Cell Cycle-regulated Phosphorylation of the Human SIX1 Homeodomain Protein*

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Human SIX1 (HSIX1) is a member of the Six class of homeodomain proteins implicated in muscle, eye, head, and brain development. To further understand the role of HSIX1 in the cell cycle and cancer, we developed an HSIX1-specific antibody to study protein expression at various stages of the cell cycle. Our previous work demonstrated that HSIX1 mRNA expression increases as cells exit S phase and that overexpression of HSIX1 can attenuate a DNA damage-induced G<sub>2</sub> cell cycle checkpoint. Overexpression of HSIX1 mRNA was observed in 44% of primary breast cancers and 90% of metastatic lesions. Now we demonstrate that HSIX1 is a nuclear phosphoprotein that becomes hyperphosphorylated at mitosis in both MCF7 cells and in Xenopus extracts. The pattern of phosphorylation observed in mitosis is similar to that seen by treating recombinant HSIX1 with casein kinase II (CK2) in vitro. Apigenin, a selective CK2 inhibitor, diminishes interphase and mitotic phosphorylation of HSIX1. Treatment of MCF7 cells with apigenin leads to a dose-dependent arrest at the G<sub>2</sub>/M boundary, implicating CK2, like HSIX1, in the G<sub>2</sub>/M transition. HSIX1 hyperphosphorylated in vitro by CK2 loses its ability to bind the MEF3 sites of the aldolase A promoter (pM), and decreased binding to pM is observed during mitosis. Because CK2 and HSIX1 have both been implicated in cancer and in cell cycle control, we propose that HSIX1, whose activity is regulated by CK2, is a relevant target of CK2 in G<sub>2</sub>/M checkpoint control and that both molecules participate in the same pathway whose dysregulation leads to cancer.

The products of homeobox genes are characterized by a 60 amino acid DNA-binding region, the homeodomain, which enables them to activate the transcription of genes that are important for the regulation of cell growth, fate, differentiation, and body patterning. HSIX1 belongs to the Six class of homeodomain proteins, which share a lysine in position 50 of the recognition helix of the homeodomain (1). These proteins can be further subdivided into three distinct families that presumably originated from three different ancestral Six genes (2). In mammals two gene members have been identified for each family, thus accounting for the six known members of this class. To date, 12 Six gene homologues have been identified in lower vertebrates (2). Of the Six proteins discovered to date, several function in the development of the forebrain, eye, and muscle (2, 3).

We previously cloned HSIX1 from late S phase 21PT mammary carcinoma cells, and demonstrated that its overexpression in MCF7 cells attenuated a DNA damage-induced G<sub>2</sub> cell cycle checkpoint. HSIX1 overexpression was observed in 44% of primary breast cancers, and 90% of metastatic lesions examined. This suggested that HSIX1 has a role in tumor progression, possibly through its cell cycle checkpoint function (4). Recently, it was speculated that the c-met gene is a potential target of Six1 (5). Additional targets that may explain the role of Six1 in the cell cycle and/or tumor progression are not known. However, myogenin was identified as a target of HSIX1 in muscle development (6).

In general, very little is known about the targets of homeodomain proteins. Although most homeodomain containing proteins bind to similar short consensus DNA sequences in vitro, they have highly specific functions in vivo. Therefore, target specificity in vivo is achieved by other elements such as interaction with cofactors, translational regulation, subcellular localization, or protein phosphorylation (7).

Protein phosphorylation regulates a number of homeodomain-containing transcription factors including Csx/Nkx2.5, Cut, Pit-1, Oct-1, and Drosophila Engrailed and Antennapedia by affecting protein-protein interactions, DNA binding, or nuclear localization (7). In some instances, phosphorylation is cell cycle-dependent (8–10). Mitotic phosphorylation of both the POU transcription factor GHF-1 and the Oct-1 homeodomain containing protein inhibits their DNA binding activity (9, 10) and may represent a general mechanism for decreasing transcription during mitosis.

Several kinases are known to phosphorylate homeodomain-containing proteins, including protein kinase casein kinase II (CK2), protein kinase C (PKC), and protein kinase A. In particular, protein kinase CK2, a serine/threonine kinase that is ubiquitously expressed, has been shown to phosphorylate transcription factors including those encoded by Csx/Nkx2.5 (7), Cut (11), Hoxb-6 (12), even-skipped (13), and Engrailed (14) homeobox genes. The phosphorylation of the Drosophila An-

mitogen-activated protein kinase; CIAP, calf intestinal alkaline phosphatase.

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tennapedia protein by CK2 was shown to be important for its role in thoracic and abdominal development (15).

To understand the regulation of the HSIX1 protein, we developed an HSIX1-specific antibody and examined protein levels and phosphorylation at various stages of the cell cycle. We find that HSIX1 is a phosphoprotein in both interphase and mitotic cells and that protein kinase CK2 is at least partly responsible for the phosphorylation of HSIX1 in both interphase and mitosis. In mitosis, the HSIX1 protein becomes hyperphosphorylated, and a concomitant loss in DNA binding activity is seen. The phosphorylation of HSIX1 by CK2 has implications for both cell cycle control and tumorigenesis.

**MATERIALS AND METHODS**

**Plasmid Constructions**

The GST C-terminal HSIX1 construct utilized for antibody production was generated by PCR amplification of the C terminus of HSIX1 (beginning from nucleotide 822, just after the homeodomain and terminating at the STOP codon) from the full-length SKMFL plasmid (wild type HSIX1 cloned into the BamHI/XbaI site of the Invitrogen pCDNA3.1/His plasmid) utilizing standard PCR conditions and a 5′ primer containing a Xhol restriction site (ACT CTC GAG GAC CAG GAA AGG GAG AAC) and 3′ primer containing an XhoI restriction site (TGG TCT AGA CCT TGA GGA CCC CAA GTC CAC-pSixXba I). The C terminus was then subcloned into an In vitropolymerase chain vector pcR2.1 according to the manufacturer’s recommendations, resulting in the pcR2.1Cterm plasmid. Partial digests (16) were performed on the pcR2.1Cterm plasmid with EcoRI to release the full-length C-terminal fragment of HSIX1. The C terminus of HSIX1 was then subcloned into the EcoRI sites of pGEX2TK (Amersham Pharmacia Biotech), and the resulting construct was sequenced to ensure the proper orientation and to ensure that no mutations were introduced. Deletion constructs were generated as follows.

ΔHD— The N terminus of HSIX1 (from the start codon to nucleotide 689, which is in the first helix of the encoded homeodomain) was amplified as the C terminus (above), using a 5′ primer containing a BamHI site (CTG GGA TCC ATG ATG CTG CCG TTG-pSixBHI) and a 3′ primer containing an Xhol site (ATC CTC GAG GAC ACC CCT CGA CTG CTT CTT). The resulting N-terminal fragment was then subcloned into the TA cloning vector pcR2.1 as above (pCcr2.1Nterm). The N-terminal and C-terminal portions of HSIX1 were then removed from pcR2.1 by digesting with BamHI/XhoI and XhoI/XbaI, respectively, and were subsequently ligated into the BamHI and XhoI sites of pCDNA3.1 (+) to generate the ΔHD plasmid. Sequencing was performed to ensure that the two portions of HSIX1 were fused in frame and that the homeodomain was intact.

ΔNterm and ΔCterm—The homeobox and C-terminal portions (for ΔNterm) of HSIX1 were amplified using standard PCR conditions from the SIXFL plasmid (4) with a 5′ primer containing a BamHI site (CTG GGA TCC ATG AAA TTT CCA CCG CCG ACC) and the pSixXbaI 3′ primer. The N-terminal and homeobox regions (for ΔCterm) were amplified as above using the pSixBHI 5′ primer and a 3′ primer containing a STOP codon as well as an ATG site (TGC TCT AGA CTA GTT AAG GAA AGG GAG AAC) and 3′ primer containing a BamHI site (CTG GGA TCC ATG ATG CTG CCG TTG-pSixXbaI) and 3′ primer containing an XhoI restriction site (ATG TCG ATG CTG CCG TTT-pSixBHI) and a 3′ primer containing an XhoI restriction site (ATC CTC GAG GAC ACC CCT CGA CTG CTT CTT). The resulting N-terminal fragment was then subcloned into the TA cloning vector pcR2.1 as above (pCcr2.1Cterm). Plasmids were then digested with BamHI and XhoI and subcloned into the pcDNA3.1 (+) plasmid. Sequencing of both constructs was performed to ensure that no mutations were introduced.

**Antibody Production**

The GST C-terminal HSIX1 fusion protein was induced and purified using glutathione beads as described previously (17). The fusion protein was released into the supernatant by adding 50 μM Tris, pH 8, containing 10 mM reduced glutathione and incubating at 4 °C for 10 min. Bradford assays were performed to determine the protein concentration in vitro.

**Western Blot Analysis and CIAP Reactions**

Transfected cells were lysed 24–48 h post-transfection in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% nadeoxycholate, 0.1% SDS, 5 μg/ml leupeptin, 1 mM phenylmethyisulfonyl fluoride, 10 μM NaF, 200 μM Na3VO4) for 20 min at 4 °C. Lysates were passed through a 25-gauge needle 5–6 times to shear the DNA and then microcentrifuged at 4 °C, 14,000 rpm for 15 min. Supernatants were treated with calf intestinal alkaline phosphatase according to Kasshara and Izumo (7), and Western blots were performed as described (18) using a 1:1000 dilution of anti-HSIX1 as the primary antibody and a 1:10000 dilution of anti-rabbit IgG horseradish peroxidase (Sigma) as a secondary antibody. Chemiluminescence with ECL (Amersham Pharmacia Biotech) was utilized to detect the HSIX1 signal.

**Immunocytochemistry**

MCF7 cells were plated in 6-well dishes on coverslips at 2.5 × 105 cells/well. 24 h later, cells were transfected with SIXFL using Fugene (Roche Molecular Biochemicals) according to the manufacturer’s protocols. 24–48 h post-transfection, cells were fixed in 0.75% formaldehyde in PBS for 10 min. followed by 5–10 min in 0.5% Triton X-100. After overnight incubation in a 1:100 dilution of anti-HSIX1 for 1 h at room temperature followed by several washes in PBS. The cells were then incubated in a 1:1000 dilution of anti-rabbit IgG-fluorescein (Calbiochem, La Jolla, CA) for 45 min at room temperature. After five washes in PBS, the cells were mounted in Vectashield (Vector Labs, Burlingame, CA) containing 0.1 μg/ml 4,6-diamidino-2-phenylindole to counterstain the nuclei.

**In Vitro HSIX1 Phosphorylation**

[S35]Methionine-labeled HSIX1 was incubated with various kinases as follows: CK2 for 30 min at 30 °C with 50 units of recombinant casein kinase II (Calbiochem) in 20 mM Tris, pH 7.5, 50 mM KCl, 10 mM MgCl2, 0.25 mM ATP; cyclin B/cdc2 for 20 min at 30 °C with 2 μl of purified Xenopus cyclin B-cdc2 (19) in 50 mM Tris, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol; PKC for 15 min at 30 °C with 20 ng of the catalytic subunit of rat brain protein kinase C (Calbiochem) in 25 mM Tris, pH 7.4, 5 mM MgCl2, 6 mM nMgCl2, 1 mM CaCl2, 1 mM ATP. Before use in EMSAs, salt concentrations were adjusted to give appropriate final molarities.

**Inhibitor Studies**

Cells were transfected with SIXFL as described above. MCF7/SIXFL cells were incubated with various inhibitors at indicated concentrations in medium for 3–5 h at 37 °C, after which lysates were isolated as above. Phosphorylation of Western blots developed with the HSIX1 antibody allowed determination of the percentage of HSIX1 phosphorylated in interphase in the presence of the various inhibitors. For assessment of kinases important for mitotic phosphorylation, mitotic Xenopus assays containing [35S]methionine-labeled HSIX1 were carried out as above by adding the indicated inhibitors at the time of HSIX1 addition. Densitometric scanning was utilized to determine the phosphorylation level.
percentage of HSIX1 that was hyperphosphorylated in the presence of various inhibitors. The inhibitors utilized were: apigenin (Sigma), a selective CK2 inhibitor; roscovitine (Calbiochem), a cdc2 kinase inhibitor; bisindolylmaleimide 1 (Calbiochem), a PKC inhibitor; and PD98059 (New England Biolabs, Beverly, MA), a mitogen-activated protein kinase kinase 1 (MEKI) and MAPK cascade inhibitor.

**Protein CK2 Activity Assays**

For kinase assays, 5 μg of protein extracted from MCF7 cells was incubated with or without 1 μM of the specific protein kinase CK2 peptide RRREETEEET (Sigma-Genosys, The Woodlands, TX) in buffer (100 mM Tris, pH 8.0, 20 mM MgCl2, 100 mM NaCl, 50 mM KCl, 0.1 μg/μl bovine serum albumin, and 100 μM Na3VO4) and 5 μCl of [γ-32P]ATP at 30 °C for 10 min (20). The kinase reaction was terminated by addition of 25 μl of 100 mM ATP in 0.4 N HCl. Samples were spotted onto a P81 Whatman filter and washed four times for 5 min each with 150 mM H3PO4 to elute unincorporated counts. Incorporated counts were quantified in an automatic scintillation counter. Samples were assayed in triplicate. Kinase activity was calculated as the subtraction of the mean of samples without peptide from the mean of samples with peptide.

**Cell Cycle Experiments**

MCF7 cells were cultured as described above. When cells reached 50–70% confluence, 20–80 μM apigenin (Sigma) or Me2SO alone was administered for 18 h. Cells were then resuspended in Nicotelli buffer (0.1% Triton X-100 and 0.1% sodium citrate) containing 0.5 mg/ml propidium iodide (Sigma), and the DNA content was analyzed on a flow cytometer (Becton Dickinson, Mountain View, CA) using the Cellquest software program.

**Electrophoretic Mobility Shift Assays**

These assays were performed as described in Spitz et al. (6) using the aldolase A MEP3 site sequence (tgaattctagggttctggttcc). The buffer utilized for protein-DNA binding contained 25 mM Hepes, pH 7.6, 5 mM MgCl2, 10% glycerol, 34 mM KCl, 1 mM dithiothreitol. Unlabeled wild type and mutant oligonucleotides (bold nucleotide changed from t to g above) were used as competitors at 50 times the radiolabeled oligonucleotide concentration.

**RESULTS**

**HSIX1 Is a Nuclear Phosphoprotein in Mammary Carcinoma Cells**—To study HSIX1 function, we generated an HSIX1-specific antibody by injecting a GST C-terminal HSIX1 fusion protein into rabbits. After affinity purifying the antibody, it was tested on lysates from MCF7 cells transiently transfected with HSIX1 (MCF7/SIXFL) and on lysates from control transfected MCF7 cells that had previously been shown to contain almost no endogenous HSIX1 mRNA. Western blot analysis identified three bands of molecular masses between 37 and 42 kDa in HSIX1 transfected MCF7 cells but not in controls, demonstrating the specificity of our antibody (Fig. 1A) and suggesting that the protein is post-translationally modified or processed. Immunocytochemistry with the HSIX1 antibody demonstrated that transfected HSIX1 is a nuclear protein (Fig. 1B).

A data-base search of the HSIX1 amino acid sequence revealed 11 potential phosphorylation sites in the protein (Fig. 2), particularly in the C terminus. To address whether HSIX1 is a phosphoprotein, lysates obtained from asynchronous MCF7/SIXFL cells were treated with calf intestinal alkaline phosphatase (CIAP), which demonstrated the existence of a phosphatase sensitive form of HSIX1 (Fig. 3A). Dephosphorylation was blocked in the presence of excess phosphate. To determine whether endogenous HSIX1 also exists as a phosphoprotein, we prepared nuclear extracts from asynchronous 21PT breast cancer cells, previously shown to contain HSIX1 mRNA, and performed the CIAP reaction as described for MCF7/SIXFL cells. Fig. 3B demonstrates that endogenous HSIX1 also exists as a phosphoprotein in an asynchronous population of 21PT cells.

**HSIX1 Is Hyperphosphorylated in Mitotic Cells**—The cdc2 kinase, which has catalytic specificity for a proline C-terminal to the site it phosphorylates, is only active in mitosis when it is partnered with its regulatory subunit, cyclin B, and is activated by various phosphorylation and dephosphorylation events (21). The HSIX1 sequence has several putative cdc2 phosphorylation sites (Fig. 2). This, in addition to previous findings that several homeodomain containing proteins are hyperphosphorylated in mitosis (8–10), prompted us to examine the phosphorylation state of HSIX1 in interphase versus mitosis. Western blot analysis on lysates from MCF7/SIXFL cells synchronized in mitosis by addition of nocodazole, as well as on lysates from mitosis- and metaphase-enriched MCF7/SIXFL cells that were sorted by flow cytometry, demonstrated the existence of a hyperphosphorylated form of HSIX1 (data not shown).

Because biochemical analysis of this form of the protein was difficult in mammalian culture cells, where drug treatments...
must be used to obtain large numbers of mitotic cells, we chose the synchronous Xenopus laevis system to carry out these studies. In vitro translated [35S]methionine-labeled HSIX1 was incubated with interphase and mitotic extracts from X. laevis and examined for phosphatase-sensitive alterations in mobility. In interphase, an HSIX1 triplet was observed, as seen in asynchronous MCF7 cells, where greater than 75–90% are generally in interphase (data not shown). HSIX1 incubated in mitotic extracts exhibited a higher molecular mass form of HSIX1, which could be eliminated by treatment with CIAP, indicating hyperphosphorylation of HSIX1 in mitosis (Fig. 4A).

To determine the region of HSIX1 that is hyperphosphorylated in mitosis, deletion constructs were generated (Fig. 4B). Proteins with deleted regions were translated in the presence of [35S]methionine and incubated in interphase and mitotic extracts. Those lacking the homeodomain (ΔHD) or the N terminus (ΔNterm) exhibited a shift to a slower mobility when incubated in mitotic extracts. However, the C-terminal deleted protein (ΔCterm) was not shifted (Fig. 4C). This suggests that the majority of the mitotic-specific phosphorylation occurs in the C terminus, in accordance with the multiple phosphorylation sites observed in this region of the protein.

HSIX1 Is Phosphorylated by Protein Kinase CK2 in Asynchronous MCF7 Cells—Data base searching revealed that HSIX1 contains potential consensus phosphorylation sites for protein kinase CK2, PKC, and cyclin B/cdc2. We set out to determine which of these kinases are responsible for HSIX1 phosphorylation. [35S]Methionine-labeled in vitro translated HSIX1 was incubated with each of these three putative HSIX1 kinases (Fig. 5A). PKC, cyclin B/cdc2, and CK2 all can phosphorylate HSIX1 in vitro, and a greater extent of hyperphosphorylation is observed when the protein is incubated with cyclin B/cdc2 or CK2 than with PKC. Phosphorylation of HSIX1
**Protein kinase CK2 phosphorylates HSIX1 in interphase and mitosis.** A. [$^{35}$S]methionine-labeled HSIX1 can be phosphorylated *in vitro* by cdc2 kinase, protein kinase CK2, and to a lesser degree by PKC. [$^{35}$S]methionine-labeled HSIX1 incubated in interphase and mitotic extracts (IE and ME, respectively) demonstrates the patterns of phosphorylation seen in the different periods of the cell cycle as compared with the *in vitro* phosphorylated proteins. The IE and ME lanes represent a longer exposure of the same gel on which the *in vitro* phosphorylated proteins were electrophoresed. B. MCF7 cells were transfected with HSIX1 and then treated with varying amounts of kinase inhibitors. api, apigenin, a selective protein kinase CK2 inhibitor; roso, roscovitine, a cdc2 kinase inhibitor; bisInd, bisindolylmaleimide, a PKC inhibitor. Only the apigenin significantly decreases the phosphorylation of HSIX1 in interphase. C, dose response of apigenin on CK2 activity in MCF7 cells parallels the decreased phosphorylation of HSIX1. In a separate experiment, inhibition of HSIX1 phosphorylation as well as inhibition of CK2 activity by apigenin was measured. conc, concentration; inh, inhibition; phosph, phosphorylation. D, protein kinase CK2 is involved in the mitotic hyperphosphorylation of HSIX1. Mitotic extracts containing exogenously added [$^{35}$S]methionine-labeled HSIX1 were assessed for the extent of hyperphosphorylation of HSIX1 in the presence of the various kinase inhibitors. Only extracts incubated with apigenin showed a statistically significant decrease in hyperphosphorylation of HSIX1. The results represent an average of three samples ± S.D. Statistical analysis was performed using a Student’s *t* test with *p* values as follows: apigenin treatment, 0.0155*; roscovitine treatment, 0.1188; bisindolylmaleimide treatment, 0.9069 (where the asterisk indicates that only apigenin treatment leads to a statistically significant decrease in HSIX1 phosphorylation).

*Fig. 5.* Protein kinase CK2 phosphorylates HSIX1 in interphase and mitosis. A. [$^{35}$S]methionine-labeled HSIX1 can be phosphorylated *in vitro* by cdc2 kinase, protein kinase CK2, and to a lesser degree by PKC. [$^{35}$S]methionine-labeled HSIX1 incubated in interphase and mitotic extracts (IE and ME, respectively) demonstrates the patterns of phosphorylation seen in the different periods of the cell cycle as compared with the *in vitro* phosphorylated proteins. The IE and ME lanes represent a longer exposure of the same gel on which the *in vitro* phosphorylated proteins were electrophoresed. B. MCF7 cells were transfected with HSIX1 and then treated with varying amounts of kinase inhibitors. api, apigenin, a selective protein kinase CK2 inhibitor; roso, roscovitine, a cdc2 kinase inhibitor; bisInd, bisindolylmaleimide, a PKC inhibitor. Only the apigenin significantly decreases the phosphorylation of HSIX1 in interphase. C, dose response of apigenin on CK2 activity in MCF7 cells parallels the decreased phosphorylation of HSIX1. In a separate experiment, inhibition of HSIX1 phosphorylation as well as inhibition of CK2 activity by apigenin was measured. conc, concentration; inh, inhibition; phosph, phosphorylation. D, protein kinase CK2 is involved in the mitotic hyperphosphorylation of HSIX1. Mitotic extracts containing exogenously added [$^{35}$S]methionine-labeled HSIX1 were assessed for the extent of hyperphosphorylation of HSIX1 in the presence of the various kinase inhibitors. Only extracts incubated with apigenin showed a statistically significant decrease in hyperphosphorylation of HSIX1. The results represent an average of three samples ± S.D. Statistical analysis was performed using a Student’s *t* test with *p* values as follows: apigenin treatment, 0.0155*; roscovitine treatment, 0.1188; bisindolylmaleimide treatment, 0.9069 (where the asterisk indicates that only apigenin treatment leads to a statistically significant decrease in HSIX1 phosphorylation).

by CK2 *in vitro* most closely resembled the hyperphosphorylation of the protein observed in mitotic extracts (Fig. 5A), although none of the kinases gave *in vitro* phosphorylation patterns of HSIX1 that were identical to those seen in interphase or mitotic extracts.

To determine which kinases were responsible for phosphorylating HSIX1 *in vitro*, MCF7/SIXFL cells were treated with inhibitors to each of these kinases. Apigenin, a selective CK2 inhibitor, diminished the phosphorylation of HSIX1 (Fig. 5B). This inhibition of HSIX1 phosphorylation was paralleled by
partial inhibition of CK2 activity (Fig. 5C). Neither roscovitine, a cyclin B/cdc2 inhibitor, nor bisindolylmaleimide, a PKC inhibitor, significantly inhibited the phosphorylation of HSIX1 in asynchronous, primarily interphase cells (Fig. 5B).

**Inhibitors of CK2, but Not Cyclin B/cdc2 or PKC, Significantly Diminish the Mitotic Hyperphosphorylation of HSIX1**—[35S]Methionine-labeled HSIX1 was incubated in *Xenopus* mitotic extracts in the absence or presence of various kinase inhibitors. 100 μM apigenin reduced CK2 activity in the extract by approximately 40% (data not shown) and decreased the ratio of the higher molecular mass (hyperphosphorylated) form of HSIX1 to the total amount of protein by an average of 27%, a statistically significant difference as assessed by a Student’s t test. However, treatment with either 100 μM roscovitine, a concentration known to inhibit MPF (cyclin B/cdc2) activity in *Xenopus* extracts, or 1 μM bisindolylmaleimide, which specifically inhibits PKC activity, did not significantly alter the extent of HSIX1 hyperphosphorylation (Fig. 5D). This suggests that CK2 is, at least in part, also responsible for the mitotic-specific hyperphosphorylation of HSIX1.

**Inhibition of CK2 Arrests MCF7 Cells at the G2/M Boundary**—Our previous work as well as that of others has implicated both HSIX1 and CK2 in the DNA damage-induced G2 cell cycle checkpoint and in tumorigenesis. Discovery of HSIX1 as a target of CK2 in both mammalian and *Xenopus* systems implies that the two proteins may cooperate in cell cycle control and tumorigenicity. Because we have already demonstrated that overexpression of HSIX1 in MCF7 cells affects the transition of cells through G2, we set out to determine the effect of CK2 on the cell cycle. MCF 7 cells treated with apigenin were arrested at the G2/M boundary in a dose-dependent manner (Fig. 6), suggesting that CK2 activity is important in the G2/M transition of mammary carcinoma cells.

**In Vitro Hyperphosphorylation of HSIX1 Inhibits DNA Binding**—To determine whether hyperphosphorylation of HSIX1 by CK2 affects its DNA binding activity, we performed EMSA (Fig. 7A) using the MEF3 sites of the aldolase A promoter (pM), which were previously demonstrated to bind the mouse Six1 protein (6). IVT HSIX1 formed three complexes when incubated with the pM oligonucleotide, which were all competed by cold wild type pM. Only the fastest migrating complex was not competed with cold mutant pM, suggesting that this complex is specific for HSIX1. The existence of HSIX1 in the complex was verified by competition with the HSIX1 antibody, and an antibody to GAL4 was not able to diminish binding. Furthermore, incubation with another in vitro translated homeodomain containing protein (Sox3) did not result in formation of the specific complex. When HSIX1 was hyperphosphorylated in vitro using...
CK2, the specific complex was diminished (Fig. 7, A and B); however, incubation of HSIX1 with heat-inactivated CK2 did not inhibit DNA binding (Fig. 7B). HSIX1 incubated with CK2 or heat-inactivated CK2 was examined on 10% SDS-polyacrylamide gels to determine the extent of HSIX1 phosphorylation under both conditions. Hyperphosphorylation of the protein was not observed when heat-inactivated CK2 was used (Fig. 7C), suggesting that hyperphosphorylation by CK2 decreases the ability of HSIX1 to bind DNA.

**DISCUSSION**

We have demonstrated that HSIX1 is a phosphoprotein that is hyperphosphorylated in mitosis and that this phosphorylation may regulate its activity. Phosphorylation regulates the activity of several *Drosophila* homeodomain containing proteins including fushi tarazu (22), Antennapedia (15), and even-skipped (Eve) (13). Such regulation is also observed in mammalian cells. The specific complex was diminished when mitotic extracts were incubated with the pM oligonucleotide, and addition of exogenous IVT HSIX1 to the extracts enhanced the binding in both interphase and mitosis, suggesting that this complex does contain Six1. The data are indicative of an endogenous form of *X. laevis* Six1 that is able to bind MEF3 sites in the aldolase A promoter to a greater extent in interphase than in mitotic extracts. This suggests that hyperphosphorylation of endogenous Six1 may decrease DNA binding in vivo. Although consistent with the hypothesis that mitotic hyperphosphorylation of HSIX1 inhibits DNA binding, a decrease in HSIX1 protein may be an alternative mechanism by which the DNA binding is reduced in mitosis. This possibility could not be examined using our HSIX1 antibody, because cross-reactivity to *Xenopus* Six1 was very low, an expected outcome because the antibody was made to the least conserved C-terminal domain of the protein.

**FIG. 7.** The DNA-protein complex formed by Six1 on the pM oligonucleotide is diminished when HSIX1 is phosphorylated in vitro by protein kinase CK2 and is also diminished in mitotic extracts. A, IVT HSIX1 forms a complex with the pM oligonucleotide that is competed by the wild type oligonucleotide (wt comp) and the HSIX1 antibody (HSIX1 Ab) but not with a mutant oligonucleotide (mut comp) or the GAL4 antibody (GAL4 Ab). When HSIX1 is phosphorylated with CK2 prior to incubation with the pM oligonucleotide (CK2), the specific binding is lost. The Six3 homeodomain containing protein cannot form the specific complex when incubated with the pM oligo. B, incubation of HSIX1 with an active form of CK2 is necessary to inhibit binding to the pM oligo. Left lane, pM oligonucleotide incubated with in vitro translated HSIX1. CK2, pM oligonucleotide incubated with HSIX1 phosphorylated by CK2; CK2/HI, pM oligonucleotide incubated with HSIX1 treated with heat-inactivated CK2. The arrow designates the specific complex in A and B. C, 35S methionine in vitro translated proteins were phosphorylated in parallel with the nonradioactive proteins utilized in the EMSA to demonstrate the effect of the kinases on the state of HSIX1 phosphorylation under the reaction conditions used for the EMSA experiment. CK2 was inactivated by incubating at 80 °C for 10 min. D, binding to the pM oligonucleotide is diminished in mitotic extracts. Interphase extracts (IE), mitotic extracts (ME), or interphase and mitotic extracts to which exogenous HSIX1 was added (IE/HSIX1 and ME/HSIX1, respectively) were incubated with the pM oligonucleotide and electrophoresed on a 5% nondenaturing polyacrylamide gel. The incubation resulted in a DNA-protein complex that was diminished in mitosis and that could be enhanced by adding exogenous HSIX1.

CK2, the specific complex was diminished (Fig. 7, A and B); however, incubation of HSIX1 with heat-inactivated CK2 did not inhibit DNA binding (Fig. 7B). HSIX1 incubated with CK2 or heat-inactivated CK2 was examined on 10% SDS-polyacrylamide gels to determine the extent of HSIX1 phosphorylation under both conditions. Hyperphosphorylation of the protein was not observed when heat-inactivated CK2 was used (Fig. 7C), suggesting that hyperphosphorylation by CK2 decreases the ability of HSIX1 to bind DNA.

**DNA Binding to MEF3 Sites Is Decreased in Mitotic Extracts**—Because we observed differential phosphorylation of HSIX1 in interphase and mitotic extracts when exogenous protein was added and because treatment of HSIX1 with CK2 inhibited DNA binding in vitro, we reasoned that endogenous *Xenopus* Six1 may also be differentially phosphorylated and that this may affect the ability of the protein to bind DNA. To test this hypothesis, we performed EMSA with interphase and mitotic extracts. When interphase extracts were incubated with the pM oligonucleotide, a complex was formed (Fig. 7D) that was competed with the wild type oligonucleotide and that migrated to the same position as the specific complex obtained with IVT HSIX1 (data not shown). This complex was diminished when mitotic extracts were incubated with the pM oligonucleotide, and addition of exogenous IVT HSIX1 to the extracts enhanced the binding in both interphase and mitosis, suggesting that this complex does contain Six1. The data are indicative of an endogenous form of *X. laevis* Six1 that is able to bind MEF3 sites in the aldolase A promoter to a greater extent in interphase than in mitotic extracts. This suggests that hyperphosphorylation of endogenous Six1 may decrease DNA binding in vivo. Although consistent with the hypothesis that mitotic hyperphosphorylation of HSIX1 inhibits DNA binding, a decrease in HSIX1 protein may be an alternative mechanism by which the DNA binding is reduced in mitosis. This possibility could not be examined using our HSIX1 antibody, because cross-reactivity to *Xenopus* Six1 was very low, an expected outcome because the antibody was made to the least conserved C-terminal domain of the protein.
malian cells, where phosphorylation of homeodomain-containing proteins such as Csx/Nkx2.5 (7), Cut (11), GHF-1 (9), TTF-1 (23), and Oct-1 (10) leads to changes in DNA binding activity, transactivation, or nuclear localization.

Inhibitor studies demonstrate that protein kinase CK2 is at least in part responsible for the in vivo phosphorylation of HSIX1 in asynchronous, primarily interphase, cells, and for the hyperphosphorylation of the protein in mitosis. Apigenin, a selective CK2 inhibitor, affected HSIX1 migration in both asynchronous cells and in mitosis. Although apigenin has been reported to inhibit cdc2 (24) and MAPK (25) as well as CK2, these results were only obtained through treatment of intact cells and may be the result of an indirect effect. In contrast, CK2 has been identified as a direct target of apigenin (26). Additionally, we ruled out the activity of the two other kinases by using PD98059, an inhibitor specific for MAPK activation (data not shown), and roscovitine, an inhibitor of the cyclin B/cdc2 kinase. Neither kinase inhibitor increased the electrophoretic mobility of HSIX1 in asynchronous MCF7 cells or in mitotic Xenopus extracts. These data strongly suggest that CK2, not cyclin B/cdc2 or MAPK, is involved in the phosphorylation of HSIX1 in vivo in both interphase and mitosis.

CK2 is a tetrameric serine/threonine protein kinase consisting of two catalytic α subunits (α and α') and two regulatory β subunits. It is ubiquitous and highly conserved in eukaryotic organisms, suggesting an essential role for the kinase. Normal CK2 activity is required for male germ cell development (27). The known substrates of CK2 include enzymes involved in metabolic processes, signal transduction mediators, cell division mediators, structural proteins, and transcription factors, including numerous homeodomain-containing proteins (28, 29).

**FIG. 8.** CK2 sites in the Six class of homeodomain containing proteins. A, alignment of the Six domains (N-terminal conserved region), homeodomains, and a small segment of the C termini of mammalian Six class members using the Clustal Method and the DNA Star software (DNA Star, Inc., Madison, WI). The three helices of the homeodomain are boxed, and CK2 phosphorylation sites are underlined. The asterisk represents the third potential consecutive CK2 site. B, diagram representing CK2 sites in the various domains of Six class proteins. A line represents a putative CK2 site. SD, Six domain; HD, homeodomain; C-term, C-terminal region.
Many studies suggest that CK2 has a role in cell cycle progression. In yeast, temperature-sensitive inactivation of CK2 results in cell cycle arrest at either the G1/S or G2/M boundary (30). In mammalian cells, progression through G2/M can be inhibited by antisense oligonucleotides or antibodies directed against CK2 (31). Additional evidence implies that CK2 has a role in cell division. Both the α and β subunits are phosphorylated in mitotic cells, levels of CK2β increase in mitosis, and the cyclin B/cdc2 mitotic kinase affects CK2 activity in vitro (32). In yeast, CK2β has been implicated in adaptation to the DNA damage-induced G2 checkpoint, a process that allows cells to override the checkpoint and continue through the cell cycle even if unable to completely repair the damaged DNA (33).

In an earlier study, we demonstrated that HSIX1, when overexpressed in mammary carcinoma cells, can attenuate the DNA damage induced G2 cell cycle checkpoint (4). Now, we demonstrate that HSIX1 is phosphorylated by CK2 and that inhibitors of CK2 cause a G2/M arrest in the same cell type. A similar G2/M arrest after apigenin treatment has been reported in keratinocytes, fibroblasts, and neuronal cells (24, 34, 35). We propose that CK2 regulates HSIX1 activity in these cells and that HSIX1 is a target for CK2 in cell cycle control at the G2/M transition, particularly in response to DNA damage. Interestingly, both CK2 (20, 36–39) and HSIX1 (4, 40) have been implicated in numerous types of cancers, including those of the mammary gland (4),2 and their role in the DNA damage response may enhance the tumorigenic potential.

The HSIX1 protein contains seven putative CK2 sites, two PKC consensus sites, and five possible cdc2 sites. An alignment of HSIX1 with the other members of the Six class of proteins demonstrates that several potential CK2 phosphorylation sites are highly conserved (Fig. 8). One highly conserved CK2 site resides in the N terminus, at the very end of the Six domain. This site is conserved in mammalian Six1–5 and Six9 in a region believed to be important for both protein-protein interactions and DNA binding (41, 42). A second conserved CK2 phosphorylation site resides immediately adjacent to the second helix of the homeodomain in Six1, Six2, Six4, and Six5, which is important for furnishing the hydrophobic core of the homeodomain and preserving the amphipathic nature of the α-helices (43). This site is also present in the NK, msh, and POU classes of homeodomain containing proteins. Position 204 in the C terminus of HSIX1 contains a CK2 site that is conserved between HSIX1 and Six5, and three potential CK2 sites at positions 214, 215, and 216 of HSIX1 are conserved between HSIX1 and two sites in Six2 and one site in Six4. Interestingly, numerous homeodomain containing proteins, including Hoxb6, Hoxc6, engrailed, and Antennapedia contain CK2 sites C-terminal to the homeodomain, in regions that otherwise are not conserved (12, 15). Members of the Six class also have numerous C-terminal CK2 sites (Fig. 8B) without any other conservation in this region. The number of conserved CK2 phosphorylation sites in the Six class members suggests an important role for CK2 in controlling their functions.

Our data suggest that mitotic hyperphosphorylation appears to be primarily in the C terminus of the protein, where most of the potential CK2 sites exist. Whether particular CK2 sites in the C terminus are important for the HSIX1 DNA binding activity is not known. Furthermore, although deletion analysis demonstrates that the mobility shift seen in mitosis is a result of phosphorylations in the C terminus, one cannot rule out sites in the N terminus and homeodomain as also important.

We are currently performing mutagenesis analysis as well as mass spectrometry to determine which of the CK2 sites are phosphorylated in vivo in interphase and mitotic cells. In addition, the role of other kinases in phosphorylating HSIX1 cannot yet be ruled out. Although no effects were seen in vivo with inhibitors of the cdc2 kinase or PKC, these kinases do have effects in vitro, and it is possible that their roles in vivo are only observed under a specific set of conditions (DNA damage, growth factor stimulation, etc.) or in specific cell types. The role of other kinases in HSIX1 regulation will be further examined.

A screen performed to isolate mitotic phosphoproteins identified numerous transcription factors, including five homeodomain containing proteins, and it was postulated that phosphorylation of transcription factors during mitosis may be a general mechanism by which regulatory proteins are removed from chromatin to decrease transcription (8). HSIX1 may be regulated by such a mechanism. Our data suggest that HSIX1 activity is confined to the G2 period of the cell cycle in some cell types, because mRNA levels do not increase until the S/G2 boundary and DNA binding, at least with respect to the aldolase A promoter, is diminished in mitosis. This tight regulation of HSIX1 activity may in part be controlled by varying levels of phosphorylation, leading to alterations in activity at different stages of the cell cycle. It is also possible that binding to additional promoters and/or other proteins in mitosis is differentially affected by the phosphorylation status of the protein.

It seems paradoxical that hyperphosphorylation of HSIX1 by CK2 may inhibit HSIX1 DNA binding activity in mitosis, yet both can promote exit from the G2 checkpoint. However, many cell cycle regulators have paradoxical effects on cell cycle progression when expressed aberrantly, and it is clearly necessary to both up- and down-regulate their activity at various stages of the cell cycle for proper transit. Cell cycle regulators such as E2F, polo-like kinase (Pxl), and cyclin B fall into this category (44–46). In this way, HSIX1 activity may be necessary for the G2/M transition, but it may also be necessary to remove that activity in mitosis for further cell cycle progression. Differential phosphorylation of HSIX1 by CK2 in interphase and mitosis may allow for both its activation and inactivation. Future studies utilizing HSIX1 CK2 phosphomutants as well as the identification of other HSIX1 target genes should determine whether these two proteins cooperate in both cell cycle control and in tumorigenicity.

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