Mapping of Amino Acid Residues in the p34 Subunit of Human Single-stranded DNA-binding Protein Phosphorylated by DNA-dependent Protein Kinase and Cdc2 Kinase in Vitro*

Hongwu Niu‡, Hediye Erdjument-Bromage‡, Zhen-Qiang Pan§, Suk-Hee Lee¶, Paul Tempst‡, and Jerard Hurwitz¶¶

From the §Graduate Program in Molecular Biology, Memorial Sloan Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, New York 10021, ¶Derald H. Ruttenberg Cancer Center, The Mount Sinai Medical Center, New York, New York 10029, and ¶Department of Virology and Molecular Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38101

Human single-stranded DNA-binding protein (HSSB, also called RPA), is a heterotrimeric complex that consists of three subunits, p70, p34, and p11. HSSB is essential for the in vitro replication of SV40 DNA and nucleotide excision repair. It also has important functions in other DNA transactions, including DNA recombination, transcription, and double-stranded DNA break repair. The p34 subunit of HSSB is phosphorylated in a cell cycle-dependent manner. Both Cdc2 kinase and the DNA-dependent protein kinase (DNA-PK) phosphate HSSB-p34 in vitro. In this study, we show that efficient phosphorylation of HSSB-p34 by DNA-PK requires Ku as well as DNA. The DNA-PK phosphorylation sites in HSSB-p34 have been mapped at Thr-21 and Ser-33. Kinetic studies demonstrated that a phosphate residue is first incorporated at Thr-21 followed by the incorporation of a second phosphate residue at Ser-33. We also identified Ser-29 as the major Cdc2 kinase phosphorylation site in the p34 subunit.

HSSB is also required for nucleotide excision repair (14). It participates in the recognition of UV-damaged DNA by forming a complex with XPA and stimulates the incision activities of XPG and XPF-ERCC1 (15–18). Formation of a protein complex between HSSB and the human homologue of Rad52 (19) and of HSSB with Ku (20) indicate that HSSB is important in DNA recombination and double-stranded DNA break repair. This is underscored by the observations that mutations within the p70 subunit of Saccharomyces cerevisiae SSB affect DNA recombination and double-str break repair (21–23). It has also been shown that HSSB binds to the acidic domains of transcription factors VP16 and p53 (24–26) and in S. cerevisiae ScSSB binds to DNA sequences that regulate transcription (27, 28). These observations suggest that HSSB also has important functions in transcription and its regulation.

The p34 subunit of HSSB is phosphorylated in a cell cycle-dependent manner (29–31). Phosphorylated forms first appear during the G1 to S transition and persist through the S phase. As the cell cycle progresses through late M phase, HSSB-p34 is dephosphorylated. In x-ray or UV-irradiated cells, the level of p34 phosphorylation also increases dramatically (32, 33). While the protein kinases responsible for the phosphorylation of p34 in vivo are not known, in vitro studies have shown that the HSSB-p34 subunit is phosphorylated by both the Cdc2 kinase and the DNA-dependent protein kinase (DNA-PK) (29, 30, 34, 35).

Although it has been suggested that the phosphorylation of HSSB-p34 may play an important role in cell cycle regulation of DNA synthesis and in coordinating DNA replication and repair (29–31), the biological significance of HSSB-p34 phosphorylation is still not clear. Several studies have shown that HSSB-p34 phosphorylation does not affect its ability to bind single-stranded DNA, support SV40 DNA replication (35–38), or nucleotide excision repair (38).

Alanine substitutions at Ser-23 and Ser-29, two putative Cdc2 kinase phosphorylation sites in HSSB-p34, do not affect the binding of HSSB to single-stranded DNA or its ability to support SV40 DNA replication (35, 37). HSSB, reconstituted with a mutant p34 subunit containing a deletion of 30–33 N-terminal amino acids, was not phosphorylated by either the Cdc2 kinase or DNA-PK, but efficiently supported SV40 DNA replication (35, 37). In contrast, a mutant HSSB, reconstituted with a C-terminal 30-amino acid truncated p34 subunit, was phosphorylated by both kinases, but did not support SV40 DNA replication (37). These studies showed that phosphorylation of the p34 subunit of HSSB has no detectable effect on the in vitro replication of SV40 DNA.

* This work was supported in part by National Institutes of Health Grant GM38559 (to J. H.), National Science Foundation Grant BIR-9402123 (to P. T.), National Institutes of Health Grant GM 52358 and ALSAC grant of St. Jude (to S.-H. L.), and National Cancer Institute 9420123 (to P. T.), National Institutes of Health Grant GM 52358 and ALSAC grant of St. Jude (to S.-H. L.).

† An American Cancer Society Professor. To whom correspondence should be addressed: Cornell University Graduate School of Medical Sciences, Memorial Sloan Kettering Cancer Center, New York, NY 10021, Tel.: 212-639-5898; Fax: 212-717-3627.

‡ The abbreviations used are: HSSB, human single-stranded DNA-binding protein; DNA-PK, DNA-dependent protein kinase; MALDI-TOF MS, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin.
The phosphorylation sites in HSSB-p34 have been inferred from the presence of putative kinase consensus sequences but have not been identified. In this study, a combination of biochemical and biophysical approaches were used to define both DNA-PK and Cdc2 kinase phosphorylation sites in the p34 subunit of HSSB. We have mapped two DNA-PK phosphorylation sites in the HSSB-p34 subunit at Thr-21 and Ser-33 and have identified Ser-29 as the major Cdc2 kinase phosphorylation site.

**MATERIALS AND METHODS**

**Enzymes and Materials—**HSSB, DNA-PK, and Ku were purified from HeLa cell as described previously (12, 39). The Cdc2-cyclin B kinase complex was purchased from Upstate Biotechnology Inc. Synthetic peptides were made using the ABI-433 peptide synthesizer (Microchemistry Core Facility, Memorial Sloan Kettering Cancer Institute).

In **In Vitro DNA-PK Phosphorylation Reaction**—Standard reaction mixtures (20 μl) contained 50 μM Hesper (pH 7.5), 10 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 4 μg of bovine serum albumin, 50 mM KCl, 1 μg of sonicated calf thymus DNA (average size ~300 base pairs), 16 ng of Ku protein, and 3 units of DNA-PK (1 unit of enzyme incorporated 1 nmol of 32P into peptide PK53 (40) in the presence of 16 ng of Ku at 30 °C after 30 min). Reactions with synthetic peptides contained 2 nmoles of each peptide and 500 μM [γ-32P]-ATP (~2000–4000 cpm/nmol). Incubation was for 2 h at 30 °C after which reaction mixtures were subjected to 15% SDS-PAGE analysis. Gels were dried using the Bio-Rad vacuum drier at 70 °C for 1 h followed by autoradiography.

In reactions containing HSSB, unless otherwise indicated, 300 ng (2.7 pmol) of HSSB were used with 100 μM [γ-32P]-ATP (~10,000–20,000 cpm/nmol). Reactions were incubated for 1 h at 30 °C followed by 12.5% SDS-PAGE analysis. Gels were dried and followed by autoradiography as described above, or proteins were transferred to a nitrocellulose membrane for Western blot analysis.

In **In Vitro Cdc2-Cyclin B Phosphorylation Reaction**—Standard reaction mixtures (20 μl) contained 40 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 50 μM [γ-32P]-ATP (~20,000 cpm/nmol), and 5 ng of Cdc2-cyclin B. Substrates used were as indicated in figure legends. Incubation was for 1 h at 37 °C followed by 12.5% (for HSSB) or 15% (for peptides) SDS-PAGE analysis. Gels were dried and subjected to autoradiography.

**Protein Structural Analysis—**Phosphorylated HSSB derivatives were resolved through a 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (0.2 mm, Schleicher & Shuell). The protein bands of interest ([32P]-phosphorylated and unphosphorylated proteins) were excised from the Ponceau S-stained nitrocellulose membrane, and peptides were generated by *in situ* proteolysis (41, 42). Briefly, digestion was carried out in reaction mixtures (25 μl) using either 0.2 μg of trypsin (Promega, Madison, WI) or chymotrypsin (sequencing grade; Boehringer Mannheim) in 100 mM NH4HCO3 (supplemented with 0.1% Triton X-100 and 0.1% Zwittergent 3–16) at 37 °C for 2 h. The resulting digest was reduced and S-alkylated with 0.1% β-mercaptoethanol (Bio-Rad) and 0.3% 4-vinylpyridine (Aldrich), respectively, and fractionated by reverse-phase HPLC. Solvents and HPLC system configuration were as described elsewhere (43), except that a 2.1-mm 214 TP54 Vydac C4 (Separations Group, Hesperia, CA) column was used with gradient elution at a flow rate of 100 μl/min. Tryptophan-containing peptides were identified by ratio analysis of UV absorbance at 297 and 277 nm, monitored in real time using an Applied Biosystems (Foster City, CA) model 1000S diode-array detector (44). Peak fractions were collected and aliquots (2 μl) were subjected to liquid scintillation counting, and all fractions were stored at −70 °C prior to further analysis.

Peak fractions that contained [32P]-labeled peptides were analyzed by a combination of automated Edman degradation and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described previously (43–45). After storage, fractions were supplemented with trifluoroacetic acid to give a final concentration of 10% before loading onto sequencer disks and mass spectrometer targets. Mass analysis (on 2% aliquots) was carried out using a model Voyager RP MALDI-TOF MS instrument (Vestec/PerSeptive, Framingham, MA) in the linear mode, with a 337-nm output nitrogen laser, a 1.3-m flight tube, and a cyano-4-hydroxy cinnamic acid (pre-made solution obtained from Linear Sci., Reno, NV) as the matrix. A 30-kV ion acceleration voltage (grid voltage at 70%; guide wire voltage at 0.1%) and −2.0-kV multiplier voltage were used (positive ion mode). Laser irradiance and number of acquisitions were adjusted as judged from

**RESULTS**

DNA-PK Catalyzed In Vitro Phosphorylation of the HSSB p34 Subunit Is Dependent on DNA and Ku—We have previously shown that the p34 subunit of HSSB (HSSB-p34) is phosphorylated by a HeLa cell fraction containing both DNA-PK and Ku in a DNA-dependent manner (34). To determine the role of Ku in the DNA-dependent phosphorylation of HSSB-p34, HSSB was incubated with highly purified DNA-PK...
in the presence or absence of Ku and calf thymus DNA. The phosphorylation of HSSB-p34 was analyzed by measuring $^{32}$P incorporation from [y-$^{32}$P]ATP into the p34 subunit (Fig. 1, upper panel) and examining the mobility changes of the p34 subunit following SDS-PAGE by immunoblot assay using a p34 monoclonal antibody (Fig. 1, lower panel). As shown in Fig. 1, lane 6, the p34 subunit was efficiently phosphorylated in the presence of DNA-PK, Ku, and calf thymus DNA. Omission of either Ku (lane 2), calf thymus DNA (lane 3), or DNA-PK (lane 4) markedly reduced $^{32}$P incorporation. The incorporation of phosphate(s) into the p34 subunit resulted in the appearance of p34-P1, a slower migrating form of p34 following SDS-PAGE (p34-P1, Fig. 1, lower panel). The incorporation of additional phosphate residues into p34 resulted in a hyperphosphorylated species of the subunit, leading to further retardation of its migration (p34-P2, Fig. 1, lower panel).

These results are consistent with our previous findings and further demonstrate that Ku and DNA are required for the efficient phosphorylation of HSSB-p34 by DNA-PK.

Amino Acid Residues Thr-21 and Ser-33 in HSSB-p34 Are Phosphorylated by DNA-PK in Vitro—We mapped the amino acid residues of HSSB-p34 that were phosphorylated by DNA-PK using the procedure outlined in Fig. 2. For this purpose, the two species of $^{32}$P-labeled phosphorylated p34 were resolved by SDS-PAGE (Fig. 1) and transferred to nitrocellulose membranes. The phosphorylated forms of p34 were subjected to trypsin and chymotrypsin digestion, and the digested mixture was fractionated by reverse-phase HPLC (as described in the text). The details concerning this procedure are described in the text.

The chromatogram (relevant section only) shows the absorption profile at 214 nm of peptides eluted from 2.1-mm Vydac C4 column (reverse-phase HPLC as described under "Materials and Methods"). Elution was carried out at 100 µl/min in a 0.7%/min acetonitrile gradient (3.5 to 70% in 0.1% trifluoroacetic acid). Numbered peaks (T1 to T4) contained $^{32}$P-labeled peptides, some of which were analyzed by chemical sequencing and mass spectrometry (Table I).

Two relatively small phosphorylated chymotryptic fragments (C1 and C2) were also isolated from the p34-P1 chymotryptic digestion mixture. As shown in Table Ib, the combined sequencing and mass spectrometry data of these peptides narrowed the phosphorylated site to an 8-amino acid peptide (C1, GAGGTYTQ; see Table I), which enabled positive identification of Thr-21 as the single phosphorylated residue in this peptide. This conclusion was further supported by the following observations. (a) Thr-21 was not observed during automated chemical sequencing of either peptide (C1 and C2 in Table I), which indicated that it was modified. Tyr-20 and Ser-23, on the other hand, were detected in amounts equivalent to the other amino acids (G, A, Q, and P) present in these peptides. Furthermore, neither phosphotyrosine nor its dephosphorylated form have been detected during noncovalent chemical sequencing (54). (b) The phosphorylated peptide C1 (residues 15–22) contains the sequence that includes a single threonine (at position 21) but no serine.
It has been shown recently that HSSB-p34 is rapidly degraded by trypsin to a ~28-kDa fragment (which contains the middle and C-terminal domain) and a ~4-kDa N-terminal fragment (48) which contains all the residues phosphorylated by DNA-PK (49). Our trypsin in situ digestion experiments showed that this ~4-kDa N-terminal fragment corresponded to amino acids 1–37 of the p34 subunit (Tables Ia and IIa). One threonine and 8 serine residues were located in this region (Thr-21, Ser-23, Ser-29, Ser-33, Ser-34, Ser-37, Ser-38, and Thr-39). Among them, two serine residues, Ser-23 and Ser-33, were contained in DNA-PK target sequence ((S/Q) or (Q/S)) in addition to Thr-21, which, as described above, was one of the DNA-PK phosphorylation sites.

To determine whether these two serine residues were also phosphorylated by DNA-PK, two peptides were synthesized (Fig. 4A) and Ku (data not shown). Since peptide II contained two serine residues (Ser-29 and Ser-33), one of these serine residues was substituted by an alanine (in chemical sequencing results), uninterpretable or no signal; IY, initial yield.

TABLE I

Analysis of tryptic and chymotryptic phosphopeptides derived from HSSB-p34 (p34-P1) phosphorylated by DNA-PK

| Peptide | Experimental mass | Chemical sequencing (IY, pmol) | Position in HSSB-P34 | Modifications | Calculated mass |
|---------|------------------|-----------------------------|----------------------|--------------|----------------|
| a. Tryptic digest | m/z | | | | |
| T4 | 3815.2 | ND | Acetyl-1–37 \(b\) | + 1 phosphate | 3814.90 |
| T3 | 3828.4 | ND | Acetyl-1–37[Met\(c\)] \(x\) | + 1 phosphate | 3830.90 |
| C1 | 789.3 | ND | 15–22 | + 1 phosphate | 790.72 |
| C2 | 1236.4 | GGGGY(QSPG) \(x\) (3.0) | 15–27 | + 1 phosphate | 1236.20 |
| B. Chymotryptic digest | | | | | |
| C1 | 1726.9 | TQQPGEKR \(x\) (0.61) | 21–37 | + 1 phosphate | 1726.74 |

a ND, not determined.
b Acetyl, N-terminus acetylated.
c Met\(x\), methionine sulfoxide.

TABLE II

Analysis of tryptic and chymotryptic phosphopeptides derived from HSSB-p34 phosphorylated by Cdc2-cyclinB

| Peptide | Experimental mass | Chemical sequencing (IY, pmol) | Position in HSSB-P34 | Modifications | Calculated mass |
|---------|------------------|-----------------------------|----------------------|--------------|----------------|
| a. Tryptic digest | m/z | | | | |
| T6 | 3814.2 | ND | Acetyl-1–37 | + 1 phosphate | 3814.90 |
| T5 | 3829.9 | ND | Acetyl-1–37[Met\(c\)] | + 1 phosphate | 3830.90 |
| C1 | 1854.0 | TQQPGEKR \(x\) (0.49) | 21–38 | + 1 phosphate | 1854.91 |
| C1–1a.a. | 1890.0 | ND | 22–38 + xPTC \(y\) | + 1 phosphate | 1888.83 |
| C1–3a.a. | 1808.4 | ND | 24–38 + xPTC \(y\) | + 1 phosphate | 1808.62 |

a No signal observed.
b Acetyl, N terminus acetylated.
c Met\(x\), methionine sulfoxide.
d 1-(3) a.a., one (or 3) amino acids removed by Edman degradation.
ND, not done.
y xPTC, phenyl thiocarbamyl derivative of lysine side chain (\(x\)-amine).

The peptides listed here were selected on the basis of 32P incorporation and were assumed to include all phosphorylation sites. Experimental mass (\(m/z\)) was obtained by MALDI-TOF MS (see “Materials and Methods”); chemical sequencing data (including initial yields in pmol) are also listed. Theoretical mass calculations for predicted peptides were carried out by summing isotopic masses of the composite amino acids and of all the modifying groups. \(\text{MH}^+\) represents molecular mass [M] plus one proton \([H^+]\). \(x\) (in chemical sequencing results), uninterpretable or no signal; IY, initial yield.

Phosphorylation of HSSB

The peptides listed here were selected on the basis of 32P incorporation and were assumed to include all phosphorylation sites. Experimental mass (\(m/z\)) was obtained by MALDI-TOF MS (see “Materials and Methods”; chemical sequencing data (including initial yields in pmol) are also listed. Theoretical mass calculations for predicted peptides were carried out by summing isotopic masses of the composite amino acids and of all the modifying groups. \(\text{MH}^+\) represents molecular mass [M] plus one proton \([H^+]\). \(x\) (in chemical sequencing results), uninterpretable or no signal; IY, initial yield.

HSSB-P34 Phosphorylation by CDC2 Kinase

| Peptide | Experimental mass | Chemical sequencing (IY, pmol) | Position in HSSB-P34 | Modifications | Calculated mass |
|---------|------------------|-----------------------------|----------------------|--------------|----------------|
| a. Tryptic digest | m/z | | | | |
| T6 | 3814.2 | ND | Acetyl-1–37 \(b\) | + 1 phosphate | 3814.90 |
| T5 | 3829.9 | ND | Acetyl-1–37[Met\(c\)] \(x\) | + 1 phosphate | 3830.90 |
| C1 | 1854.0 | TQQPGEKR \(x\) (0.49) | 21–38 | + 1 phosphate | 1854.91 |
| C1–1a.a. | 1890.0 | ND | 22–38 + xPTC \(y\) | + 1 phosphate | 1888.83 |
| C1–3a.a. | 1808.4 | ND | 24–38 + xPTC \(y\) | + 1 phosphate | 1808.62 |

a No signal observed.
b Acetyl, N terminus acetylated.
c Met\(x\), methionine sulfoxide.
d 1-(3) a.a., one (or 3) amino acids removed by Edman degradation.
ND, not done.
y xPTC, phenyl thiocarbamyl derivative of lysine side chain (\(x\)-amine).
Phosphorylation of HSSB

DNA-PK were also carried out. As shown in Fig. 6B, the phosphorylated HSSB-p34 form, p34-P1, was detected after 15 min of incubation. The level of p34-P1 increased as the phosphorylation reaction proceeded and plateaued after 90 min. In contrast, the hyperphosphorylated product, p34-P2, was not clearly visible until after 30 min of incubation, and the amount of p34-P2 continued to increase up to 2 h. In the presence of a saturating amount of DNA-PK and lower levels of substrate, all of the HSSB-p34 subunit was converted to the hyperphosphorylated form, p34-P2 (data not shown). These observations suggest that DNA-PK initially phosphorylated Thr-21, resulting in the p34-P1 species, followed by phosphorylation of Ser-33 and conversion to the p34-P2 species.

Ser-29 is the Predominant Cdc2-Cyclin B Phosphorylation Site within the HSSB-p34 Subunit in Vitro—Previously we and others have shown that, in addition to DNA-PK, the Cdc2 kinase also phosphorylates HSSB-p34 in vitro (29–31, 34). This reaction yielded two forms of p34 that migrated slower than the unphosphorylated p34 subunit following SDS-PAGE, a predominant phosphorylated form p34-P1 and a minor band, p34-PII (see Fig. 7, inset). Approximately one phosphate residue was incorporated into each molecule of p34-P1 (34). p34-P1 was isolated and subjected to trypsin digestion followed by reverse phase HPLC fractionation. Six $^{32}$P-radiolabeled peptides were isolated (one major peak, T6, and five minor peaks, T1 to T5) (Fig. 7). These peptides were characterized by chemical sequencing and MALDI-TOF MS (Table II). All six peptides contained overlapping sequences, an identical C-terminal residue (Lys-37), and variable truncated N termini, most likely due to the action of a contaminating chymotryptic-like protease activity (Table IIa). Added diversity was derived from the oxidative state of the acetylated, N-terminal Met (methionine sulfoxide). The combined mass data indicated the presence of a single phosphate group situated between residues 20 and 37 of HSSB-p34, a region that contains three serines (Ser-23, -29, -33, and Thr-21). The level of p34-P1 increased as the phosphorylation reaction proceeded and plateaued after 90 min. In contrast, the hyperphosphorylated product, p34-P2, was not clearly visible until after 30 min of incubation, and the amount of p34-P2 continued to increase up to 2 h. In the presence of a saturating amount of DNA-PK and lower levels of substrate, all of the HSSB-p34 subunit was converted to the hyperphosphorylated form, p34-P2 (data not shown). These observations suggest that DNA-PK initially phosphorylated Thr-21, resulting in the p34-P1 species, followed by phosphorylation of Ser-33 and conversion to the p34-P2 species.

Ser-29 is the Predominant Cdc2-Cyclin B Phosphorylation Site within the HSSB-p34 Subunit in Vitro—Previously we and others have shown that, in addition to DNA-PK, the Cdc2 kinase also phosphorylates HSSB-p34 in vitro (29–31, 34). This reaction yielded two forms of p34 that migrated slower than the unphosphorylated p34 subunit following SDS-PAGE, a predominant phosphorylated form p34-P1 and a minor band, p34-PII (see Fig. 7, inset). Approximately one phosphate residue was incorporated into each molecule of p34-P1 (34). p34-P1 was isolated and subjected to trypsin digestion followed by reverse phase HPLC fractionation. Six $^{32}$P-radiolabeled peptides were isolated (one major peak, T6, and five minor peaks, T1 to T5) (Fig. 7). These peptides were characterized by chemical sequencing and MALDI-TOF MS (Table II). All six peptides contained overlapping sequences, an identical C-terminal residue (Lys-37), and variable truncated N termini, most likely due to the action of a contaminating chymotryptic-like protease activity (Table IIa). Added diversity was derived from the oxidative state of the acetylated, N-terminal Met (methionine sulfoxide). The combined mass data indicated the presence of a single phosphate group situated between residues 20 and 37 of HSSB-p34, a region that contains three serines (Ser-23, -29, -33, and Thr-21). The level of p34-P1 increased as the phosphorylation reaction proceeded and plateaued after 90 min. In contrast, the hyperphosphorylated product, p34-P2, was not clearly visible until after 30 min of incubation, and the amount of p34-P2 continued to increase up to 2 h. In the presence of a saturating amount of DNA-PK and lower levels of substrate, all of the HSSB-p34 subunit was converted to the hyperphosphorylated form, p34-P2 (data not shown). These observations suggest that DNA-PK initially phosphorylated Thr-21, resulting in the p34-P1 species, followed by phosphorylation of Ser-33 and conversion to the p34-P2 species.

Ser-29 is the Predominant Cdc2-Cyclin B Phosphorylation Site within the HSSB-p34 Subunit in Vitro—Previously we and others have shown that, in addition to DNA-PK, the Cdc2 kinase also phosphorylates HSSB-p34 in vitro (29–31, 34). This reaction yielded two forms of p34 that migrated slower than the unphosphorylated p34 subunit following SDS-PAGE, a predominant phosphorylated form p34-P1 and a minor band, p34-PII (see Fig. 7, inset). Approximately one phosphate residue was incorporated into each molecule of p34-P1 (34). p34-P1 was isolated and subjected to trypsin digestion followed by reverse phase HPLC fractionation. Six $^{32}$P-radiolabeled peptides were isolated (one major peak, T6, and five minor peaks, T1 to T5) (Fig. 7). These peptides were characterized by chemical sequencing and MALDI-TOF MS (Table II). All six peptides contained overlapping sequences, an identical C-terminal residue (Lys-37), and variable truncated N termini, most likely due to the action of a contaminating chymotryptic-like protease activity (Table IIa). Added diversity was derived from the oxidative state of the acetylated, N-terminal Met (methionine sulfoxide). The combined mass data indicated the presence of a single phosphate group situated between residues 20 and 37 of HSSB-p34, a region that contains three serines (Ser-23, -29, -33, and Thr-21). The level of p34-P1 increased as the phosphorylation reaction proceeded and plateaued after 90 min. In contrast, the hyperphosphorylated product, p34-P2, was not clearly visible until after 30 min of incubation, and the amount of p34-P2 continued to increase up to 2 h. In the presence of a saturating amount of DNA-PK and lower levels of substrate, all of the HSSB-p34 subunit was converted to the hyperphosphorylated form, p34-P2 (data not shown). These observations suggest that DNA-PK initially phosphorylated Thr-21, resulting in the p34-P1 species, followed by phosphorylation of Ser-33 and conversion to the p34-P2 species.

Ser-29 is the Predominant Cdc2-Cyclin B Phosphorylation Site within the HSSB-p34 Subunit in Vitro—Previously we and others have shown that, in addition to DNA-PK, the Cdc2 kinase also phosphorylates HSSB-p34 in vitro (29–31, 34). This reaction yielded two forms of p34 that migrated slower than the unphosphorylated p34 subunit following SDS-PAGE, a predominant phosphorylated form p34-P1 and a minor band, p34-PII (see Fig. 7, inset). Approximately one phosphate residue was incorporated into each molecule of p34-P1 (34). p34-P1 was isolated and subjected to trypsin digestion followed by reverse phase HPLC fractionation. Six $^{32}$P-radiolabeled peptides were isolated (one major peak, T6, and five minor peaks, T1 to T5) (Fig. 7). These peptides were characterized by chemical sequencing and MALDI-TOF MS (Table II). All six peptides contained overlapping sequences, an identical C-terminal residue (Lys-37), and variable truncated N termini, most likely due to the action of a contaminating chymotryptic-like protease activity (Table IIa). Added diversity was derived from the oxidative state of the acetylated, N-terminal Met (methionine sulfoxide). The combined mass data indicated the presence of a single phosphate group situated between residues 20 and 37 of HSSB-p34, a region that contains three serines (Ser-23, -29, -33, and Thr-21). The level of p34-P1 increased as the phosphorylation reaction proceeded and plateaued after 90 min. In contrast, the hyperphosphorylated product, p34-P2, was not clearly visible until after 30 min of incubation, and the amount of p34-P2 continued to increase up to 2 h. In the presence of a saturating amount of DNA-PK and lower levels of substrate, all of the HSSB-p34 subunit was converted to the hyperphosphorylated form, p34-P2 (data not shown). These observations suggest that DNA-PK initially phosphorylated Thr-21, resulting in the p34-P1 species, followed by phosphorylation of Ser-33 and conversion to the p34-P2 species.

Ser-29 is the Predominant Cdc2-Cyclin B Phosphorylation Site within the HSSB-p34 Subunit in Vitro—Previously we and others have shown that, in addition to DNA-PK, the Cdc2 kinase also phosphorylates HSSB-p34 in vitro (29–31, 34). This reaction yielded two forms of p34 that migrated slower than the unphosphorylated p34 subunit following SDS-PAGE, a predominant phosphorylated form p34-P1 and a minor band, p34-PII (see Fig. 7, inset). Approximately one phosphate residue was incorporated into each molecule of p34-P1 (34). p34-P1 was isolated and subjected to trypsin digestion followed by reverse phase HPLC fractionation. Six $^{32}$P-radiolabeled peptides were isolated (one major peak, T6, and five minor peaks, T1 to T5) (Fig. 7). These peptides were characterized by chemical sequencing and MALDI-TOF MS (Table II). All six peptides contained overlapping sequences, an identical C-terminal residue (Lys-37), and variable truncated N termini, most likely due to the action of a contaminating chymotryptic-like protease activity (Table IIa). Added diversity was derived from the oxidative state of the acetylated, N-terminal Met (methionine sulfoxide). The combined mass data indicated the presence of a single phosphate group situated between residues 20 and 37 of HSSB-p34, a region that contains three serines (Ser-23, -29, -33, and Thr-21). The level of p34-P1 increased as the phosphorylation reaction proceeded and plateaued after 90 min. In contrast, the hyperphosphorylated product, p34-P2, was not clearly visible until after 30 min of incubation, and the amount of p34-P2 continued to increase up to 2 h. In the presence of a saturating amount of DNA-PK and lower levels of substrate, all of the HSSB-p34 subunit was converted to the hyperphosphorylated form, p34-P2 (data not shown). These observations suggest that DNA-PK initially phosphorylated Thr-21, resulting in the p34-P1 species, followed by phosphorylation of Ser-33 and conversion to the p34-P2 species.
32P-phosphate moiety was still associated with the truncated peptide and that the single phosphorylated site was either Ser-29 or Ser-33 (Table IIb; peptide C1-1a.a. and C1-3a.a.). Though only Ser-29 within this peptide is located in a Cdc2 kinase target sequence (serine-proline), direct evidence was needed to further define which of these two serine residues was phosphorylated by the Cdc2-cyclin B kinase. Synthetic peptides were constructed to include these two serine residues (Fig. 8, top, wild type). Peptides in which either one of these two serines was changed to alanine (mutant 1 and mutant 2, see Fig. 8, top) were also synthesized. As shown in Fig. 8, bottom, the Cdc2-cyclin B kinase phosphorylated both the wild-type and mutant peptide 2, which contained an alanine substitution at Ser-29 but not Ser-33 is the major Cdc2 kinase phosphorylation site in HSSB-p34.

Other residue(s) within HSSB-p34 may be targets for phosphorylation by the Cdc2 kinase, as indicated by the presence of a minor hyperphosphorylated p34 species, p34-PII (Fig. 7, inset). Mutant HSSB-p34 in which the Ser-29 was changed to an alanine residue was phosphorylated by Cdc2-cyclin B kinase, although with much reduced efficiency (36). These results indicate that HSSB-p34 probably contains multiple Cdc2 phosphorylation sites, with Ser-29 being the predominant one.

DISCUSSION

We have shown that the p34 subunit of HSSB is efficiently phosphorylated at residues Thr-21 and Ser-33 by DNA-PK in the presence of Ku and calf thymus DNA. In this reaction, Thr-21 was more rapidly phosphorylated than Ser-33. We also

FIG. 7. Reversed phase-HPLC tryptic peptide map of HSSB-p34 phosphorylated by Cdc2-cyclin B kinase. The chromatogram (relevant section only) shows the absorption profile of 214 nm of peptides eluting from an 2.1-mm Vydac C4 column at 100 µl/min in 0.7%/min acetonitrile gradient (3.5 to 70% in 0.1% trifluoroacetic acid). Numbered peaks (T1 to T6) contained 32P-labeled peptides, all of which were analyzed by chemical sequencing and mass spectrometry (see Table II). The inset shows a silver-stained protein gel of HSSB-p34. Lane 1, untreated p34 (P-0); lane 2, p34 treated with Cdc2 kinase; P-I and P-II represent Cdc2-cyclin B phosphorylated forms of the p34 subunit.
identified Ser-29 as the primary Cdc2 phosphorylation site in HSSB-p34 (Fig. 9).

DNA-PK preferentially phosphorylates serine or threonine residues that are followed or preceded by glutamine residues (S/T+Q or Q-S/T) (50). Such target sequences are found within the HSSB-p34 subunit, including five serine residues (Ser-23, -33, -52, -72, and -174) and one threonine residue (Thr-21). However, of the six potential target sites, only Thr-21 and Ser-33 were efficiently phosphorylated by DNA-PK. Earlier studies have shown that, in addition to the target sequences described above, poorly characterized additional sequences and/or tertiary protein structures may be required for efficient DNA-PK phosphorylation (40). These additional considerations may explain why only a few of the synthetic peptides that contain Ser-23, Ser-33, Ser-52, Ser-72, or Ser-174 were efficiently phosphorylated by DNA-PK (data not shown). Since only Thr-21 and Ser-33 in HSSB-p34 were phosphorylated efficiently, the other potential phosphorylation sites may not meet the poorly defined additional requirements. Another consideration is that the sites not phosphorylated may be inaccessible to DNA-PK due to the association of p34 with p70 and p11 subunit. It has been shown that the p34 subunit is tightly associated with the p70 and p11 subunits via interactions with the middle and C-terminal regions of p34 (51), although the last 33 amino acid residues in the C-terminal region of p34 are not essential for this interaction (37). This association may sterically block the phosphorylation sites (Ser-52, -72, and -174) located within these regions of p34. Proteolysis experiments done by Gomes et al. (48) and protease digestion results shown in this report suggest that the N-terminal region of HSSB-p34 may be more accessible for enzymatic modification.

The association of p34 with p70 and p11 enabled a more efficient phosphorylation of Thr-21 and Ser-33 in the N-terminal region. As we have previously shown, whereas the p34 subunit alone was a poor substrate for DNA-PK, its association with the p11 subunit dramatically increased phosphorylation of the p34 subunit to a level equivalent to that observed with the p70-p34-p11 trimeric complex (39). This suggests that, although only the p34 subunit contains DNA-PK phosphorylation sites, the other two associated subunits, especially the p11 subunit, are important for the efficient phosphorylation of HSSB-p34. The mechanism underlying this observation is not known. One possibility is that the interaction between the p11 subunit and the p34 subunit renders the substrate more accessible to DNA-PK.

It has been shown recently that HSSB interacts with DNA in at least two different modes. Different physical changes occur in the HSSB protein structure when HSSB binds to single-stranded DNA, including a decrease in replication activity (32, 33). This suggests that the phosphorylation of HSSB-p34 may also play a role in coordinating the repair of damaged DNA with normal DNA synthesis and cell cycle progression.

Currently the construction of HSSB containing mutations in both the DNA-PK and the Cdc2 kinase phosphorylation sites is underway. Biochemical characterization of this mutant protein may allow us to determine directly the role of the phosphorylation of HSSB-p34 in the cell.

Acknowledgments—We thank Scott Geromano, San-San Yi, Mary Lui, and Lynne Lacomis for technical support. We also thank Dr. Anthony Amin and Emma Gibbs for critical reading of the manuscript.

REFERENCES

1. Wobbe, C. R., Weissbach, L., Borowiec, J. A., Dean, F. B., Murakami, Y., Bullock, P., and Hurwitz, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1834–1838

2. Wold, M. S., Li, J. J., and Kelly, T. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3643–3647

3. Fairman, M. P., and Stillman, B. (1988) EMBO J. 7, 1211–1218

4. Hurwitz, J., Dean, F. B., Kwong, A. D., and Lee, S.-H. (1990) J. Biol. Chem. 265, 18043–18046

5. Dean, F. B., Borowiec, J. A., Ishimi, Y., Deb, S., Tegtmeyer, P., and Hurwitz, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8267–8271

6. Dedon, M., Dean, F., Bullock, P., Echols, H., and Hurwitz, J. (1987) Science 236, 964–967

7. Collins, K. L., and Kelly, T. J. (1991) Mol. Cell. Biol. 11, 2108–2115

8. Erdile, L. F., Heyer, W.-D., Kolodner, R., and Kelly, T. J. (1991) J. Biol. Chem. 266, 12090–12098

9. Donreiter, I., Erdile, L. F., Gilbert, I. U., Winkler, D., Kelly, T. J., and Fanning, E. (1992) EMBO J. 11, 769–776

10. Tsurimoto, T., and Stillman, B. (1989) EMBO J. 8, 3883–3889

11. Kenny, M. K., Lee, S.-H., and Hurwitz, J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9757–9761

12. Kenny, M. K., Schlegel, U., Furneaux, H., and Hurwitz, J. (1990) J. Biol. Chem. 265, 7693–7700

13. Lee, S.-H., Pan, Z.-Q., Kwong, A. D., Burgers, P. M. J., and Hurwitz, J. (1991) J. Biol. Chem. 266, 22707–22711

14. Coverty, D., Kenny, M. K., Mane, M., Rupp, W. D., Lane, D. P., and Wood, R. D. (1991) Nature 349, 538–541

15. Matuda, T., Saijo, M., Kuraoka, I., Kobayashi, T., Nakatsu, Y., Nagai, A., Enjoji, T., Masutani, C., Sugawara, K., Hanaka, F., Yasui, A., and...
16. He, Z., Henricksen, L. A., Wold, M. S., and Ingles, C. J. (1995) Nature 374, 566–569
17. Li, L., Lu, X., Peterson, C. A., and Legerski, R. J. (1995) Mol. Cell. Biol. 15, 5396–5402
18. Matsunaga, T., Park, C.-H., Bessho, T., Mu, D., and Sancar, A. (1996) J. Biol. Chem. 271, 11047–11050
19. Park, M. S., Ludwig, D. L., Stigger, E., and Lee, S.-H. (1996) J. Biol. Chem. 271, 18996–19000
20. Moore, S. P., Erdile, L., Kelly, T., and Fishel, R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9067–9071
21. Firmenich, A. A., Elias-Arnanz, M., and Berg, P. (1995) Mol. Cell. Biol. 15, 1620–1631
22. Longhese, M. P., Plevani, P., and Lucchini, G. (1994) Mol. Cell. Biol. 14, 7884–7890
23. Smith, J., and Rothstein, R. (1995) Mol. Cell. Biol. 15, 1632–1641
24. He, Z., Brinton, B. T., Greenblatt, J., Hassell, J. A., and Ingles, C. J. (1993) Cell 73, 1223–1232
25. Li, R., and Botchan, M. R. (1993) Cell 73, 1207–1221
26. Dutta, A., Ruppert, J. M., Aster, J. C., and Winchester, E. (1993) Nature 365, 79–82
27. Luche, R. M., Smart, W. C., Marion, T., Tillman, M., Sumrada, R. A., and Cooper, T. G. (1993) Mol. Cell. Biol. 13, 5749–5761
28. Singh, K. K., and Samson, L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4907–4911
29. Fotedar, R., and Roberts, J. M. (1992) EMBO J. 11, 2177–2187
30. Dutta, A., and Stillman, B. (1992) EMBO J. 11, 2189–2199
31. Din, S.-U., Brill, S. J., Fairman, M. P., and Stillman, B. (1990) Genes Dev. 4, 968–977
32. Chomczynski, P., Zernik-Kobak, M., McGrath, S., and Dixon, K. (1994) EMBO J. 13, 2114–2123
33. Liu, V. F., and Weaver, D. T. (1993) J. Cell Sci. 106, 983–994
34. Pan, Z.-Q., Amin, A. A., Gibbs, E., Niu, H., and Hurwitz, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8343–8347
35. Brush, G. S., Anderson, C. W., and Kelly, T. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12520–12524
36. Henricksen, L. A., Umbricht, C. B., and Wold, M. S. (1994) J. Biol. Chem. 269, 11121–11126
37. Lee, S.-H., and Kim, D. K. (1995) J. Biol. Chem. 270, 12801–12807
38. Pan, Z.-Q., Park, C.-H., Amin, A. A., Hurwitz, J., and Sancar, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4636–4640
39. Gibbs, E., Pan, Z.-Q., Niu, H., and Hurwitz, J. (1996) J. Biol. Chem. 271, 22847–22854
40. Lees-Miller, S. P., Sakaguchi, K., Ullrich, S., Appella, E., and Anderson, C. W. (1992) Mol. Cell. Biol. 12, 5041–5049
41. Tempst, P., Link, A. J., Riviere, L. R., Fleming, M., and Elicone, C. (1990) Electrophoresis 11, 537–553
42. Lui, M., Tempst, P., and Erdjument-Bromage, H. (1996) Anal. Biochem. 241, 156–166
43. Elicone, C., Lui, M., Geromanos, S., Erdjument-Bromage, H., and Tempst, P. (1994) J. Chromatogr. 676, 121–137
44. Erdjument-Bromage, H., Lui, M., Sabatini, D. M., Snyder, S. H., and Tempst, P. (1994) Protein Sci. 3, 2435–2446
45. Geromanos, S., Casteels, P., Elicone, C., Powell, M., and Tempst, P. (1994) Techniques in Protein Chemistry V (Crabb, J. W., ed) pp. 143–150, Academic Press, San Diego, CA
46. Tempst, P., Geromanos, S., Elicone, C., and Erdjument-Bromage, H. (1994) Methods 6, 248–261
47. Kamps, M. P., and Selton, B. M. (1989) Anal. Biochem. 176, 22–27
48. Gomes, X. V., Henricksen, L. A., and Wold, M. S. (1996) Biochemistry 35, 5586–5595
49. Henricksen, L. A., Carter, T., Dutta, A., and Wold, M. S. (1996) Nucleic Acids Res. 24, 3117–3122
50. Anderson, C. W. (1993) Trends Biochem. Sci. 18, 433–437
51. Lin, Y.-L., Chen, C., Keshav, K. F., Winchester, E., and Dutta, A. (1996) J. Biol. Chem. 271, 17190–17198
52. Blackwell, L. J., Borrow, J. A., and Mastrangelo, I. A. (1996) Mol. Cell. Biol. 16, 4798–4807
53. Henricksen, L. A., and Wold, M. S. (1994) J. Biol. Chem. 269, 24203–24208
54. Azam, M., Erdjument-Bromage, H., Kreider, B. L., Xia, M., Quelle, P., Basu, R., Saris, C., Tempst, P., Ihle, J. E., and Schindler, C. (1995) EMBO J. 14, 1402–1411
Mapping of Amino Acid Residues in the p34 Subunit of Human Single-stranded DNA-binding Protein Phosphorylated by DNA-dependent Protein Kinase and Cdc2 Kinase in Vitro

Hongwu Niu, Hediye Erdjument-Bromage, Zhen-Qiang Pan, Suk-Hee Lee, Paul Tempst and Jerard Hurwitz

J. Biol. Chem. 1997, 272:12634-12641.
doi: 10.1074/jbc.272.19.12634

Access the most updated version of this article at http://www.jbc.org/content/272/19/12634

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 53 references, 32 of which can be accessed free at http://www.jbc.org/content/272/19/12634.full.html#ref-list-1