Cyclin-dependent kinases (Cdks) are required for cell cycle progression. Two potentially significant Cdk substrates in human cells are the human single-stranded binding protein (HSSB or RPA), which plays an essential role in DNA replication, repair, and recombination, and the tumor suppressor p107 which acts to negatively regulate cell growth.

In this report we describe the in vitro phosphorylation of these two proteins by Cdks in an attempt to understand how cyclin-substrate interactions direct phosphorylation efficiencies. We show that cyclin A-Cdk2 efficiently phosphorylates the p34 subunit of HSSB (HSSB-p34) alone or as a part of the heterotrimeric complex. In contrast, cyclin E-Cdk2 that is active in phosphorylating histone H1, does not support the phosphorylation of the p34 subunit of HSSB. We provide evidence that this differential phosphorylation results from a specific interaction between HSSB-p34 and cyclin A, but not cyclin E. Thus the observed cell cycle-dependent phosphorylation of HSSB-p34 at the G1 to S transition is most likely catalyzed by cyclin A-Cdk2 initiated by the direct interaction between cyclin A and the HSSB-p34 subunit.

These studies are consistent with our previous observation that p107, which directly binds cyclin A, is efficiently phosphorylated by cyclin A-Cdk2 but not cyclin B-associated kinases. Here we further demonstrate that cyclin A only complexes with p107 in its unphosphorylated form. These data suggest a catalytic mechanism by which Cdk acts: substrate targeting by a cyclin-substrate interaction followed by dissociation of the Cdk upon phosphate incorporation allowing the Cdk to become available for the next cycle of phosphorylation.

Two important events of the eukaryotic cell cycle are the S and M phases, when chromosomes are replicated and when replicated chromosomes are segregated into dividing cells, respectively. Progression through the cell cycle is controlled by several factors including cyclin-dependent kinases (Cdks), cyclin inhibitors, Cdk-activating kinase, Cdk phosphatases, and the checkpoint proteins (see Refs. 1–3 for reviews). It is likely that these mechanisms serve to regulate downstream pathways leading to coordinated DNA replication, DNA repair, and chromosomal segregation.

Key regulators of the G1 to S phase transition in mammalian cells include three D-type cyclins and cyclin E (4). In the presence of growth factors, the D-type cyclins exhibit moderate oscillations during the cell cycle reaching a peak at the G1/S transition (5). Maximal levels of cyclin E-dependent kinase activity are also apparent at this stage (6). Once cells enter S phase, cyclin E is degraded and its kinase partner (Cdk2) complexes with cyclin A concomitantly with the onset of DNA synthesis. In order for cells to enter mitosis after DNA replication, cyclin B must accumulate above a threshold level and complex with Cdc2 (7, 8). Following completion of division this cyclin is proteolyzed and the cell progresses to G1 (9).

The mechanism by which cyclins activate Cdks has recently been revealed by the crystal structure of the cyclin A-Cdk2-ATP complex (10). Cyclin A binds to one side of the Cdk2 catalytic cleft inducing large conformational changes that realign active site residues and relieve the steric block at the entrance to the catalytic cleft.

It is thought that formation of each successive cyclin-Cdk complex is responsible for phosphorylating substrates essential for any given cell cycle event. Thus the G1/S phase cyclin E or A/Cdk2 complex may phosphorylate and activate proteins necessary for DNA replication, and the G2/M phase cyclin B/Cdc2 phosphorylates proteins necessary for mitosis and cytokinesis (see Ref. 3 for a review). How these cyclin/Cdk complexes activate replication at the G1/S transition and what their physiological substrates are, is not known. In this report we have utilized two Cdk substrates in human cells, the human single-stranded binding protein (HSSB) and the tumor suppressor protein p107, to investigate the mechanism by which a Cdk targets its substrate.

HSSB, a complex containing three polypeptides of 70, 34, and 11 kDa, was originally identified as a HeLa cell protein required for the in vitro replication of SV40 DNA (12). While the p70 subunit of HSSB binds to single-stranded DNA (16), the biological functions of the p34 and p11 subunits are not known. However a direct role for the HSSB-p34 and -p11 in this reaction is likely as antibodies against these subunits block SV40 DNA replication (16, 17). The trimeric HSSB is an essential component of the replication initiation complex functioning to stabilize unwound regions of DNA as well as complexing with T antigen and DNA polymerase α-prime to catalyze RNA primer synthesis (18). HSSB also plays multiple roles in the elongation stages of replication stimulating DNA polymerases.
δ and ε to catalyze elongation of DNA chains (19).

In addition to its role in replication, HSSB is essential for nucleotide excision repair (20–25) and may play important roles in recombination, double-stranded break repair and transcription (26–30).

The p34 subunit of HSSB is phosphorylated at the G1/S boundary of the cell cycle (12) or following exposure to DNA damage-inducing agents, including ultraviolet light (31) or ionizing radiation (32). We have demonstrated that cyclin A-dependent hyperphosphorylation of HSSB-p34 in G1 extracts is dependent on p350 protein kinase (DNA-PK) (33). During G1 to S phase progression, cyclin A expression leads to the formation of active cyclin protein kinase A complexes that may initiate efficient phosphorylation of the p34 subunit of HSSB allowing further phosphorylation by DNA-PK that converts HSSB into a hyperphosphorylated form. The biological significance of these findings is currently not clear as the phosphorylation state of HSSB does not alter its in vitro activity in either SV40 DNA replication or nucleotide excision repair (34–36). However it has been suggested that HSSB may be part of a cellular signal transduction mechanism for sensing that DNA synthesis is ongoing (34).

Both cyclins E and A are active during the cell cycle phase at which HSSB is phosphorylated indicating that cyclin E could also be involved in this phosphorylation event. However in vitro experiments presented here suggest that it is exclusively cyclin A/Cdk2 that comprises the cyclin/kinase complex responsible for phosphorylation of the p34 subunit of HSSB during the G1/S transition as a result of a specific interaction between cyclin A and its HSSB-p34 substrate. Cyclin E cannot participate in such a protein/protein interaction and is unable to phosphorylate HSSB. This finding is consistent with our previous observation that p107 is phosphorylated by cyclin A/Cdk2 as the result of its interaction with cyclin A (42). We provide evidence that cyclin A only interacts with unphosphorylated p107 and following substrate modification becomes dissociated.

### MATERIALS AND METHODS

#### Preparation of Protein Reagents

GST-tagged cyclins A, D1, D2 and D3 (constructs were kind gifts from Dr. Yue Xiong, University of North Carolina) were overexpressed under the control of T7 RNA polymerase in Escherichia coli (BL21-DE3). Freshly transformed bacteria were grown overnight in Luria broth containing 0.4% glucose and 0.5 mg/ml ampicillin at 37°C. The overnight culture was diluted 1:100 with the same medium (1 liter) and grown to an A600 of 0.6 at 37°C in the presence of ampicillin (0.5 mg/ml). The cells were then shifted to 25°C, and expression was induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) overnight. The cells were pelleted and lysed in 10 ml of buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 2 mM PMSE, 4 μg/ml antipain, and 2 μg/ml leupeptin) plus 0.1 mM NaCl and 1 mg/ml lysozyme for 30 min at 0°C. Following sonication (six repetitive 20-s treatments) and centrifugation at 39,000 × g for 15 min, the soluble supernatant protein was loaded onto glutathione-Sepharose beads, washed, and eluted as recommended by the manufacturer (Pharmacia Biotech Inc.).

E. coli cells (BL21-DE3-ly-PlyS) transformed with a plasmid expressing GST-cyclin E (pGex-cycE and pGex-2T-cycE; generously provided by Dr. A. Koff, Sloan-Kettering Cancer Institute) were overexpressed as described above for the other cyclins except that the IPTG-induced expression step was carried out in the absence of ampicillin. The soluble GST-cyclin E protein (630 mg, 70 ml) was loaded onto a 1-ml glutathione-Sepharose column (1 × 1.3 cm, 1 ml) equilibrated in buffer A plus 0.1 mM NaCl. Following a wash step with buffer A plus 0.8 mM NaCl (40 ml), 15 mg of GST-cyclin E were eluted from the column with 20 mM glutathione in 5 ml of the same buffer. However, GST-cyclin E was rapidly degraded and only approximately 10% of the glutathione eluted material was full-length, while 90% comprised a 30-kDa GST-cyclin E proteolyzed product that cross-reacted with the anti-cyclin E antibody.

In order to remove this degraded protein, the eluted material (15 mg) was loaded onto a 25-ml fast protein liquid chromatography-Superose 6 sizing column equilibrated in buffer A plus 0.5 mM NaCl. Elution with the equilibration buffer resulted in the isolation of 0.3 mg of full-length GST-cyclin E.

Extracts from HeLa cells fixed in G1 (G1 extracts), cyclin A, Cdk2, and HSSB were prepared as described previously (16, 37, 38). Proteins resulting from the coexpression of the HSSB-p34 and -p11 subunits in E. coli (used in Fig. 5) was kindly provided by M. Wold, University of Iowa (41).

#### Purification of DNA-dependent Kinase

Both components of the DNA-dependent kinase (DNA-PK) enzyme, the Ku autoantigen and the p350 catalytic subunit, are expressed from HeLa cell nuclear extracts. The assay used for the purification of each of these proteins was the phosphorylation of the serine residue present within the synthetic peptide (PK53) Glu-Pro-Pro-Leu-Glu-Glu-Ala-Phe-Ala-Asp-Leu-Trp-Lys-Lys. The phosphorylation assay was carried out in a reaction (20 μl) containing 50 mM HEPES, pH 7.5, 0.5 mM DTT, 10 mM MgCl2, 50 mM KCl, 0.1 mM EDTA, 100 μM γ-[32P]ATP, 0.2 μg of bovine serum albumin, in 25 mM Tris-HCl, pH 7.5, 1.77 mM MgCl2. The reaction was incubated at 30°C for 30 min. The assay that scored for the p350 subunit of DNA-PK, each reaction contained 1500 units of Ku autoantigen (16 ng). In assays used for the measurement of the Ku autoantigens, 325 units of p350 protein were added. The reactions were then incubated for 30 min at 30°C before being stopped by the addition of 20 μl of 30% acetic acid. Aliquots were spotted on phosphocellulose filters that were washed four times with 15% acetic acid before being counted by liquid scintillation.

**Purification of the p350 Subunit of DNA-PK—**

HelA cell nuclear extract (840 mg, 76.4 ml, 1.65 × 108 units) was loaded onto a phosphocellulose column (2.5 × 4 cm, 20 ml) equilibrated in buffer D (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.02% Tween 20, 1 mM DTT, 1 μg/ml each of pepstatin A, leupeptin, and soybean trypsin inhibitor, 10 μg/ml PMSE and 5% glycerol) plus 0.1 M KCl. The column was washed with 15 column volumes of buffer D plus 0.1 mM KCl and active fractions (as determined by the assay described above) were then eluted with 47 ml of buffer D containing 0.3 mM KCl. This pooled fraction (122 mg, 47 ml, 1.77 × 106 units) was diluted to 0.1 mM KCl with buffer D and loaded onto a DEAE-cellulose column (2.5 × 2 cm, 10 ml) equilibrated with buffer D plus 0.2 mM KCl. Following a 10-column volume wash step using the same buffer, the p350 DNA-PK activity was eluted with buffer D plus 0.2 mM KCl (31.9 mg, 82 ml, 9.3 × 107 units) and applied to a 25-ml Superose 6 gel filtration column equilibrated with buffer D plus 0.2 mM KCl. Active fractions were eluted with buffer D plus 0.2 mM KCl (82 mg, 4.3 ml, 8.0 × 106 units), pooled, concentrated 5-fold using an Amicon CF 25 cone and reapplied to a Superose 6 gel filtration column equilibrated with buffer D plus 1.0 mM KCl. Active fractions eluting with buffer D plus 1.0 mM KCl (0.225 mg, 11.25 ml, 5.0 × 106 units) were diluted to 50 mM KCl in buffer D and passed through an SP-Sepharose column (0.7 × 3 cm, 0.5 ml) equilibrated in the same buffer. The column was then subjected to a 10-column linear gradient of 0.05–0.5 mM KCl in buffer D; DNA-PK-p350 was eluted at 0.25 mM KCl (0.1 mg, 6.7 ml, 2.0 × 106 units). Overall, 1.3% of the activity was recovered and 110-fold purification was achieved resulting in >90% homogeneity as assayed by SDS-PAGE analysis and silver staining (data not shown).

**Purification of Ku—**

In the phosphocellulose chromatographic step used for the isolation of the DNA-PK p350 described above, all of the Ku activity was recovered in the 0.1 mM KCl flow-through fraction (196.5 mg, 135 ml, 4.4 × 105 units). This material was diluted to 0.05 mM KCl with buffer D and applied to a DEAE-cellulose column (1.5 × 28 cm, 5 ml) equilibrated with buffer D plus 0.05 mM KCl. The column was washed with 10 ml of the same buffer, and active fractions were eluted with buffer D containing 0.2 mM KCl (59.5 mg, 141.67 ml, 2.4 × 106 units). This fraction was diluted to 0.05 mM KCl and loaded onto a double-stranded DNA cellulose column (0.5 × 2 cm, 10 ml) which was washed consecutively with 10 ml of buffer D plus 0.2 mM KCl and 20 ml of buffer D plus 0.3 mM KCl, prior to elution of Ku activity with buffer D plus 0.5 mM KCl. Active fractions were pooled (1.1 mg, 0.62 ml, 5.6 × 105 units), diluted 10-fold and then applied to a Q-Sepharose column (0.7 × 2.7 cm, 0.5 ml). This column was washed with 2 ml of buffer D plus 0.05 mM KCl, and the Ku autoantigen was then eluted at 0.25 mM KCl using a 0.05 to 0.5 mM KCl
transfected into *E. coli* containing 0.4% glucose and 0.5 mg/ml ampicillin. This culture was

**Cloning and Purification of GST-p34 HSSB**

PCR primers 5'-TAAGACAGATCTGGGAAGATCCGAGTTCATGTTTG-3' and 3'-TCCGGAAGATCTGGTACCT-TATTCTCATCTGGAAGTAAATGGTC-3' to the coding sequence of the HSSB-p34 subunit, were designed to include 5' BamHI and 3' KpnI restriction sites (both underlined). The DNA product resulting from 35 cycles of amplification, after electrophoretic separation, was excised from a 1% agarose gel, digested with BamHI and KpnI, and ligated into a Pet 19b vector that included a GST fusion followed by thrombin, BamHI, and KpnI cleavage sites. The resulting plasmid was transfected into *E. coli* cells (BL21-DE3) and grown overnight in L-broth containing 0.4% glucose and 0.5 mg/ml ampicillin. This culture was diluted 1:100 with the same medium and grown to an A590 of 0.6 at 37°C and induced with 0.4 mM IPTG for 3 h. Cells from 1 liter of medium (5 g) were pelleted and then lysed in buffer A plus 1 mg/ml lysozyme for 30 min at 0°C. After sonication and centrifugation at 12,000 × g for 15 min, the supernatant was loaded onto a glutathione-Sepharose column (0.7 × 2.7 cm, 0.5 ml) which was washed and eluted as recommended by the manufacturer. The yield of the GST-p34 was 0.6 mg of protein from each L of cells.

**Purification of Cyclin A/Cdk2 and Cyclin E/Cdk 2**

G1 extracts (5 mg) were incubated with human GST-cyclin A or GST-cyclin E (0.05 mg each) for 20 min at 30°C in the presence of 40 mM creatine phosphate (dTriT salt, pH 7.7), 25 μg/ml of creatine kinase, 7 mM MgCl2, 0.5 mM DTT and 4 mM ATP. The reaction mixtures (1 ml) were each loaded onto a glutathione-Sepharose column (0.7 × 2.7 cm, 0.5 ml) equilibrated in buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.01% Nonidet P-40, 0.1 mM PMSF, 0.2 mg/liter antipain, and 0.1 mg/liter leupeptin) plus 0.2 mM NaCl. After washing successively with 2 ml of equilibration buffer and 2 ml of buffer A containing 0.8 M NaCl, bound protein was eluted with 1.5 ml of buffer A plus 0.05 M NaCl and 20 mM glutathione. The resulting fractions contained cyclin A or E/Cdk2 activity as determined by immunoblot analysis and H1 kinase assays. One unit of kinase activity was defined as the amount of cyclin/kinase bound protein was eluted with buffer A plus 0.8 M NaCl (2.5 ml). These fractions were concentrated 10-fold using Amicon CF25 Centricon cones. Further bound protein was eluted with buffer A plus 0.1 M NaCl and 10 mM glutathione (2.5 ml). These fractions were dialyzed for 3 h at 0°C against 2 liters of buffer A plus 50 mM NaCl and concentrated 15-fold using Amicon cones. The proteins present in these fractions were visualized by both Coomassie staining and Western blot analyses with antibodies against cyclin A or p107 following SDS-PAGE.

In order to assay for the dephosphorylation of p107, the material eluted with glutathione (100 ng, 2 μl) was added to reaction mixtures (10 μl) containing 20 mM Heps (pH 7.5), 7 mM MgCl2, 1 mM DTT, 0.1 mM NaCl, and 0.05 unit of phosphatase PP2A (Upstate Biotechnology Inc.). After 20 min of incubation at 30°C, the phosphorylation state of p107 was analyzed by examining the alteration of its electrophoretic mobility by SDS-PAGE and Western blot analyses with polyclonal antibodies against p107.

**RESULTS**

Cyclin E, although Capable of Activating Cdk2 and Cdc2 in Vitro, Cannot Support the Phosphorylation of HSSB, as Cyclin A Does—As both cyclins A and E are expressed during the G1/S transition phase of the cell cycle, either one or both of these cyclin-dependent kinase activities could be responsible for the phosphorylation of HSSB which occurs at the G1/S transition. In order to examine and compare the effects of these cyclins, as well as the D type cyclins, on the phosphorylation of HSSB, bacterial expressed GST-cyclins A, and D1–3 were solubilized and purified as described under “Materials and Methods.” To ensure that these purified cyclins were active, they were examined for their ability to activate Cdk2 or Cdc2 kinases in the phosphorylation of histone H1. Cdk2 and Cdc2 were isolated from HeLa cell cytosolic extracts that harbor the Cdk-activating enzyme (CAK) that phosphorylates these kinases on residues Thr-160 or Thr-161, respectively (38). As shown in Fig. 1, whereas Cdk2 alone exhibited little H1 kinase activity, it was stimulated 350-fold following addition of cyclin A or...
equilibrated in buffer A plus 0.2 M NaCl. After washing with buffer A, 1 ml of glutathione-Sepharose columns containing 0.8 M NaCl (10 ml) was eluted with buffer A plus 0.05 M NaCl and 20 mM glutathione.

FIG. 2. Cyclins A, E, D1, D2, or D3-dependent phosphorylation of HSSB-p34. As described under “Kinase Assays”, reaction mixtures (10 μl) containing 250 ng of GST-cyclins A, E, D1, D2, or D3, were preincubated with 50 μg of G1 extract for 30 min at 30°C. 1.0 μg of exogenous underphosphorylated HSSB was added to each reaction followed by a further 60-min incubation at 37°C. An aliquot (2.0 μl) of each reaction was subjected to immunoblot analysis using monoclonal antibodies against the p34 subunit of HSSB. In lanes 9–11, 0.75 μg of each of the cyclins, E, D1, or D2 was preincubated with 250 ng of cyclin A and 50 μg of G1 extract prior to HSSB addition. The positions at which the unphosphorylated and phosphorylated species of HSSB-p34 migrate are indicated to the right.

130-fold by the addition of cyclin E. Similar levels of stimulation were observed when Cdc2 was used in place of Cdk2 (data not shown). While reconstituted cyclin A or B/kinase complexes have been shown to phosphorylate p107 (42) and HSSB (33, 34), the activity of cyclin E has not previously been reconstituted in vitro. Here we have shown for the first time that purified cyclin E is able to stimulate the activity of Cdc2 and Cdk2 in an in vitro reconstituted reaction.

The D type cyclins did not stimulate Cdk2 or Cdc2 kinase activities in the assays used here (data not shown), consistent with the notion that these cyclins form complexes with Cdk4 and Cdk6 which are either present at very low levels or not present in HeLa cell extracts.

Having shown that cyclin E was active in supporting histone H1 phosphorylation by Cdk2, we then investigated whether this cyclin also stimulated the phosphorylation of HSSB. It has previously been shown in vitro that the p34 subunit of HSSB is phosphorylated by kinase activities present in G1 extracts preincubated with cyclin A (33). This hyperphosphorylated HSSB product contained four species of p34 that migrated more slowly through denaturing polyacrylamide gels than the hypophosphorylated form. Following fractionation of the G1 extracts, these hyperphosphorylated species of p34 were formed by the combined action of purified cyclin A-dependent Cdk2 and DNA PK (33).

In order to ascertain whether cyclin E contributed to this phosphorylation we used similar assays. Addition of either GST-cyclin A or E stimulated the histone H1 kinase activity of G1 extracts by ∼4–5-fold (from 2.7 pmol in the absence of cyclins to 12.5 or 14.2 pmol following cyclin A or E addition, respectively) indicating that active cyclin/kinase complexes were formed. The phosphorylation of the p34 subunit of HSSB by these cyclin E or A-activated G1 extracts was then analyzed by examining the electrophoretic mobility changes of p34-HSSB using monoclonal antibodies against the p34 subunit. In the absence of cyclins, only limited amounts of the HSSB-p34 were converted into slow migrating forms (Fig. 2, lanes 1–3). However, following incubation for 60 min in the presence of cyclin A, nearly all of the p34 subunit had been converted into multiple slow migrating phosphorylated forms (lane 8), as reported (33). In contrast, incubation with cyclins D1, D2, D3, or cyclin E-activated G1 extract resulted in conversion of limited amounts of p34 into slow migrating forms (lanes 4–7, respectively). Furthermore, preincubation of cyclin A with any of cyclins D1, D2, or E did not significantly alter the cyclin A-dependent phosphorylated forms of HSSB observed (lanes 9–11, respectively).

The specific cyclin-activated kinases present in the G1 extracts following addition of each cyclin were examined. For this purpose cyclin A and E/kinase complexes were purified by selecting for proteins bound to each of the cyclins in the G1 extracts by virtue of their GST fusions (as detailed under “Materials and Methods”). This procedure yielded both cyclin A- and E-specific Cdk2 kinase complexes (as shown in Fig. 3A). Cdk2 but not Cdc2 was detected in the cyclin/kinase complexes isolated from G1 extracts. The mobilities of both cyclins A and E were retarded following incubation with G1 extracts due to their phosphorylation. Calf intestinal phosphatase treatment converted the slower migrating forms of each of these cyclins into a single fast migrating, hypophosphorylated form (data not shown). Both the kinases responsible for phosphorylating these cyclins and the physiological significance are, however, unknown.

The isolated cyclin A-Cdk2 and cyclin E-Cdk2 complexes were examined for their ability to phosphorylate histone H1. As
indicated in Fig. 4A, the addition of 2.0 μl of the cyclin E/Cdk2 eluate resulted in the incorporation of 15 pmol of phosphate into histone H1, while the addition of 1.0 μl of the cyclin A/Cdk2 eluate resulted in a similar amount of histone H1 phosphorylation. To ascertain whether HSSB was also a substrate for cyclin E-dependent kinase activity, the amount of 32P-labeled phosphate incorporated into the HSSB-p34 subunit by cyclin A/Cdk2 and cyclin E/Cdk2 was quantitated and compared. As shown in Fig. 4B, the addition of 1.0 μl of cyclin A/Cdk2 (which catalyzed the incorporation of 15 pmol of 32P into histone H1) resulted in the transfer of 400 fmol of 32P-labeled phosphate into the HSSB-p34 subunit; the addition of 2.0 μl of cyclin E/Cdk2 (which catalyzed the incorporation of 15 pmol of 32P into histone H1) resulted in the incorporation of 13 fmol of 32P into the HSSB substrate. Thus, cyclin E/Cdk2 phosphorylated HSSB-p34 about 30-fold less efficiently than cyclin A/Cdk2.

Cyclin A/Cdk2, but Not DNA-PK, Efficiently Phosphorylates the HSSB-p34 Subunit Alone—The influence of the HSSB-p70 and -p11 subunits on the phosphorylation of the p34 subunit was examined. For this experiment, the HSSB-p34 subunit was cloned into a vector that overexpresses this protein in bacteria. As shown in Fig. 5A, lane 3, incubation of cyclin A-activated G1 extracts with the HSSB-p34 subunit resulted in the extensive conversion of the p34 subunit into multiple slow migrating forms, similar to that seen with the trimeric HSSB (Fig. 2). This finding indicates that both the cyclin A-dependent kinase and DNA-PK present in these extracts efficiently phosphorylated the HSSB-p34 subunit in the absence of the p11 and p70 subunits. This experiment was repeated using both purified cyclin A/Cdk2 and DNA-PK. As indicated in Fig. 5B, the level of cyclin A/Cdk2 catalyzed 32P incorporation into trimeric HSSB and the p34 subunit alone, were similar (0.65 and 0.5 pmol, respectively). Thus, HSSB-p34 phosphorylation by cyclin A/Cdk2 is independent of the p70 and p11 subunits. Purified DNA-PK catalyzed efficient phosphorylation of the p34 subunit in the presence of DNA (Fig. 5C, lane 10). However, in contrast to the efficient phosphorylation of the p34 subunit alone catalyzed by DNA-PK present in extracts, purified DNA-PK catalyzed phosphorylation of the HSSB-p34 subunit poorly in the absence of the other HSSB subunits (Fig. 5C, lanes 3 and 4; Fig. 5D, quantitation of results). Efficient phosphorylation of the HSSB-p34 subunit was restored following addition of the p34-p11 complex isolated after coexpression of these two subunits (Fig. 5C, lane 8). Thus a conformational change in the p34 subunit brought about by its interaction with the p11 subunit (with which it has been shown to interact, prior to the addition of the p70 subunit) (40, 41) may result in a more suitable substrate for phosphorylation by DNA-PK.

Cyclin A, but Not Cyclin E, Interacts Specifically with HSSB-p34—The results outlined above indicate that cyclin A/Cdk2 phosphorylates HSSB-p34 while cyclin E/Cdk2 is unable to catalyze this reaction. To test the hypothesis that this substrate specificity is dictated by the ability of a cyclin to directly interact with its substrate, we examined whether cyclin A directly interacts with the HSSB-p34 protein (see scheme in Fig. 4A). As seen in Fig. 6B, cyclin A was retained on a GST-p34 column at salt concentrations up to 0.4 M NaCl, suggesting that there was an interaction between this cyclin and the GST-p34 subunit. Furthermore, this GST-p34-cyclin A complex was efficiently phosphorylated following addition of human Cdc2 (data not shown). In order to determine whether this interaction was specific, a control column was used in which the GST protein was coupled to glutathione-Sepharose beads. When

---

**Fig. 5. Phosphorylation of the p34 subunit of HSSB by cyclin A-activated G1 extracts.** A, as described under “Kinase Assays”, 250 ng of cyclin A were preincubated with 40 μg of G1 extract. HSSB-p34 (1 μg) was added followed by a further 60 min incubation. An aliquot of the reaction (2 μl) was subjected to immunoblot analysis using monoclonal antibodies against the p34 subunit of HSSB (lane 3). Lane 1 shows the signal detected with the HSSB-p34 substrate, and lane 2 is a control that shows the effects of incubating G1 extracts with HSSB-p34 in the absence of cyclin A. B, comparison of the phosphorylation of trimeric HSSB and the HSSB-p34 subunit by cyclin A/Cdk2. The cyclin A/Cdk2 complex was added (in amounts as indicated) to reactions (10 μl) containing 40 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT, and 50 μM ATP with either 1 μg of trimeric HSSB (9 pmol) or 300 ng of the HSSB-p34 subunit (9 pmol), as indicated. Following incubation for 45 min incubation at 37 °C, the products were separated by SDS-PAGE and the 32P-phosphorylated forms of p34 were visualized by Coomassie Brilliant Blue staining, excised, and counted by liquid scintillation. Quantitation of the results is shown. C, comparison of the DNA-PK catalyzed phosphorylation of trimeric HSSB and the HSSB-p34 subunit. Assay conditions used were as described under “Materials and Methods.” Equivalent amounts of substrate were added, i.e. 0.3 μg of HSSB (2.7 pmol), 79 ng of HSSB-p34 subunit (2.7 pmol), 29 ng of HSSB-p11 subunit (2.7 pmol), or 0.12 μg of the HSSB-p34-p11 complex (2.7 pmol) in the presence or absence of DNA, as indicated. Quantitation of the results are summarized in D.
Phosphorylation of HSSB-p34 and -p107 by Cdns

Fig. 7. p107 is hyperphosphorylated by cyclin A-activated G1 extracts. Anti-p107 immunoblot, lanes 1 and 2. 100 ng of GST-p107 incubated in the absence and presence of cyclin A, respectively; lane 3, 1 μl from the 200-μl reaction mixture containing 1 mg of G1 extract; lane 4, 1 μl from the 200-μl reaction mixture containing 10 μg of cyclin A and 1 mg of G1 extract; lanes 5 and 6, 1 μl from the 200-μl reaction mixture containing 20 μg of GST-p107 and 1 mg of G1 extract in the absence or presence of 10 μg of cyclin A, respectively. The positions at which the phosphorylated and unphosphorylated species of p107 migrate are indicated to the right.

In order to isolate p107 in its phosphorylated or unphosphorylated form and examine whether these p107 species interact with cyclin A, GST-p107 was incubated with G1 extracts in the presence of cyclin A, with or without EDTA, a Mg2+ chelating agent that acts to inhibit kinase activity. GST-p107 was reisolated after each reaction and its phosphorylation status as well as its association with cyclin A were analyzed as diagrammed in Fig. 8A. As shown in Fig. 8B and D, GST-p107 eluted from the glutathione-Sepharose column exclusively in the phosphorylated slow migrating form following incubation with cyclin A-activated G1 extracts, nearly all of the GST-p107 was converted into a slower migrating form (lane 6).

To confirm that the SDS-PAGE mobility alterations of p107 result from its phosphorylation, aliquots of the GST-p107 glutathione eluates were treated with phosphatase PP2A (Fig. 8E). Following phosphatase treatment, the slower migrating GST-p107 (Fig. 8E, lane 1) was converted to the faster migrating species (Fig. 8E, lane 2). Incubation of phosphatase PP2A with EDTA-treated GST-p107 did not alter its mobility (Fig. 8E, compare lanes 3 and 4). Thus, the change in mobility of p107 following incubation with cyclin A-activated G1 extracts was due to the phosphorylation of p107, and the addition of EDTA inhibited this modification.

In order to investigate whether cyclin A was stably associated with p107 before and/or after p107 phosphorylation, the material isolated following incubation of GST-p107 with cyclin A-activated G1 extracts was analyzed for protein by Coomassie staining (Fig. 8B) and for cyclin A by immunoblot analysis (Fig. 8C). Only limited amounts of cyclin A were retained on the glutathione columns from reactions carried out with either GST alone (Fig. 8C, lane 2) or reactions that generated phosphorylated GST-p107 (Fig. 8C, lane 3). In contrast, cyclin A present in the extracts was retained with unphosphorylated GST-p107 and eluted from the glutathione column with high salt (Fig. 8C, lane 4) or with 10 mM glutathione (Fig. 8C, lane 7). This suggests that cyclin A interacted with unphosphorylated p107, but following p107 phosphorylation, cyclin A was no longer bound.

In contrast to the retention of cyclin A by the GST-p34, cyclin E was detected in the flow through fraction (Fig. 6C, lane 2), suggesting that cyclin A interacts specifically with the p34 subunit of HSSB.

By guest on July 23, 2018http://www.jbc.org/Downloaded from
DISCUSSION

Reconstituted cyclin A or B/kinase complexes have been shown to phosphorylate p107 (42, 52) and HSSB (33) but the activity of cyclin E and the D-type cyclins has not been reconstituted in vitro, and no substrates for these complexes have been identified. Results described here demonstrate that in vitro purified cyclin E is able to activate Cdk2 or Cdc2 kinases to phosphorylate histone H1 by 130-fold, which is 3-fold less than the observed stimulation by cyclin A/Cdk2. However, cyclin E was unable to stimulate the phosphorylation of HSSB or to affect cyclin A-dependent HSSB phosphorylation in vitro. Thus, cyclin A/Cdk2 is most likely the major cyclin-dependent kinase activity responsible for phosphorylation of this protein at the G1/S phase transition.

Furthermore, substrate structure may also determine the efficiency of cyclin/Cdk-mediated phosphorylation. We have shown that the cyclin A-dependent HSSB-p34 phosphorylation is independent of the p70 or p11 HSSB subunits using both cyclin A-activated G1 extracts and purified cyclin A/Cdk2 preparations. Incubation with cyclin A-activated extracts results in the appearance of multiple slow migrating phosphorylated forms of HSSB-p34 due to the combined action of cyclin A/Cdk and DNA-PK (33). Following incubation of cyclin A-activated extracts with either trimeric HSSB or the p34 subunit, all the slower migrating p34 bands that result from phosphorylation by both cyclin A/Cdk and DNA-PK were observed. This indicates that under these conditions the p11 and p70 subunits are not required for efficient phosphorylation by either the cyclin A/Cdk2 or DNA-PK present in these extracts. However, when purified DNA-PK was used in these experiments, a paradox
was apparent. Purified DNA-PK, in the presence of Ku and DNA, can efficiently phosphorylate trimeric HSSB, but was unable to phosphorylate the HSSB-p34 subunit in the absence of the HSSB-p11 and -p70 subunits. Efficient phosphorylation of the HSSB-p34 subunit was observed when the p34 subunit was complexed with the p11 subunit. It is unclear why DNA-PK present in extracts is able to phosphorylate the HSSB-p34 subunit in the absence of the p11 and p70 subunits while purified DNA-PK is not. It is possible that a conformational change in the p34 subunit brought about through its interaction with the p11 subunit may be necessary in order for the HSSB-p34 to constitute a suitable substrate for phosphorylation by DNA-PK. The interaction of the HSSB-p34 with other proteins present when extracts are used may allow it to be in the correctly folded form for phosphorylation by DNA-PK in the absence of either the HSSB-p70 or -p11 subunits.

The increasing number of cyclin and Cdk family members identified indicates that multiple distinct cyclin-Cdk complexes may be assembled in vivo. The role of each of these complexes is thought to be the phosphorylation of a specific cellular substrate(s) but the identity of very few of these is known. During S phase, cyclin A forms a multiprotein complex with Cdk2, the transcription factor E2F, and a retinoblastoma gene product, p107 (43–46). Cyclin A has been shown to bind p107 (47, 48), and when overexpressed, cyclin A/Cdk2 hyperphosphorylates pRB overcoming its ability to suppress proliferation (49). Cyclin E also forms a complex with p107 and E2F (50), while cyclin D1 has been shown to associate with pRB-related protein, p107 (43–46). Cyclin A has been shown to interact with unphosphorylated p107, but not with pRB-phosphorylated p107 (49). Consistent with this, we have shown that cyclin A interact with unphosphorylated p107, but following phosphorylation, the association between these two proteins was markedly reduced. Thus the cyclins may target Cdk to their substrates, bringing about substrate phosphorylation and destabilization of the complexes. In this way cyclins can be recycled and become available for interactions with other proteins. 

Acknowledgments—We thank Dr. A. Amin of the Memorial Sloan-Kettering Cancer Center for providing the p107 protein, Dr. M. S. Wold of the University of Iowa for providing the HSSB-p34-p11 protein complex, Dr. A. Koff of the Memorial Sloan-Kettering Cancer Center for providing the vector for cyclin E expression, and Dr. Y. Xiong of the University of North Carolina for providing vectors for cyclin A, D1, D2, and D3 expression.

REFERENCES
1. Sherr, C. J. (1994) Cell 79, 551–555
2. Heichman, K. A., and Roberts, J. M. (1994) Cell 79, 557–562
3. O’Connell, M. J., and Nurse, P. (1994) Curr. Opin. Cell Biol. 6, 867–871
4. Lew, D. J., Dulio, V., and Reed, S. I. (1991) Cell 66, 1197–1206
5. Xiong, Y., Connolly, T., Fletcher, B., and Beach, D. (1991) Cell 66, 691–699
6. Koff, A., Cross F., Fisher, A., Schumacher, J., Leguennec, K., Philippe, M., and Roberts, J. M. (1991) Cell 66, 1217–1228
7. Dunphy, W. G., Brizuela, L., Beach, D., and Newport, J. (1988) Cell 54, 423–431
8. Gautier, J., Norbury, C., Nurse, P., and Maller, J. (1988) Cell 55, 433–439
9. Pines, J., and Hunter, T. (1989) Science 245, 833–846
10. Jeffrey, P. D., Russel, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. (1995) Nature 376, 313–320
11. Deleted in proof
12. Deleted in proof
13. Deleted in proof
14. Deleted in proof
15. Deleted in proof
16. Kenny, M., Schlegel, U., Fumexaux, H., and Hurwitz, J. (1990) J. Biol. Chem. 265, 7693–7700
17. Umbricht, C. B., Erdile, L. F., Jabs, E. W., and Kelly, T. J. (1993) J. Biol. Chem. 268, 1631–1638
18. Melendez, T., and Stillman, B. (1993) J. Biol. Chem. 268, 3389–3395
19. Lee, S.-H., Pan, Z.-Q., Kwon, A. D., Burgers, P. M. J., and Hurwitz, J. (1991) J. Biol. Chem. 266, 2271–2277
20. Longmore, M. P., Plevani, P., and Luchini, G. (1994) Mol. Cell. Biol. 14, 7884–7890
21. Matsuda, T., Saito, M., Kurasato, I., Kobayashi, T., Nakatsu, Y., Nagai, A., Enjoji, T., Marutani, C., Sugawasa, K., Hanaoka, F., Yasui, A., and Tanaka, K. (1995) J. Biol. Chem. 270, 4152–4157
22. He, Z., Henrichsen, L. A., Wold, M. S., and Ingle, C. J. (1995) Nature 374, 566–569
23. Li, H., Peterson, C. A., and Legerski, R. J. (1995) Mol. Cell. Biol. 15, 5396–5402
24. Mu, S., Park, C.-H., Matsunaga, T., Hau, D. S., Reardon, J. T., and Sancar, A. (1995) J. Biol. Chem. 270, 2415–2418
25. Coverly, D., Kenny, M. K., Munn, M., Rupp, W. D., Lane, D. P., and Wolf, R. D. (1991) Nature 349, 538–541
26. Firmenich, A. A., Elias-Arranz, M., and Berg, P. (1995) Mol. Cell. Biol. 15, 1629–1630
27. Li, R., and Botchan, M. R. (1993) Cell 73, 1207–1211
28. He, Z., Brinton, B. T., Greenblatt, J., Hassell, J. A., and Ingels, C. J. (1993) Cell 73, 2114–2123
29. Expereiments carried out in our laboratory (42) and by Peeper et al. (52) have illustrated that both the cyclin and the catalytic subunit are important factors in determining kinase substrate specificity. These authors have proposed that cyclin activated kinases target their cellular substrates through cyclin mediated protein-protein interactions. The demonstration that cyclin A and HSSB-p34 form a complex in the absence of kinase or ATP is consistent with this hypothesis. Cyclin E did not participate in such a protein/protein interaction and was unable to phosphorylate HSSB.

Using 597 cells coinfected with baculovirus vectors encoding the product of the human retinoblastoma gene (pRB) and murine D-type cyclins, Kato et al. (53) demonstrated that cyclins D2 and D3 bind pRB. However, introduction of a vector encoding Cdk4 induced pRB hyperphosphorylation and the dissociation of cyclins D2 and D3. Consistent with this, we have shown that cyclin A interacts with unphosphorylated p107, but following phosphorylation, the association between these two proteins was markedly reduced. Thus the cyclins may target Cdk to their substrates, bringing about substrate phosphorylation and destabilization of the complexes. In this way cyclins can be recycled and become available for interactions with other proteins.

Downloaded from http://www.jbc.org/ by guest on July 22, 2018
Studies on the in Vitro Phosphorylation of HSSB-p34 and -p107 by Cyclin-dependent Kinases: CYCLIN-SUBSTRATE INTERACTIONS DICTATE THE EFFICIENCY OF PHOSPHORYLATION

Emma Gibbs, Zhen-Qiang Pan, Hongwu Niu and Jerard Hurwitz

J. Biol. Chem. 1996, 271:22847-22854.
doi: 10.1074/jbc.271.37.22847

Access the most updated version of this article at http://www.jbc.org/content/271/37/22847

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 26 of which can be accessed free at http://www.jbc.org/content/271/37/22847.full.html#ref-list-1