Cell, Vol. 113, 507–517, May 16, 2003, Copyright ©2003 by Cell Press

Apoptotic Phosphorylation of Histone H2B Is Mediated by Mammalian Sterile Twenty Kinase

Wang L. Cheung,[1,2] Kozo Ajiro,[2] Kumiko Samejima,[3] Malgorzata Kloc,[4] Peter Cheung,[2] Craig A. Mizzen,[2] Alexander Beeser,[5] Laurence D. Etkin,[4] Jonathan Chernoff,[5] William C. Earnshaw,[3] and C. David Allis[1,2,6,*]

[1]Department of Microbiology
[2]Department of Biochemistry and Molecular Genetics
University of Virginia Health System
Charlottesville, Virginia 22908
[3]Wellcome Trust Centre for Cell Biology
ICMB
University of Edinburgh
Swann Building
Mayfield Road
Edinburgh EH9 3JR
Scotland, United Kingdom
[4]Department of Molecular Genetics
University of Texas M.D. Anderson Cancer Center
1515 Holcombe Boulevard.
Houston, Texas 77030
[5]Fox Chase Cancer Center
7701 Burholme Avenue
Philadelphia, Pennsylvania 19111

Summary

DNA in eukaryotic cells is associated with histone proteins; hence, hallmark properties of apoptosis, such as chromatin condensation, may be regulated by posttranslational histone modifications. Here we report that phosphorylation of histone H2B at serine 14 (S14) correlates with cells undergoing programmed cell death in vertebrates. We identify a 34 kDa apoptosis-induced H2B kinase as caspase-cleaved Mst1 (mammalian sterile twenty) kinase. Mst1 can phosphorylate H2B at S14 in vitro and in vivo, and the onset of H2B S14 phosphorylation is dependent upon cleavage of Mst1 by caspase-3. These data reveal a histone modification that is uniquely associated with apoptotic chromatin in species ranging from frogs to humans and provide insights into a previously unrecognized physiological substrate for Mst1 kinase. Our data provide evidence for a potential apoptotic "histone code."

Introduction

Apoptosis is an active process of cell death important for normal development and homeostasis in multicellular organisms. Formation of pycnotic and condensed chromatin bodies and digestion of DNA into oligonucleosomal fragments are hallmarks for apoptosis (Wyllie, 1980; Wyllie et al., 1980, 1984). At present, the biochemical and molecular mechanisms for these nuclear events are unclear, whereas upstream cytoplasmic pathways (involving caspases and kinases) that promote or prevent cell death are better characterized (reviewed in Kaufmann and Hengartner, 2001; Metzstein et al., 1998; Johnstone et al., 2002).

The fundamental unit of eukaryotic chromatin is the nucleosome, a particle containing 146 base pairs of DNA wrapped around a histone core octamer (two copies each of histone H3, H4, H2A, and H2B; see Luger et al., 1997 for details). Our understanding of how nucleosomal arrays are packaged into higher-order chromatin fibers, and how distinct functional domains are formed is poor. An increasing body of evidence suggests that posttranslational histone modifications (acetylation, phosphorylation, methylation, etc.) influence chromatin folding and have clear functional consequences (reviewed in van Holde, 1988; Wolffe and Hayes, 1999). In addition, distinct patterns of covalently modified histones, by acting as a histone code, may serve as signaling platforms to recruit and bind nuclear factors that mediate downstream functions by unknown mechanisms (reviewed in Strahl and Allis, 2000; Turner, 2000; Cheung et al., 2000). Given the drastic changes in the integrity of DNA and the state of chromatin compaction during apoptosis, histone modifications may play a functional role in promoting these changes.

To date, histone modifications that are specifically induced during apoptosis have not been clearly defined (reviewed in Cheung et al., 2000; Th'ng, 2001; Jason et al., 2001). Mitotic chromatin condensation, for example, is associated with histone H3 phosphorylation at serine 10, but this modification has not been consistently observed during apoptotic-induced chromatin condensation (reviewed in Cheung et al., 2000; Hendzel et al., 1998). Phosphorylation of a relatively minor histone variant, H2A.X, increases during the early stages of DNA fragmentation in apoptosis (Rogakou et al., 1998, 2000). However, H2A.X phosphorylation at serine 139 correlates with double-stranded DNA breaks induced by numerous stimuli, suggesting that this mark acts more as a DNA-damage sensor rather than an event specifically linked to the apoptotic process. In mammalian cells, the only core histone modification that has been uniquely associated with apoptosis is histone H2B phosphorylation. While earlier work showed that H2B phosphorylation occurs at the N-terminal tail, the specific sites of phosphorylation were not known (Ajiro, 2000; reviewed in Cheung et al., 2000). Furthermore, H2B phosphorylation and the H2B, but not other histones, amino-terminal tail is essential for chromatin condensation in *Xenopus* cell-free systems (de la Barre et al., 2001). These data are consistent with the idea that H2B phosphorylation may be important for apoptotic chromatin condensation.

Here, using a new phosphospecific antibody (hereafter referred to as α-Phos (S14) H2B), we demonstrate that H2B S14 phosphorylation specifically correlates with the onset of apoptosis in human HL-60 cells. This correlation extends to cells undergoing programmed cell death during *Xenopus* tail resorption. Using an in-gel kinase assay, we identified an apoptotic-induced 34

*Correspondence: alliscd@mail.rockefeller.edu
[6]Present address: Laboratory of Chromatin Biology, The Rockefeller University, 1230 York Avenue, New York, New York 10021

kDa H2B S14 kinase as the caspase-cleaved form of Mst1 (mammalian sterile twenty). Furthermore, H2B S14 phosphorylation is not dependent on caspase-activated DNase (CAD) suggesting that this modification is associated with the apoptotic chromatin condensation pathway. Collectively, these studies define what may be an apoptotic histone code conserved among vertebrates and cast new light on physiological substrates of Mst1 kinase.

Results

Phosphorylation of H2B at S14 Is Strongly Induced in HL-60 cells Undergoing Apoptosis

Previously, in vivo labeling studies showed that H2B phosphorylation occurs specifically at the amino-terminal tail during apoptosis (Ajiro, 2000). Other in vitro studies indicated that the H2B amino-terminal tail is essential for chromatin condensation (de la Barre et al., 2001). Preliminary phosphoamino acid analyses suggested that serine 14 (S14) of H2B was a potential site of phosphorylation during apoptosis (Ajiro, 2000; K. Ajiro, unpublished data). Hence, we generated a site-specific, H2B S14 phosphospecific antibody (α-Phos (S14) H2B) to examine the phosphorylation status at this site during apoptosis (see Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/113/4/507/DC1 for demonstration of antibody specificity). A human leukemia cell line, HL-60, was treated with either etoposide (VP16) or UV to induce apoptosis, and histones were then isolated by acid extraction and examined by Western blotting using α-Phos (S14) H2B. As shown in Figure 1A, the H2B phosphorylation signal was strongly enhanced by VP16 and UV treatment. To confirm that apoptosis had been induced, DNA was extracted from parallel samples. The appearance of a DNA ladder indicative of apoptotic DNA fragmentation coincides with the onset of H2B S14 phosphorylation (Figure 1B); therefore, these data demonstrate that the two events could be linked.

To confirm that phosphorylation of H2B at serine 14 occurs specifically in apoptotic cells, VP16-treated HL-60 cells were examined by immunofluorescence microscopy with α-Phos (S14) H2B immunostaining (Figure 1C). This antibody only stained apoptotic cells and strikingly, the majority of the α-Phos (S14) H2B signal was found localized to characteristic apoptotic chromatin bodies identified by DAPI staining (see downward arrows in Figures 1C and 1D). In contrast, control histone antibodies, such as the mitotic H3 phospho (S10) antibody, do not stain the apoptotic nuclei (data not shown; Hendzel et al., 1998). Also, α-Phos (S14) H2B failed to stain condensed mitotic chromosomes, suggesting that these distinct histone phosphorylation modifications are associated with different biological events (Supplemental Figure S2 available at http://www.cell.com/cgi/content/full/113/4/507/DC1). To rule out any unexpected effects of VP16, other apoptotic inducers were tested in different cell lines. As summarized in Table 1, a variety of well-known apoptotic inducers led to H2B S14 phosphorylation, albeit to variable extents, in different cell lines. Furthermore, immunostaining of HeLa cells induced to undergo apoptosis with VP16 showed that H2B S14 phosphorylation positive cells also contain DAPI-dense bodies and show TUNEL staining, hallmarks of apoptosis (Figure 1E). Hence, these data demonstrate that histone H2B phosphorylation at S14 is associated with condensed apoptotic chromatin in multiple mammalian cell lines.

H2B (S14) Phosphorylation Is Increased during Programmed Cell Death at *Xenopus laevis* Tail Resorption

To determine whether H2B (S14) phosphorylation is associated with apoptosis in a more physiological model, we examined this histone modification in a *Xenopus laevis* system where programmed cell death is well characterized and where serine 14 is conserved in the H2B sequence (Figure 2A). During metamorphosis, resorption occurs in *Xenopus* tails, and clusters of these cells die by developmentally programmed apoptosis (Yoshizato, 1989; Usuku and Gross, 1965; Kerr et al., 1974). Degenerating tails from stage 64 froglets were examined by immunofluorescence using α-Phos (S14) H2B antibody. Pockets of apoptotic cells in the tail were stained (Figure 2B, middle image); these colocalize precisely with the Hoechst-dense apoptotic condensed nuclei (Figure 2B, left and right images). These data indicate that H2B S14 phosphorylation also occurs in apoptotic cells during normal *Xenopus* development.

Nuclear Extracts from Apoptotic Cells Contain a 34 kDa H2B Kinase that Is the Caspase-Cleaved Form of Mst1

To search for the kinase responsible for H2B S14 phosphorylation, we first determined that apoptotic nuclear extracts contain an H2B S14 kinase activity by in vitro kinase assays and Western blotting with α-Phos (S14) H2B (data not shown; see Supplemental Figure S3 available at http://www.cell.com/cgi/content/full/113/4/507/DC1). We then used an in-gel kinase assay to determine the molecular weight(s) of potential apoptotic H2B kinases in apoptotic nuclear extracts. Using either H2B or mixtures of core histones (data not shown) as substrates, we assayed for apoptotic-induced proteins that have H2B kinase activity. In addition to the several bands that were found in both H2B-containing and the "no substrate" gels (likely due to autophosphorylation), one band that had an apparent molecular weight of $\sim$34 kDa was consistently detected in the H2B-containing gel only (Figure 3, arrow, lane 6, and data not shown). This band was only found in the apoptotic extracts, and therefore, this 34 kDa protein is likely the apoptotic-specific H2B kinase (hereafter called 34H2BK).

A search of the literature for kinases known to be activated in the apoptotic pathway showed that Mst1 (mammalian sterile twenty) is a caspase-activated Ser/Thr protein kinase of approximately 56 kDa with a 34 kDa caspase-cleavage product (Creasy and Chernoff, 1995; Creasy et al., 1996; Graves et al., 1998, 2001; Ura et al., 2001). To determine if 34H2BK was Mst1, nuclear extracts prepared from VP16-stimulated HL-60 cells were analyzed with an antibody to the N-terminal catalytic domain of Mst1 (α-N-Mst1; Graves et al., 1998). Western blotting showed that the cleaved form of Mst1 was present in the VP16-stimulated nuclear extract (Fig-

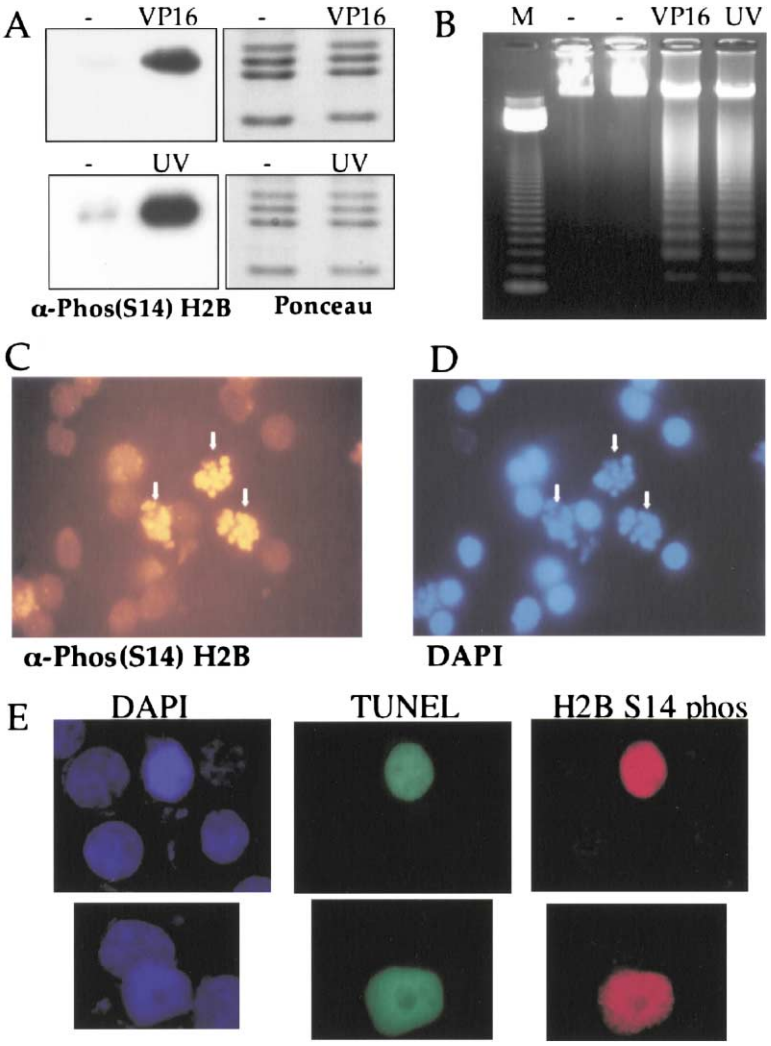

Figure 1. Antibodies to phospho (S14) H2B Selectively Stain Pycnotic Apoptotic HL-60 Cells and TUNEL Positive HeLa Cells

(A) Bulk histones were isolated from HL-60 cells mock-treated or treated with etoposide (VP16) or with 100 J/m^2 of UV. Western analyses show a dramatic increase in H2B S14 phosphorylation as cells undergo apoptosis.

(B) DNA harvested from parallel samples as in (A) was resolved on an agarose gel. The DNA laddering assay confirms that VP16 or UV-treated HL-60 cells were undergoing apoptosis.

(C) HL-60 cells treated with VP16 were immunostained using the H2B S14 phospho antibody (α-Phos (S14) H2B). Cells that have fragmented apoptotic nuclei (see arrows) display a striking increase in H2B S14 phosphorylation. Note that HL-60 cells undergoing mitotic division fails to stain with α-Phos (S14) H2B, but are easily stained with α-Phos (S10) H3 (see text and Supplemental Figure S2 available at http://www.cell.com/cgi/content/full/113/4/507/DC1).

(D) The same cells in (C) were counterstained with DAPI for DNA. The arrows point to apoptotic cells, as indicated by fragmented DAPI-stained nuclei.

(E) HeLa cells were treated with VP16 and then costained with α-Phos (S14) H2B and TUNEL. These same cells were counterstained with DAPI for DNA. H2B S14 phosphorylation positive cell also contains TUNEL staining indicative of apoptotic cells. Note that the double H2B S14 phospho and TUNEL positive cells are smaller and more DAPI-dense.

ure 3, lanes 7 and 8), with an apparent molecular weight that was similar, if not identical, to the apoptotic-induced H2B kinase (arrows).

To further purify the apoptotic-induced H2B kinase, we fractionated the apoptotic nuclear extract using size-exclusion chromatography. Examination of the H2B kinase elution profiles from "apoptotic" and "normal" nuclear extracts revealed that fraction 18 of the apoptotic extract was significantly enriched in H2B S14 kinase activity compared to the same fraction from mock-treated extracts (Supplemental Figure S4A and S4B available at http://www.cell.com/cgi/content/full/113/4/507/DC1; see downward arrows). Using an antibody specific to Mst1 for Western blot analyses and an in-gel kinase assay, we found that only fraction 18 from apoptotic extracts contained the 34 kDa truncated form of Mst1 (Supplemental Figure S4C available at http://www.cell.com/cgi/content/full/113/4/507/DC1) and the 34H2BK (Supplemental Figure S4D available at http://www.cell.com/cgi/content/full/113/4/507/DC1). Taken together, these data strongly suggest that the Mst1-cleavage product is 34H2BK.

Table 1. H2B Ser14 Phosphorylation in Different Cell Lines Treated with Inducers of Apoptosis

Cell Lines Tested	Cytotoxic Agent	Level of H2B phos
HL-60	Etoposide (VP16)	high
	UV	high
	anti-Fas	high
	anisomycin	high
NIH3T3	UV	high
HeLa	UV	medium
IMR90	UV	medium
HepG2	MMS	medium
293T	UV	low
Chicken DT40	VP 16	medium

After cytotoxic agent treatments, cells were harvested. The level of H2B S14 phosphorylation is determined by Western blots using the H2B S14 phos antibody.

Mst1 Can Phosphorylate Histone H2B at S14 In Vitro

To directly test whether Mst1 can phosphorylate histone H2B, we transfected 293T cells with CMV promoter-driven plasmids containing either a myc-tagged full-length (FL) or kinase dead (KD) form of Mst1 (Creasy et

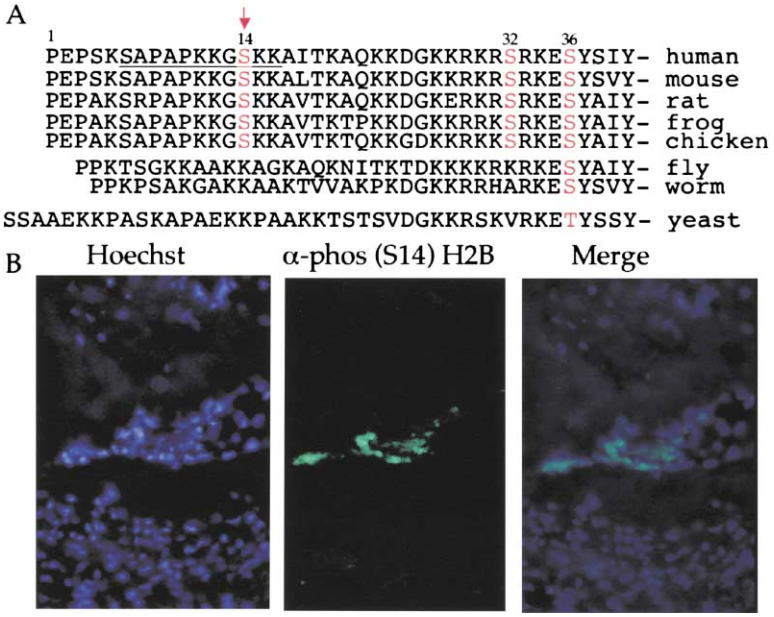

Figure 2. H2B S14 Phosphorylation Occurs in Programmed Cell Death during *Xenopus* Tail Resorption

(A) Primary sequence alignment of H2B amino-terminal "tails" from different species shows that only vertebrates have well-conserved sequence surrounding S14 (see downward arrow) and S32. In contrast, a highly conserved S/T is evident in the H2B tails of most species from yeast to human at position of S36 in human H2B. A peptide containing the underlined sequence with S14 phosphorylated was used to immunize rabbits for antibody production (see Supplemental Data available at http://www.cell.com/cgi/content/full/113/4/507/DC1 for more details). (B) A *Xenopus* tail was immunostained with α-phos (S14) H2B (middle image), and the DNA was stained with Hoechst (left). Zones of apoptotic-condensed nuclei in the degenerating frog tail have increased H2B S14 phosphorylation whereas nuclei with normal morphology do not.

al., 1996). Transfected Mst1 was immunoprecipitated using an antibody against the myc tag and tested for kinase activity using histone H2B (Figure 4, lanes 3 and 4), free core histones (lanes 5 and 6), or nucleosomes (lanes 1 and 2) as substrates. Incorporation of the γ[^{32}P] label on the substrates was detected in the reactions with full-length Mst1 when purified H2B and free core histones were used as substrates (Figure 4B, lanes 3 and 5). Although weaker, Mst1 FL can still phosphorylate H2B in a nucleosomal context (Figure 4B, lane 1). In contrast, the kinase dead (KD) form of Mst1 (which contains a single point mutation changing lysine to arginine at amino acid 59) did not phosphorylate H2B (Figure 4B, lanes 2, 4, and 6), arguing against the possibility that other histone kinases are associated with the Mst1 IPs. To test whether phosphorylation of H2B occurs at S14, parallel IP kinase reactions were performed with nonradioactive ATP and analyzed by Western blotting. As predicted, Mst1 FL, but not Mst1 KD, phosphorylated H2B as detected by α-Phos (S14) H2B (Figure 4C). Taken

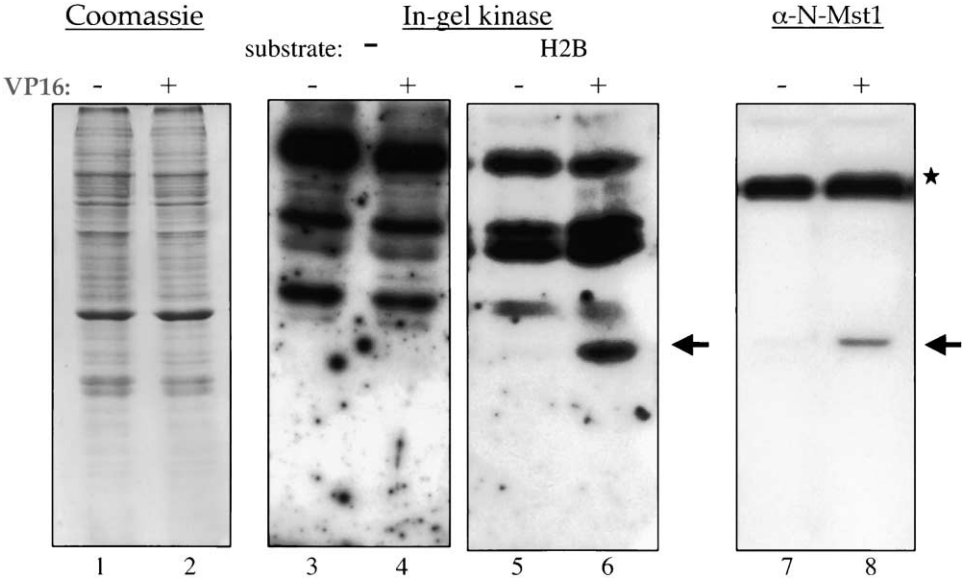

Figure 3. Apoptotic HL-60 Nuclear Extracts Contain an Induced 34 kDa H2B S14 Kinase Activity that Is Reactive with Antibodies Generated Against the Caspase-Cleaved Form of Mst1 Kinase

An in-gel kinase assay using histone H2B as substrate (lanes 5 and 6) shows that apoptotic, but not normal, extracts contain a 34 kDa kinase (see arrow). Parallel in-gel assay without substrate (lane 3 and 4) shows that the signal seen with the 34 kDa protein is not due to autophosphorylation. Western blot of the nuclear extracts with an antibody against the N terminus of Mst1 (lanes 7 and 8) shows that the caspase-cleaved form of Mst1 has a similar size to the apoptotic induced H2B kinase. The antibody also detects the full-length, uncleaved Mst1 (see star next to lane 8).

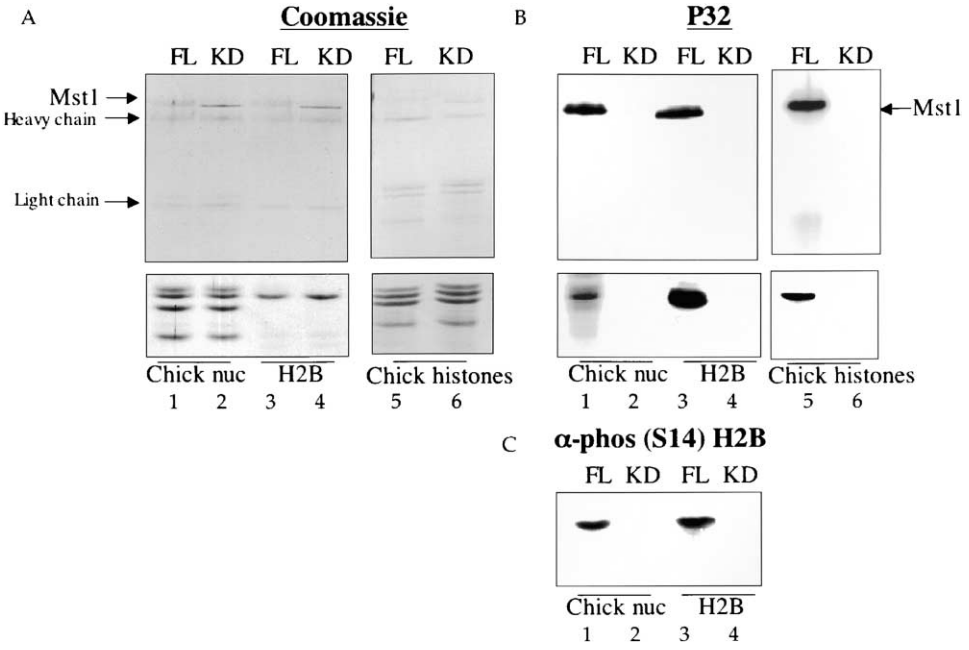

Figure 4. Mst1 Phosphorylates Histone H2B at S14 in In Vitro Kinase Assays

Myc-tagged full-length (FL) and kinase dead (KD) of Mst1 were transfected into 293T cells, immunoprecipitated with α-myc, and assayed for kinase activity using purified H2B, core histones, or nucleosomes as substrate. In vitro assays were done in presence of γ-[^{32}P] ATP (A, B) or with nonradioactive ATP (C). "Cold" reactions were assayed by Western blotting with α-phos (S14) H2B.

together, these data suggest that H2B may be a physiologically relevant nuclear substrate for Mst1.

The Kinetics of H2B S14 Phosphorylation Are Similar to Cleavage of Mst1 during Apoptosis

During apoptosis Mst1 is cleaved by caspases, and only the cleaved form of this kinase is localized to the nucleus. If Mst1 cleavage is important for establishing H2B S14 phosphorylation during apoptosis, the kinetics of both events should be similar. To determine the time courses of these events, HL-60 cells induced to undergo apoptosis with VP16 were harvested at half-hour intervals for Western blot analyses. Significantly, time course analyses (probing Western blots with α-Phos (S14) H2B and α-N-Mst1) indicated that the onset of H2B phosphorylation coincided with the initial appearance of caspase-cleaved Mst1 at 2 hr postinduction (Figure 5A, arrow). As additional markers for apoptosis, identical blots were probed with α-PARP (poly (ADP-ribose) polymerase), which is cleaved as cells undergo apoptosis (reviewed in Duriez and Shah, 1997), and genomic DNA isolated from parallel samples was evaluated for the appearance of characteristic DNA laddering (Figures 5A and 5B). Interestingly, cleavage of PARP and the appearance of a DNA ladder (approximately 2.5 hr postinduction) were not detected until after H2B phosphorylation and cleavage of Mst1 had initiated. While these timing differences may reflect differences in detection sensitivity for these apoptotic markers, the data suggest that H2B phosphorylation precedes DNA laddering (Figure 5B) and hence, may play a role in establishing apoptotic DNA fragmentation (see Discussion).

Comparison between the Kinetics of H2B S14 Phosphorylation and Other Histone Modifications during Apoptosis

H2A.X phosphorylation has been correlated with the occurrence of double-strand DNA breaks (Rogakou et al., 1998, 2000), and since VP16 causes DNA breaks, we also evaluated the kinetics of H2A.X phosphorylation. As expected, H2A.X phosphorylation occurred within one hour of VP16 treatment (Figure 5A), suggesting that DNA breaks, induced by VP16, occur early during the apoptotic pathway. Since H2B S14 phosphorylation occurs later than H2A.X phosphorylation, but before (or concurrent with) other well-known apoptotic markers, these data suggest that H2B phosphorylation is not simply associated with DNA breaks. Rather, H2B phosphorylation appears to be associated with a distinct aspect of apoptosis. In addition, since H2B acetylation at lysines 12 and 15 is not changed, the increase of H2B S14 phosphorylation as detected by antibody staining is not due to a decrease in acetylation at nearby residues.

H2B S14 Phosphorylation and Cleavage of Mst1 Are Caspase-3 Dependent

Since caspases are integral components of many apoptotic pathways, we sought to determine whether H2B S14 phosphorylation is controlled by activated-effector caspases. Mst1 cleavage is dependent on caspase-3 (Graves et al., 1998, 2001; Lee et al., 2001), and the cleaved form of Mst1, missing nuclear export signals, is retained in the nucleus (Ura et al., 2001). If H2B S14 phosphorylation is dependent upon cleavage of Mst1, then this modification should be sensitive to documented caspase-3 inhibitors such as DEVD peptide (re-

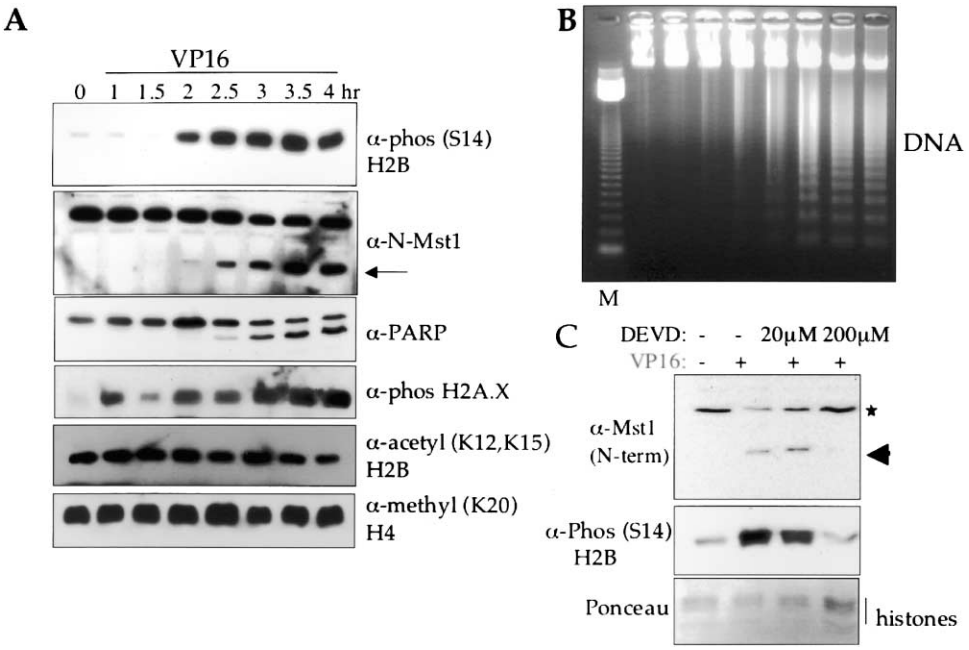

Figure 5. H2B S14 Phosphorylation and Cleavage of Mst1 Coincide with Each Other during an Apoptotic Time Course and Are Dependent on Caspase-3

HL-60 cells were harvested after treatment with VP16 at the indicated times.

(A) Half of the cells were harvested in SDS-loading buffer and then subjected to Western analysis using the indicated antibodies. The first appearance of cleaved Mst1 (arrow) coincides with the first detectable H2B S14 phosphorylation at around the 2 hr time point. Poly (ADP ribose) polymerase is well documented to be cleaved during apoptosis and is used as an internal apoptotic marker. H2A.X antibody recognizes a phosphorylation event that is associated with DNA breaks which occurs early in VP16 treatment. Histone H2B acetylation is unchanged during these time points suggesting that H2B S14 phosphorylation enhancement is not due to a loss of H2B acetylation. The status of H4 K20 methylation and ponceau staining of histones (data not shown) are constant during these time points and are used to demonstrate equal loading.

(B) The other half of these cells was harvested for genomic DNA and then ran on a 1.2% agarose TAE gel. The formation of DNA laddering, a hallmark of apoptosis, starts around 2–2.5 hr after VP16 treatment. (M) denotes 123 bp DNA marker (Gibco).

(C) HL-60 cells were pretreated with caspase-3 inhibitor (DEVD) and then treated with VP16 for 4 hr. Western blots using Mst1 N-terminal and H2B S14 phospho antibodies show DEVD inhibited the cleavage of Mst1 as well as H2B S14 phosphorylation (arrow point to the cleaved product of Mst1).

viewed in Grutter, 2000; Earnshaw et al., 1999). To test this prediction, HL-60 cells were preincubated with varying amounts of z-DEVD-fmk prior to treatment with VP16 for 4 hr and cell extracts were harvested for Western analyses. As shown in Figure 5C, incubation with 200 μM z-DEVD-fmk inhibited both Mst1 cleavage (see arrow) and H2B S14 phosphorylation. At a lower dose (20 μM z-DEVD-fmk), we observed only a modest decrease in the proportion of truncated Mst1 (see arrow) over full-length Mst1 (see star) and H2B phosphorylation still occurred. These data demonstrate that H2B S14 phosphorylation, like cleavage of Mst1 itself during apoptosis, is dependent on caspase-3.

Mst1 ΔC Can Phosphorylate H2B In Vivo

Next, we sought to determine if Mst1 could phosphorylate H2B when expressed in cells without apoptotic inducers. FL, KD, and ΔC (Mst1 amino acids 1–330, a C-terminal truncation mimicking the caspase-cleaved form) versions of myc-tagged Mst1 were transfected into HeLa cells, and immunofluorescence was performed using α-Phos (S14) H2B, and α-myc. It has been shown recently that Mst1 can induce apoptotic-like chromatin condensation when overexpressed in HeLa cells (Lee et al., 2001). In contrast to Lee et al. (2001), we detected Mst1 FL mostly in the cytoplasm, and this form did not induce apoptotic phenotypes. As expected, we also found the same result using Mst1 KD (data not shown). Whereas none of the Mst1 FL or KD positive cells contain any significant H2B S14 phosphorylation signal (Figure 6D), Mst1 ΔC was primarily localized in the nucleus and 20%–40% of the Mst1 ΔC expressing cells are shrunken and become apoptotic as confirmed by TUNEL assays (Figure 6E). Furthermore, 10%–35% of the Mst1 ΔC positive cells contain H2B S14 phosphorylation (Figures 6A and 6D). Since 90%–95% of H2B S14 phosphorylation positive cells were TUNEL positive (Figure 6F), we concluded that Mst1 ΔC is sufficient to induce H2B S14 phosphorylation and apoptosis in the same cell.

To determine if the above effect was due to the kinase activity of Mst1 ΔC, we made a point mutation (lysine 59 to arginine; K59R) that abolishes the kinase activity as measured by in vitro kinase assay (Figure 6C). The Mst1 ΔC K59R mutant did abolish the effect Mst1 ΔC had on the formation of shrunken cells and the increase in H2B S14 phosphorylation (Figures 6B and 6D), sug-

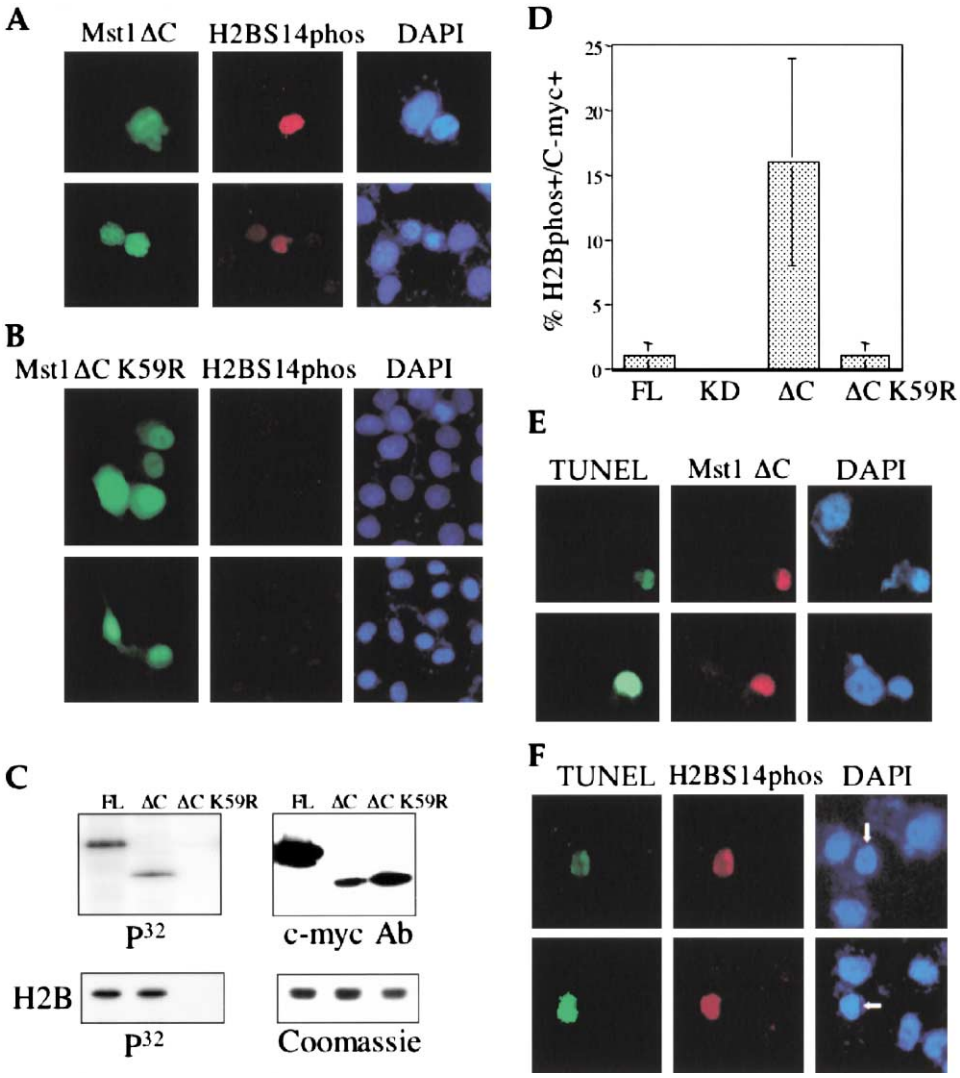

Figure 6. Caspase-Cleaved and Catalytically Mst1 Can Phosphorylate H2B at S14 In Vivo

(A) HeLa cells transfected with myc-tagged Mst1 ΔC (a C-terminal truncation), or (B) myc-tagged Mst1 ΔC K59R (kinase dead version of Mst1ΔC). At 24–36 hr posttransfection, cells were immunostained using α-Phos (S14) H2B and α-myc (9E10), and counterstained with DAPI for DNA.

(C) Myc-tagged Mst1 full-length (FL), Mst1 ΔC, and Mst1 ΔC K59R were transfected into 293T cells, immunoprecipitated with α-myc, and assayed for kinase activity using purified H2B as substrate.

(D) Graphical representation for the percentage of Mst1 positive cells that are positive for H2B S14 phosphorylation. For each Mst1 construct, at least 4 independent experiments were done and at least 200 Mst1 positive cells were counted for each experiment.

(E) Myc-tagged Mst1 ΔC transfected cells were stained with TUNEL and α-myc. 20%–40% of Mst1 ΔC positive cells are TUNEL positive as well. Both of these Mst1 ΔC containing cells are positive for TUNEL staining.

(F) Myc-tagged Mst1 ΔC transfected cells were stained with TUNEL and α-phos (S14) H2B. 90%–95% of H2B S14 phosphorylation positive cells are TUNEL positive. Shown are several examples of cells that are positive for both H2B S14 phosphorylation and TUNEL staining (see white arrows). Note that the morphology of these double-positive cells are often smaller and pycnotic. For (E) and (F), at least 3 independent experiments were performed, and 200 Mst1 ΔC positive cells were counted for each experiment.

gesting that the kinase activity for H2B is important for inducing apoptosis. Furthermore, the data suggest that the caspase-cleaved form of Mst1 is able to phosphorylate H2B at S14 in vivo.

H2B S14 Phosphorylation Is Not Dependent on DNA Fragmentation but Instead Correlates with Apoptotic Chromatin Condensation

To determine if H2B phosphorylation is involved in the apoptotic DNA fragmentation or chromatin condensation pathway, we used $CAD^{-/-}$ (caspase-activated DNase) chicken cells that are defective in DNA fragmentation but still undergo chromatin condensation. Chicken DT40 WT and $CAD^{-/-}$ cells were treated with 10 μM VP16 and were then immunostained with α-phos (S14) H2B; the DNA was counterstained with DAPI. As the apoptotic program progresses (from top to bottom images in Figure 7), the H2B S14 phosphorylation level increases and the phosphorylated H2B colocalizes to the DAPI-dense regions. Interestingly, in early apoptotic

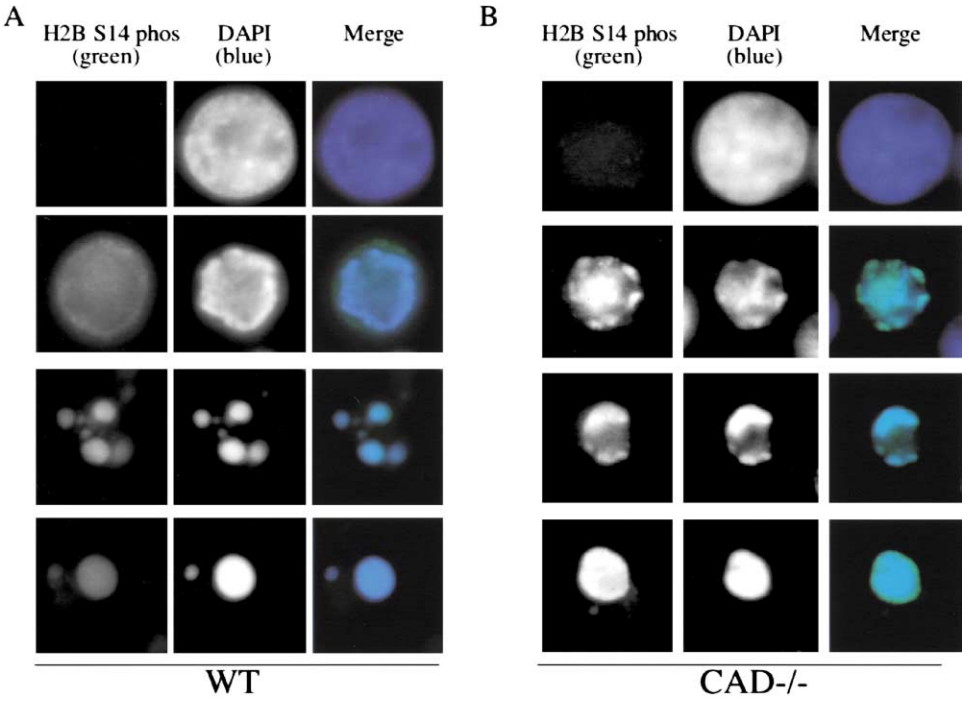

Figure 7. H2B Phosphorylation Correlates Closely with Apoptotic Chromatin Condensation

Chicken DT40 WT (A) and CAD$^{-/-}$ (caspase activated DNase) (B) cells were treated with 10 μM of VP16. Then cells were immunostained with α-phos (S14) H2B (green) and the DNA counterstained with DAPI (blue). As the apoptotic program progresses (from top to bottom images in A and B), H2B S14 phosphorylation increases and it colocalizes to the DAPI-dense regions. In CAD$^{-/-}$ cells (B), DNA fragmentation is defective while chromatin condensation still occurs. H2B S14 phosphorylation colocalizes to the DAPI dense regions in CAD$^{-/-}$ cells suggesting that this modification is closely linked to the chromatin condensation event during apoptosis. The increase of H2B S14 staining in CAD$^{-/-}$ nucleus and some cytoplasmic staining in some cells were observed and the significance of these is unclear.

cells, H2B S14 phosphorylation staining is more extensive than the DAPI-dense region, suggesting that H2B S14 phosphorylation might precede the condensation event. Interestingly, H2B S14 staining in the CAD$^{-/-}$ nucleus is higher than in the WT nucleus, but the significance of this is unclear (see below).

Discussion

Chromatin condensation and DNA fragmentation are long-standing hallmark features of apoptosis (Kerr et al., 1972), but the underlying molecular mechanisms that govern these nuclear events remain unclear, in part due to an incomplete understanding of the substrates targeted by upstream apoptotic signals. Here, we report that enhanced H2B phosphorylation at serine 14 is observed in HL-60 (human leukemia) cells undergoing apoptosis, as well as in cells undergoing programmed cell death during *Xenopus* tail resorption. In addition, we find that the 34 kDa caspase-3 cleaved form of Mst1 is likely playing an important, and previously unrecognized, role in bringing about this phosphorylation event. Together, these data shed new light on the function of this kinase and reinforce the view that histone modifications play an important role in governing a wide range of DNA-templated processes.

Mst1 Governs H2B S14 Phosphorylation in a Pathway Known to Induce Apoptotic-Like Chromatin Condensation

Mst1 is known to cause apoptotic-like chromatin condensation and induce apoptosis under some experimental conditions (Graves et al., 2001; Ura et al., 2001). We favor the view that the caspase-cleaved form of Mst1 phosphorylates histone H2B as one of its physiological targets during apoptosis. Phosphorylation of H2B, possibly in conjunction with other covalent modifications of histones, would then facilitate apoptotic chromatin condensation, DNA fragmentation, and cell death.

H2B S14 phosphorylation consistently colocalizes to the DAPI-dense regions in other cell lines undergoing apoptosis (Figures 1, 2, 6, 7, and Supplemental Figure S5A available at http://www.cell.com/cgi/content/full/113/4/507/DC1). Previous work has shown that the phosphorylation of the H2B amino-terminal tail is essential for chromatin condensation in vitro (de la Barre et al., 2001). Thus, H2B S14 phosphorylation could play a more direct role in mediating chromatin condensation and/or aggregation. Peptide analyses show that H2B S14 phosphorylation might have some intrinsic property to self-aggregate under reasonably harsh denaturing conditions (Supplemental Figures S5C and S5D available at http://www.cell.com/cgi/content/full/113/4/507/DC1). Hence, H2B S14 phosphorylation might be directly

involved in regulating apoptotic chromatin condensation through regulation of higher-order chromatin structure. In addition, our time course studies indicate that histone H2B S14 phosphorylation occurs immediately prior to DNA laddering, suggesting that H2B phosphorylation might act as a "trigger" for DNA fragmentation during apoptosis. Several nucleases that cleave internucleosomal DNA during apoptosis have been identified (reviewed in Robertson et al., 2000). At least in vitro, histone H1 and HMG-1 enhance caspase-activated nuclease (CAD) function (Liu et al., 1998), and therefore phosphorylated H2B may also enhance DNase activities on chromatin and/or facilitate their recruitment to the chromatin. Whether CAD recruitment and docking to the H2B S14 phosphorylation site explains enhanced staining of H2B S14 phosphorylation in $CAD^{-/-}$ cells is not known.

Besides CAD, phosphorylated H2B may serve as a docking site for yet unknown specific factors as shown for the interaction of bromodomains and chromodomains with acetylated and methylated histones, respectively (Dhalluin et al., 1999; Jacobson et al., 2000; Lachner et al., 2001; Bannister et al., 2001; Jacobs et al., 2001). As far as we are aware, phosphospecific histone binding factors that influence mitotic or apoptotic chromatin condensation have yet to be identified (see Jenuwein and Allis, 2001). Our data demonstrate that unique, and seemingly nonoverlapping, patterns of histone phosphorylation correlate with mitotic (e.g., S10 phosphorylation of H3) versus apoptotic (e.g., S14 phosphorylation of H2B) chromosome/chromatin condensation. These data provide support for the histone code hypothesis (Strahl and Allis, 2000) and may lend support for other histone phosphorylation events that are tied to other DNA-templated processes (reviewed in Cheung et al., 2000).

Conservation of H2B Phosphorylation and an Mst1-Mediated Apoptotic Response

Interestingly, the function(s) of H2B S14 phosphorylation with respect to apoptosis appears to apply only to vertebrate species since Ser14 and surrounding sequences that are well conserved from frogs to humans are not obvious in invertebrates or other lower eukaryotes. Although apoptosis is not widely accepted in yeast, an apoptotic-like phenotype has been observed in *S. cerevisiae* when induced with oxygen radicals, a plant antifungal agent osmotin, and pheromones (Madeo et al., 1999; Yun et al., 1998; Severin and Hyman, 2002). Interestingly, sterile 20 kinase was shown to be important for osmotin and pheromone-induced cell death in yeast (Yun et al., 1998; Severin and Hyman, 2002), suggesting the intriguing possibility that this kinase family may participate in a previously unrecognized apoptotic function that is conserved from yeast to man.

Conclusion and Perspective

Mst1 is a member of sterile 20-like superfamily represented by approximately thirty related kinases in humans (reviewed in Sells and Chernoff, 1997; Dan et al., 2001). Kinases contained in this superfamily are most often regarded as upstream regulators of MAPK pathways with roles in cellular morphogenesis and cytoskeletal rearrangements, as well as apoptotic cell death. Our finding that the cleaved-form of Mst1 is likely a nuclear kinase directly responsible for H2B S14 phosphorylation suggests that members of the family might represent new potential drug targets for therapy. Considerable evidence exists suggesting that many cells die under stress by undergoing apoptosis (reviewed in Mattson et al., 2001; Martin, 2001; Haunstetter and Izumo, 2000). However, caspase inhibitors have not been effective in decreasing cell death after the initial stress (such as ischemia) has occurred (reviewed in Loetscher et al., 2001). Perhaps, after effector caspases initiate the death pathway leading to defined chromatin changes, caspases are no longer needed. It remains an intriguing possibility that effective prevention of cell death may be best brought about by combining caspase inhibitors with drugs that target downstream activities such as Mst1 which affect chromatin changes during apoptosis.

Experimental Procedures

Cell Culture, Drug and UV Treatment, and Harvesting for Protein and DNA

HL-60 cells were cultured in RPMI with 10% FBS, whereas 293T, HeLa, HepG2, and IMR90 cells were grown in DMEM with 10% FBS. Etoposide, anisomycin, and anti-Fas were purchased from Sigma. To induce apoptosis, drugs were used in the following concentrations: 20 μg/ml for etoposide (VP16), 25 ng/ml for anisomycin, and 15 ng/ml for anti-Fas. For UV induction of apoptosis, 40–100 J/m^2 was used (BioRad GS Gene Linker UV chamber). The growth media were changed and cell lysates were harvested at various times afterward. Control cells were either DMSO or mock-treated. For z-DEVD-fmk (Trevigen), the indicated amount was added to cells prior to 4 hr of VP16 treatment.

After treatment, cells were subjected to one of the following procedures: resuspension in SDS-lysis buffer (Laemmli's sample buffer), nuclear extraction, acid extraction for histones, or genomic DNA extraction. Nuclei were isolated by lysis in detergent and low-speed spin as described in Strahl et al. (2001). These nuclei were either salt extracted (see below) or acid extracted in 0.4 N H_2SO_4. After incubating on ice for 2–4 hr, acid extracts were centrifuged to remove the insoluble pellet. A total of 5.4% of percholic acid was added to the supernatant and the precipitated core histones recovered were resuspended in water.

Genomic DNA was harvested by lysing the cell pellet in 10 mM Tris [pH 9.0], 1 mM EDTA, 10 mM NaCl, 1% w/v SDS, and 1 mg/ml of proteinase K at 50°C for 4–5 hr. Proteins were removed by phenol/chloroform extraction and the DNA precipitated in 0.3 M sodium acetate and 70% ethanol. After centrifugation, we resuspended the pellet in TE and digested RNA by adding 1 mg/ml RNase A for 1 hr at room temperature. DNA was separated on a 1.2% agarose TAE gel.

Salt Extraction of Nuclei and In-Gel Kinase Assay

Nuclei from HL-60 cells were extracted in 20 mM HEPES [pH 7.8], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 25% (v/v) glycerol for 3–4 hr on ice. After centrifugation at 14,000 rpm for 20 min, the soluble fraction was isolated and used for in vitro and in-gel kinase assays and Western blotting (see below).

SDS-PAGE was done as described (Laemmli, 1970). For in-gel kinase assays, 10%–12% SDS-PAGE gels contained 0.1 mg/ml chicken core histones or 0.1 mg/ml purified chicken H2B. In-gel assays were performed as essentially described in Sassone-Corsi et al. (1999). Following electrophoresis, gels were washed in 30 mM Tris-HCl [pH 7.4], 1 mM DTT, 0.1 mM EDTA, 20% (v/v) isopropanol for 20 min, and this was repeated three times. Gels were then incubated in 8 M urea, 30 mM Tris-HCl [pH 7.4], 1 mM DTT, and 0.1 mM EDTA for 1 hr. The gel was then immersed in 30 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 100 mM NaCl, and 0.05% Tween-40 at 4°C overnight to renature the proteins. After

renaturation, the in vitro kinase reaction was performed using 50 μCi of γ-[^{32}P] ATP in 30 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 2 mM $MnCl_2$, and 1 mM DTT at 30°C for 2 hr. Gels were then stained with Coomassie Blue, destained, and dried for autoradiography.

Nuclear Extract Fractionation, In Vitro Kinase Assay, and Western Blotting

Nuclear extracts were fractionated on a Superose 6 PC 3.2/300 column (Amersham Pharmacia) using the Pharmacia SMART system. The eluent consisted of 200 mM NaCl, 50 mM $NaPO_4$ [pH 7.0], 5 mM $MgCl_2$, 20% v/v glycerol. The flow rate was 40 μl/min at 4°C. The apoptotic fraction containing the caspase-cleaved Mst1 eluted with an apparent molecular weight of approximately 66 kDa whereas the full-length Mst1 was estimated to be 150 kDa.

Ten μl aliquots of fractions were used for in vitro kinase reactions and Western blotting. For in vitro kinase assay, 0.5 μg of histone H2B was used in 30 mM Tris [pH 7.5], 5 mM $MgCl_2$, 10 mM DTT, 0.1 μg/ml microcystin LR, 10 μM ATP plus 0.5 μCi of γ-[^{32}P] ATP. The reaction was incubated for 20 min at 30°C and spotted on P81 filter paper. Filters were washed in 0.75% H_3PO_4 and scintillation countering used to measure [^{32}P] incorporation. For nonradioactive (cold) reactions, no [^{32}P]-γ-ATP was used and the reactions were run in an SDS-PAGE gel for Western blot analysis. Of the peptides used in this paper, all contain a cysteine residue and were resolved in 15% SDS-PAGE gels.

Western blot analysis was performed as described in Briggs et al. (2001). α-phos (S14) H2B, α-N-Mst1, α-myc (9E10 from Santa Cruz), α-PARP (UBI), α-phos H2A.X (UBI), α-acetyl (K12, K15) H2B (Abcam), and α-acetyl H4 primary antibodies were used. HRP-conjugated rabbit secondary antibody (Amersham Pharmacia) was used. For chemiluminescence, the ECL plus kit (Amersham Pharmacia) was used for detection.

Immunofluorescence on Apoptotic Cell Culture and *Xenopus* Tails

Cells grown on coverslips were fixed in 4% paraformaldehyde and then washed in PBS. Following permeabilization with 0.2% Triton X-100, cells were blocked in 2% goat serum PBS (block). Cells were incubated with α-phos (S14) H2B in block at 37°C. Following incubation, cells were washed three times with block and incubated for 1 hr in goat anti-rabbit Cy3-conjugated secondary antibody (Jackson). After more washes, coverslips were mounted in Vectashield mounting media containing DAPI. 9E10 antibody (Santa Cruz) was used to detect myc-tagged proteins and anti-mouse FITC-conjugated secondary antibody (Jackson) was used. As for TUNEL staining, kits were purchased from Promega and used as recommended by the manufacturer.

Degenerating tails were cut off from stage 64 froglets and fixed in Bouin fixative (Sigma) for 2 hr, washed overnight in 70% ethanol, dehydrated, cleared in Histoclear, and embedded in paraplast. Ten μm sections were deparaffinated, hydrated, and blocked for 30 min in 0.1% BSA in PBS-0.05% Tween 20. Sections were incubated for 30 min with α-phos (S14) H2B (1:5000 dilution in 1% BSA in PBS-0.05% Tween 20), washed twice for 10 min each in PBS-Tween. After washing sections were incubated for 30 min with anti-rabbit fluorescein conjugated secondary antibody (1:200 dilution in 1% BSA in PBS-Tween) and washed twice for 10 min each in PBS-Tween. Sections were mounted in Antifade (Molecular Probes) containing Hoechst (2 μg/ml).

Transient Transfection, Immunoprecipitation, and Kinase Assays

Transfection was done according to the lipofectamine plus kit (Gibco Invitrogen). After 24–48 hr of transfection, 293T cells were harvested in 40 mM HEPES [pH 7.5], 1% Triton X-100, 0.05% NP-40, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10% v/v glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml microcystin, 1 μg/ml aprotinin, 1 μg/ml pepstain, and 1 μg/ml leupeptin. Supernatant was saved after 20 min of centrifugation at 14,000 rpm. For immunoprecipitation (IP), 1 μg of α-myc (9E10 from Santa Cruz) was incubated with supernatant for 2 hr at 4°C and then precipitated with protein G-Sepharose (Amersham Pharmacia) for another 2 hr at 4°C. IPs were washed 5–6 times in TBST (20 mM Tris [pH 7.5], 150 mM NaCl, and 0.1% Tween-20), then the beads were incubated in 1 μg of H2B, 40 mM HEPES [pH 7.5], 20 mM $MgCl_2$, 0.1 μg/ml microcystin, 10 μM ATP, and 1 μCi γ-^{32}P ATP for 30 min at 30°C. Reactions were separated on a 15% SDS-PAGE gel and dried down for autoradiography.

Transfection for immunofluorescence studies with HeLa cells was done according to the lipofectamine 2000 kit (Gibco Invitrogen). Cells were fixed 24–36 hr posttransfection with 4% paraformaldehyde. Immunostaining was done as described above.

Acknowledgments

We thank members of the Allis lab for discussions, Upstate Biotechnology Inc. (UBI; Lake Placid, New York) for assistance in the initial development of the H2B (S14) antibody, Rueyling Lin (UT Southwestern, Dallas, Texas) for testing our antibodies in *C. elegans*, and Linda Langman for laboratory managerial support. W.L.C. was supported by the NIH Medical Scientist Training Grant. Initial stages of this work were carried out by K.A., who was supported by a Visiting Scientist gift from UBI. Work in the W.C.E. lab is supported by the NIH and The Wellcome Trust, of which W.C.E. is a Principal Research Fellow. L.D.E. is supported by grants from the NIH and the NSF. This work is supported by NIH grants GM54168 to J.C. and GM40922 to C.D.A.

Received: May 2, 2002
Revised: April 22, 2003
Accepted: April 25, 2003
Published: May 15, 2003

References

Ajiro, K. (2000). Histone H2B phosphorylation in mammalian apoptotic cells. An association with DNA fragmentation. J. Biol. Chem. *275*, 439–443.

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature *410*, 120–124.

Briggs, S.D., Bryk, M., Strahl, B.D., Cheung, W.L., Davie, J.K., Dent, S.Y., Winston, F., and Allis, C.D. (2001). Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. Genes Dev. *15*, 3286–3295.

Cheung, P., Allis, C.D., and Sassone-Corsi, P. (2000). Signaling to chromatin through histone modifications. Cell *103*, 263–271.

Creasy, C.L., and Chernoff, J. (1995). Cloning and characterization of a human protein kinase with homology to Ste20. J. Biol. Chem. *270*, 21695–21700.

Creasy, C.L., Ambrose, D.M., and Chernoff, J. (1996). The STE20-like kinase, Mst1, dimerizes and contains an inhibitory domain. J. Biol. Chem. *271*, 21049–21053.

Dan, I., Watanabe, N.M., and Kusumi, A. (2001). The Ste20 group kinases as regulators of MAP kinase cascades. Trends Cell Biol. *11*, 220–230.

de la Barre, A.E., Angelov, D., Molla, A., and Dimitrov, S. (2001). The N-terminus of histone H2B, but not that of histone H3 or its phosphorylation, is essential for chromosome condensation. EMBO J. *20*, 6383–6393.

Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K., and Zhou, M.M. (1999). Structure and ligand of a histone acetyltransferase bromodomain. Nature *399*, 491–496.

Duriez, P.J., and Shah, G.M. (1997). Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. Biochem. Cell Biol. *75*, 337–349.

Earnshaw, W.C., Martins, L.M., and Kaufmann, S.H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. *68*, 383–424.

Graves, J.D., Draves, K.E., Gotoh, Y., Krebs, E.G., and Clark, E.A. (2001). Both phosphorylation and caspase-mediated cleavage contribute to regulation of the Ste20-like protein kinase Mst1 during CD95/Fas-induced apoptosis. J. Biol. Chem. *276*, 14909–14915.

Graves, J.D., Gotoh, Y., Draves, K.E., Ambrose, D., Han, D.K., Wright, M., Chernoff, J., Clark, E.A., and Krebs, E.G. (1998). Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. EMBO J. *17*, 2224–2234.

Grutter, M.G. (2000). Caspases: key players in programmed cell death. Curr. Opin. Struct. Biol. *10*, 649–655.

Haunstetter, A., and Izumo, S. (2000). Future perspectives and potential implications of cardiac myocyte apoptosis. Cardiovasc. Res. *45*, 795–801.

Hendzel, M.J., Nishioka, W.K., Raymond, Y., Allis, C.D., Bazett-Jones, D.P., and Th'ng, J.P. (1998). Chromatin condensation is not associated with apoptosis. J. Biol. Chem. *273*, 24470–24478.

Jacobs, S.A., Taverna, S.D., Zhang, Y., Briggs, S.D., Li, J., Eissenberg, J.C., Allis, C.D., and Khorasanizadeh, S. (2001). Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. EMBO J. *20*, 5232–5241.

Jacobson, R.H., Ladurner, A.G., King, D.S., and Tjian, R. (2000). Structure and function of a human $TAF_{II}250$ double bromodomain module. Science *288*, 1422–1425.

Jason, L.J., Moore, S.C., Ausio, J., and Lindsey, G. (2001). Magnesium-dependent association and folding of oligonucleosomes reconstituted with ubiquitinated H2A. J. Biol. Chem. *276*, 14597–14601.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293*, 1074–1080.

Johnstone, R.W., Ruefli, A.A., and Lowe, S.W. (2002). Apoptosis: a link between cancer genetics and chemotherapy. Cell *108*, 153–164.

Kaufmann, S.H., and Hengartner, M.O. (2001). Programmed cell death: alive and well in the new millennium. Trends Cell Biol. *11*, 526–534.

Kerr, J.F., Harmon, B., and Searle, J. (1974). An electron-microscope study of cell deletion in the anuran tadpole tail during spontaneous metamorphosis with special reference to apoptosis of striated muscle fibers. J. Cell Sci. *14*, 571–585.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239–257.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116–120.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Lee, K.K., Ohyama, T., Yajima, N., Tsubuki, S., and Yonehara, S. (2001). MST, a physiological caspase substrate, highly sensitizes apoptosis both upstream and downstream of caspase activation. J. Biol. Chem. *276*, 19276–19285.

Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W.T., and Wang, X. (1998). The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc. Natl. Acad. Sci. USA *95*, 8461–8466.

Loetscher, H., Niederhauser, O., Kemp, J., and Gill, R. (2001). Is caspase-3 inhibition a valid therapeutic strategy in cerebral ischemia? Drug Discov. Today *6*, 671–680.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. Nature *389*, 251–260.

Madeo, F., Frohlich, E., Ligr, M., Grey, M., Sigrist, S.J., Wolf, D.H., and Frohlich, K.-U. (1999). Oxygen stress: a regulator of apoptosis in yeast. J. Cell Biol. *145*, 757–767.

Martin, L.J. (2001). Neuronal cell death in nervous system development, disease, and injury. Int. J. Mol. Med. *7*, 455–478.

Mattson, M.P., Duan, W., Pedersen, W.A., and Culmsee, C. (2001). Neurodegenerative disorders and ischemic brain diseases. Apoptosis *6*, 69–81.

Metzstein, M.M., Stanfield, G.M., and Horvitz, H.R. (1998). Genetics of programmed cell death in *C. elegans*: past, present and future. Trends Genet. *14*, 410–416.

Pike, C.J., Walencewics, A.J., Glabe, C.G., and Cotman, C.W. (1991). In vitro aging of beta-amyloid proteins causes peptide aggregation and neurotoxicity. Brain Res. *563*, 311–314.

Robertson, J.D., Orrenius, S., and Zhivotovsky, B. (2000). Nuclear events in apoptosis. J. Struct. Biol. *129*, 346–358.

Rogakou, E.P., Nieves-Neira, W., Boon, C., Pommier, Y., and Bonner, W.M. (2000). Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. J. Biol. Chem. *275*, 9390–9395.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S., and Bonner, W.M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J. Biol. Chem. *273*, 5858–5868.

Samejima, K., Tone, S., and Earnshaw, W.C. (2001). CAD/DFF40 nuclease is dispensable for high molecular weight DNA cleavage and stage I chromatin condensation in apoptosis. J. Biol. Chem. *276*, 45427–45432.

Sassone-Corsi, P., Mizzen, C.A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A., and Allis, C.D. (1999). Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. Science *285*, 886–891.

Sells, M.A., and Chernoff, J. (1997). Emerging from the Pak: the p21-activated protein kinase family. Trends Cell Biol. *7*, 162–167.

Severin, F.F., and Hyman, A.A. (2002). Pheromone induces programmed cell death in *S. cerevisiae*. Curr. Biol. *12*, R233–R235.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41–45.

Strahl, B.D., Briggs, S.D., Brame, C.J., Caldwell, J.A., Koh, S.S., Ma, H., Cook, R.G., Shabanowitz, J., Hunt, D.F., Stallcup, M.R., and Allis, C.D. (2001). Methylation of histone H4 at arginine 3 occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1. Curr. Biol. *11*, 996–1000.

Th'ng, J.P. (2001). Histone modifications and apoptosis: cause or consequence? Biochem. Cell Biol. *79*, 305–311.

Turner, B.M. (2000). Histone acetylation and an epigenetic code. Bioessays *22*, 836–845.

Ura, S., Masuyama, N., Graves, J.D., and Gotoh, Y. (2001). Caspase cleavage of MST1 promotes nuclear translocation and chromatin condensation. Proc. Natl. Acad. Sci. USA *98*, 10148–10153.

Usuku, G., and Gross, J. (1965). Morphologic studies of connective tissue resorption in the tail fin of metamorphosing bullfrog tadpole. Dev. Biol. *11*, 352–370.

van Holde, K.E. (1988). Chromatin. (New York: Springer) pp. 111–148.

Wolffe, A.P., and Hayes, J.J. (1999). Chromatin disruption and modification. Nucleic Acids Res. *27*, 711–720.

Wyllie, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature *284*, 555–556.

Wyllie, A.H., Kerr, J.F., and Currie, A.R. (1980). Cell death: the significance of apoptosis. Int. Rev. Cytol. *68*, 251–306.

Wyllie, A.H., Morris, R.G., Smith, A.L., and Dunlop, D. (1984). Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J. Pathol. *142*, 67–77.

Yoshizato, K. (1989). Biochemistry and cell biology of amphibian metamorphosis with a special emphasis on the mechanism of removal of larval organs. Int. Rev. Cytol. *119*, 97–149.

Yun, D.-J., Ibeas, J.I., Lee, H., Coca, M.A., Narasimhan, M.L., Uesono, Y., Hasegawa, P.M., Pardo, J.M., and Bressan, R.A. (1998). Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. Mol. Cell *1*, 807–817.