Molecular Cell *23*, 121–132, July 7, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.molcel.2006.05.023

Cell Apoptosis: Requirement of H2AX in DNA Ladder Formation, but Not for the Activation of Caspase-3

Chengrong Lu,[1] Feng Zhu,[1] Yong-Yeon Cho,[1] Faqing Tang,[1] Tatyana Zykova,[1] Wei-ya Ma,[1] Ann M. Bode,[1] and Zigang Dong[1,*]
[1] Hormel Institute
University of Minnesota
Austin, Minnesota 55912

Summary

Immunofluorescence studies have revealed that H2AX is phosphorylated at the sites of DNA double-strand breaks induced by ionizing radiation and is required for recruitment of repair factors into nuclear foci after DNA damage. Therefore, the function of H2AX is believed to be associated primarily with repair of DNA damage. Here, we report a function of H2AX in cellular apoptosis. Our data showed that H2AX is phosphorylated by UVA-activated JNK. We also provided evidence showing that UVA induces caspase-3 and caspase-activated DNase (CAD) activity in both H2AX wild-type and H2AX knockout mouse embryonic fibroblasts (MEFs). However, DNA fragmentation occurred only in H2AX wild-type MEFs. Furthermore, H2AX phosphorylation was critical for DNA degradation triggered by CAD in vitro. Taken together, these data indicated that H2AX phosphorylation is required for DNA ladder formation, but not for the activation of caspase-3; and the JNK/H2AX pathway cooperates with the caspase-3/CAD pathway resulting in cellular apoptosis.

Introduction

The nucleosome is the fundamental unit of chromatin in eukaryotes, consisting of DNA wrapped around an octamer of two pairs each of H2A, H2B, H3, and H4. Linker histone H1 compresses linear nucleosome arrays into a 30 nm chromatin fiber (Fernandez-Capetillo et al., 2004). The histone globular domains comprise the nucleosome core, from which the histone tails protrude, providing potential sites for histone modifications such as phosphorylation and acetylation (Bode and Dong, 2005; Taneja et al., 2004). Functional isoforms of histones are scattered throughout the mammalian genome. Reports have indicated that some of the histone variants have specialized biological functions such as DNA repair (Fernandez-Capetillo et al., 2004; Rogakou et al., 1998).

H2AX is a variant of the histone H2A family comprised of three distinct subfamilies: H2A1-H2A2, H2AZ, and H2AX (Rogakou et al., 2000). The sequence differentiating H2AX from the other H2A subfamilies is located in the C-terminal motif (KATQAS*QEY-COOH) (Cheung et al., 2000; Taneja et al., 2004). Ser139 in this motif is the site of γ-phosphorylation. Ionizing radiation (IR) induces a rapid phosphorylation of H2AX at Ser139 that is mediated by an unknown signaling pathway (Rogakou et al., 1998, 2000). Phosphorylated H2AX (γH2AX) forms nuclear foci at the sites of IR-induced double-strand breaks (DSBs) and might play an important role in recruiting repair factors to nuclear foci after DNA damage (Celeste et al., 2003; Chen et al., 2000; Fernandez-Capetillo et al., 2002; Paull et al., 2000). But the migration of repair and signaling proteins to DSBs is not affected in H2AX-deficient or $H2AX^{-/-}$ cells, nor is H2AX phosphorylation needed for the initial recognition of DNA breaks (Celeste et al., 2003). In addition, the enzyme activation-induced cytidine deaminase (AID)-dependent γH2AX foci do not reflect the presence of DSBs (Casali and Zan, 2004). The fact that H2AX deficiency is not detrimental to life further indicates that γH2AX foci formation does not necessarily reflect the presence of DSBs. Furthermore, the in vitro DNA end-joining reaction for the repair of DSBs was reported to occur independently of H2AX phosphorylation (Siino et al., 2002). Thus, the function of γH2AX remains elusive.

Members of the phosphatidylinositol (PI) 3-kinase family, including ataxia telangiectasia mutated protein (ATM), AT and Rad3-related protein (ATR), and DNA-dependent protein kinase (DNA-PK), were reported to be involved in the responses of mammalian cells to DSBs (Burma et al., 2001; Park et al., 2003; Stiff et al., 2004; Ward and Chen, 2001; Ward et al., 2004). A fraction of nuclear ATM has been shown to colocalize with γH2AX at the sites of DSBs in response to DNA damage. Further evidence suggested that ATM is required for H2AX phosphorylation induced by low doses of IR (Fernandez-Capetillo et al., 2002). In addition, H2AX phosphorylation was reported to be regulated by ATR in response to DNA replication stress (Ward and Chen, 2001; Ward et al., 2004). However, no published data have shown that ATM, ATR, or DNA-PK phosphorylates H2AX directly in vivo, and conflicting conclusions have been drawn as to the involvement of these kinases in H2AX phosphorylation. For example, H2AX phosphorylation was detected in individual kinase dead mutants ($ATM^{-/-}$, $DNA\text{-}PK^{-/-}$, and $ATR^{-/-}$) (Fernandez-Capetillo et al., 2004), and ATM did not contribute to IR-induced phosphorylation of H2AX in primary fibroblasts (Stiff et al., 2004). Furthermore, several cell lines deficient in DNA-PK did not exhibit a deficit in γH2AX formation after exposure to IR (Rogakou et al., 1998). Thus, some other as yet unknown kinases possibly phosphorylate H2AX.

Here, we report that ultraviolet (UV) A irradiation strongly induced H2AX phosphorylation that was mediated by c-Jun N-terminal kinase (JNK) and phosphorylation of H2AX by JNK was associated with induction of apoptosis. These data show that H2AX phosphorylation by JNK is required for apoptosis occurring through the caspase-3/caspase-activated DNase (CAD) pathway.

Results and Discussion

UV Induces Phosphorylation of H2AX

UV is an important etiological factor in human skin cancer, and we used mouse skin epidermal JB6 cells to study the phosphorylation of H2AX. We found that UVA

*Correspondence: zgdong@hi.umn.edu

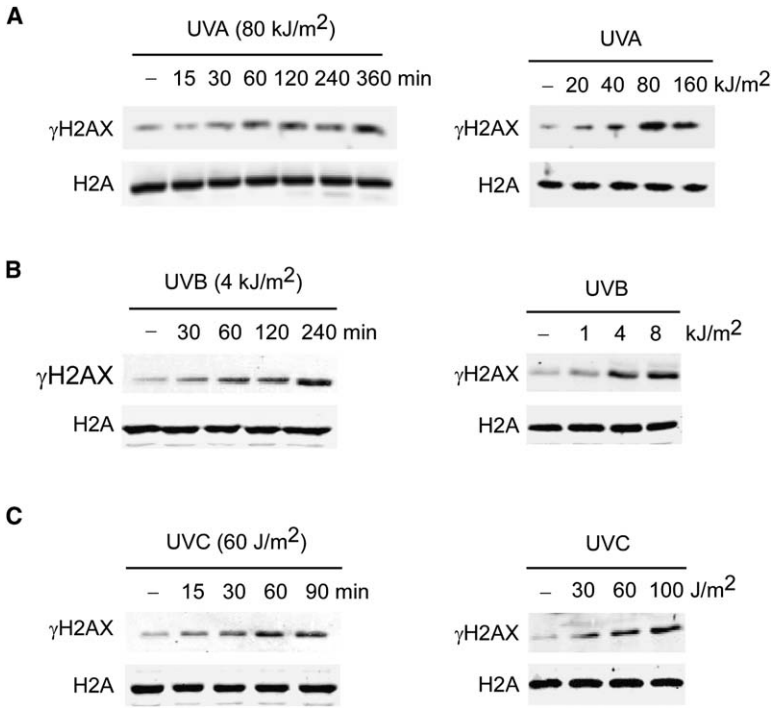

Figure 1. UVA, UVB, or UVC Induces Phosphorylation of H2AX

JB6 cells were exposed to (A) UVA, (B) UVB, or (C) UVC, and histones were extracted as described in the Experimental Procedures after the incubation time indicated (left panels) or after 60 min following indicated doses of UVA (right panels). Cells not exposed to UV served as negative controls (−). For all experiments, histones were resolved by 15% SDS-PAGE followed by Western analysis with antibodies against γH2AX or total H2A.

(320–400 nm), UVB (290–320 nm), or UVC (200–290 nm) induced strong phosphorylation of H2AX at Ser139 (γH2AX) in a time- and dose-dependent manner (Figures 1A–1C). Phosphorylation of H2AX is one of the first cellular responses when DSBs are induced by IR (Kobayashi et al., 2002; Woo et al., 2002). But some types of damage, and in particular that induced by UV exposure, do not elicit DSBs (Paull et al., 2000). Thus, the significance and mechanism of H2AX phosphorylation resulting from IR-induced DSBs appears to be distinct from UV-induced H2AX phosphorylation. UV-induced phosphorylation of H2AX might be involved in other as yet unidentified cellular functions. Because UVA is a main component of solar UV reaching the earth's surface and a major contributor to skin cancer (Zhang et al., 2002), we used UVA to treat cells in the remaining experiments.

JNK Phosphorylates H2AX

The mitogen-activated protein kinases (MAPKs) are a family of proteins that mediate distinct signaling cascades that are targets for diverse extracellular stimuli, including UV. These pathways are important in the regulation of a multitude of cellular functions, including proliferation, differentiation, apoptosis, development, growth, and inflammation (Bode and Dong, 2003; Cheung et al., 2000; Dent et al., 2003). ERKs-activated RSK2 has been shown to be directly involved in histone H3 phosphorylation in vivo (Cheung et al., 2000; Sassone-Corsi et al., 1999). We used MAPK chemical inhibitors and dominant-negative mutants (DNMs) to investigate whether MAPKs are involved in the regulation of H2AX phosphorylation induced by UVA. Our data showed that the MEK inhibitor PD98059 and the p38 inhibitor SB202190 did not affect H2AX phosphorylation, although each inhibited phosphorylation of their respective target proteins, ERKs and ATF2 (Figure S1 available in the Supplemental Data with this article online). On the other hand, SP600125, a JNK inhibitor, strongly suppressed H2AX phosphorylation and its normal target, c-Jun (Figure S1). Some reports (Bain et al., 2003; Davies et al., 2000) indicated that SP600125 is a nonspecific inhibitor in vitro and affects other kinases such as p70 S6K. However, our data showed that SP600125 had no effect on UVA-induced phosphorylation of p70 S6K (Thr421/424) (Figure S1, bottom right), suggesting that the inhibitory effect of SP600125 in vitro may be different from its effect in vivo. To address the nonspecificity of chemical inhibitors, we used various DNM-MAPK cell lines, including DNM-JNK1 stably transfected cells, to determine whether JNK was involved in H2AX phosphorylation. Results indicated that DNM-JNK1 also strongly inhibited UVA-induced phosphorylation of H2AX (Figure 2A), which was consistent with the inhibition by SP600125. In contrast, DNM-ERK2 or DNM-p38β had little effect on H2AX phosphorylation. The DNM-MAPKs cell lines were each confirmed to suppress their respective targeted kinase activity after UVA exposure (Figure 2A, bottom panels). To further support a role for JNK in H2AX phosphorylation, we transfected siRNA JNK1 into JNK2 knock out cells ($JNK2^{-/-}$) to create a double knockout of JNK. Results indicated that siRNA JNK1 effectively suppressed the level of JNK1 in $JNK2^{-/-}$ cells (Figure 2B, right panels), and siRNA JNK1 dramatically decreased UVA-induced phosphorylation of H2AX (Figure 2B, left panels). Overall these data strongly indicated that JNK is involved in UVA-induced phosphorylation of H2AX.

Next, we used in vitro kinase assays to determine whether JNK phosphorylated Ser139 of H2AX directly. The results showed that commercially active JNK1 or JNK2 phosphorylated H2AX at Ser139 (Figure 3A, lanes 2 and 4), whereas active ERK1 or p38α had no effect (Figure 3A, lanes 6 and 8). UV is known to strongly and