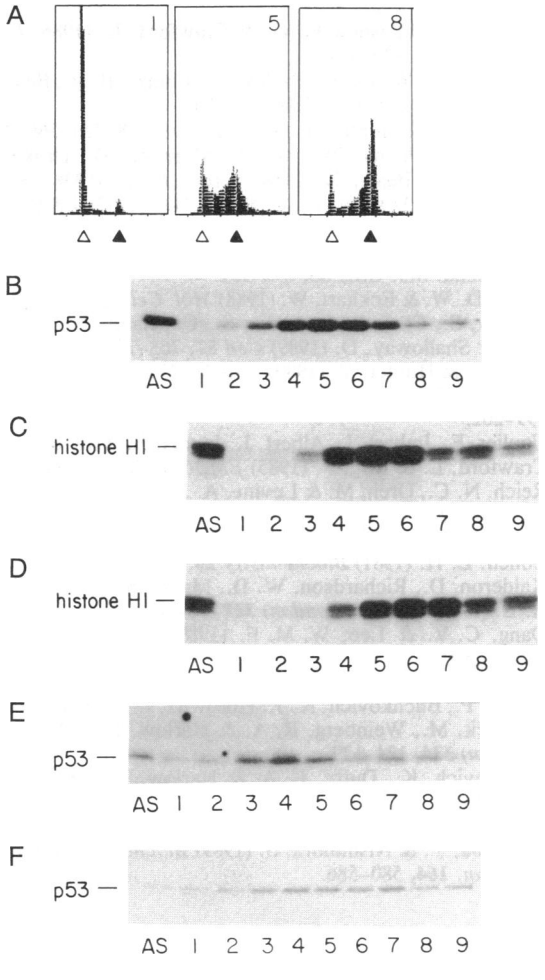

FIG. 5. Cell cycle regulation of p53 and cdc2 kinases. (*A*) Flow-cytometric profiles of CEM cells fractionated by centrifugal elutriation. Fractions 1, 5, and 8 (as indicated) are enriched in G_1, S, and G_2/M populations, respectively. Open and solid triangles signify 2*N* and 4*N* DNA content (where *N* is the haploid amount of DNA). (*B–F*) AS indicates asynchronous cells and the elutriation fractions are indicated as lane labels. (*B*) *In vitro* phosphorylation of p53 in whole cell lysates of elutriated cells. After incubation with [γ-^{32}P]ATP, p53 was immunoprecipitated with PAb421. (*C*) Phosphorylation of histone H1 by C160 immunoprecipitates of elutriated fractions. (*D*) Phosphorylation of histone H1 by G6 immunoprecipitates of elutriated fractions. Equal amounts of protein (250 μg) were assayed in all cell lysates. (*E*) Total cell protein (300 μg) from elutriated fractions was analyzed by an immunoblot with PAb421. (*F*) Elutriated fractions were inoculated into fresh medium and allowed to recover for 1 hr at 37°C. The cells were then pulse-labeled with [^{32}P]orthophosphate (1 mCi) in phosphate-free DMEM for 1 hr at 37°C. Cell lysates were immunoprecipitated with PAb421.

In a separate but similar experiment, we measured the level of p53 protein in crude lysates in each elutriated fraction and also the incorporation of [^{32}P]orthophosphate into p53 in cells briefly returned to culture after elutriation. The total amount of p53 protein, detected by immunoblot analysis, varied substantially in the various fractions. The protein was scarce in those containing predominantly G_1 populations and peaked in fraction 4 (Fig. 5*E*). In the later fractions the protein became less abundant. There was likewise a differential rate of incorporation of inorganic phosphate into p53 in each elutriated fraction. About 2- to 3-fold more ^{32}P was incorporated into p53 during the S and G_2/M phases of the cell cycle by comparison with G_1-enriched fractions (Fig. 5*F*). This subtle difference is probably due to the fact that cdc2 is only one kinase that phosphorylates p53 and it may be that the other kinase(s) involved is not cell-cycle regulated.

It is well-established that p53 accumulates after mitogenic activation of quiescent cells (32); however, in continuous culture the level of p53 has been reported to be constant throughout the cell cycle in cells that are not virally transformed (33). The cell cycle oscillation of p53 in CEM cells implies that the timing of maximal incorporation of phosphate into p53, either in cell-free lysates or *in vivo* (Fig. 5), need not precisely reflect the time of maximal activation of the kinases of which p53 is a substrate. However, the results in Fig. 5 are consistent with the likelihood that p53 is a substrate of both p60–cdc2 and cyclin B–cdc2.

DISCUSSION

Although it increasingly appears that p53 normally acts as an inhibitor of cell proliferation, its precise biochemical role is not well-understood. However, it is of great interest that the protein is phosphorylated by the cdc2 cell cycle-regulatory kinase.

The ability of both p60–cdc2 and cyclin B–cdc2 to phosphorylate p53 suggests that the protein is likely to be phosphorylated at serine-315 for much of the cell cycle, except in G_1. There is little p53 in cells that have just completed division and newly synthesized protein is expected to accumulate in an underphosphorylated state during early G_1, during which no known form of cdc2 is catalytically active. Since p60–cdc2 readily phosphorylates p53, serine-315 phosphorylation is expected to follow activation of this enzyme, close to the onset of DNA replication. Further phosphorylation may occur during M phase, during which cyclin B–cdc2 is active. Whether serine-315 retains phosphate during the G_2 phase of the cell cycle is likely to depend on the time of inactivation of p60–cdc2, which has not been accurately determined, on the half-life of the p53 protein itself, which is known to be short especially in nontransformed cells (34), and also on phosphatase-dependent dephosphorylation of serine-315. Determining the exact time of phosphorylation of p53 by cdc2 in CEM cells is further complicated by the presence of other kinases that phosphorylate p53.

It is perhaps surprising that p60–cdc2 and cyclin B–cdc2 phosphorylate p53 in such a similar manner. One very probable role of cdc2-associated proteins is to regulate the substrate specificity of the protein kinase (17) and some differences in the specificity of these two enzymes may be expected. However, histone H1, which is an M-phase substrate of cdc2 (10), contains a subset of sites that are phosphorylated during both interphase and M phase (35). These might be sites susceptible to phosphorylation by both p60–cdc2 and cyclin–cdc2.

p53 was originally identified as a protein that complexes with TAg of SV40. It is intriguing, therefore, that TAg has also been shown to be a cdc2 substrate (16). Both TAg and p53 are nuclear proteins and in each case a region of the molecule required for nuclear localization has been identi-

fied. It is of interest that in TAg and p53 the site of cdc2 phosphorylation is in a very similar relative position to the karyophylic site. Thus in TAg, threonine-124 is the site of cdc2 phosphorylation (16) and the primary nuclear localization signal lies between residues 126 and 133 (36). In p53, cdc2 phosphorylates serine-315 and the primary karyophilic site is at residues 316–322 (37). For the TAg, deletion of residues 111–125 markedly alters the kinetics of nuclear transport (38). It is thus possible that phosphorylation by cdc2 might affect the subcellular localization of TAg and p53.

In addition to p53, the product of the retinoblastoma gene (Rb) is an antioncoprotein. There are interesting parallels between the two polypeptides. Both are nuclear phosphoproteins that associate with proteins encoded by DNA tumor viruses. SV40 TAg binds to both Rb and p53. For adenovirus, E1A binds to Rb and E1B binds to p53 (2, 39). Furthermore, both are phosphorylated in a cell cycle-dependent manner (present study and ref. 40). It is likely that Rb is also a substrate of cdc2 (41).

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