The Dynamic Alterations of H2AX Complex during DNA Repair Detected by a Proteomic Approach Reveal the Critical Roles of Ca\(^{2+}\)/Calmodulin in the Ionizing Radiation-induced Cell Cycle Arrest*\(^{§}\)

Yu-Chun Du‡, Sheng Gu‡, Jianhong Zhou‡, Tianyi Wang‡, Hong Cai‡, Mark A. MacInnes‡, E. Morton Bradbury§, and Xian Chen‡¶

By using DNA nuclease digestion and a quantitative “dual tagging” proteomic approach that integrated mass spectrometry, stable isotope labeling, and affinity purification, we studied the histone H2AX-associating protein complex in chromatin in mammalian cells in response to ionizing radiation (IR). In the non-irradiated control cells, calmodulin (CaM) and the transcription elongation factor facilitates chromatin transcription (FACT) were associated with H2AX. Thirty minutes after exposing cells to IR the CaM and FACT complexes dissociated, whereas two DNA repair proteins, poly(ADP-ribose) polymerase-1 and DEAH box polypeptide 30 isoform 1, interacted with H2AX. Two hours and 30 min after exposure, none of the above proteins were in the complex. H2B, nucleophosmin/B23, and calreticulin were associated with H2AX in both non-irradiated and irradiated cells. The results suggest that the H2AX complex undergoes dynamic changes upon induction of DNA damage and during DNA repair. The genuine interactions between H2AX and H2B, nucleophosmin/B23, calreticulin, poly(ADP-ribose) polymerase-1, and CaM under each condition were validated by immunoprecipitation/Western blotting and mammalian two-hybrid assays. Because multiple Ca\(^{2+}\)-binding proteins were found in the H2AX complex, the roles of Ca\(^{2+}\) were examined. The results indicate that Ca\(^{2+}\)/CaM plays important roles in regulating IR-induced cell cycle arrest, possibly through mediating chromatin structure. The dataset presented here demonstrates that sensitive profiling of the dynamics of functional cellular protein-protein interactions can successfully lead to the dissection of important metabolic or signaling pathways. *Molecular & Cellular Proteomics 5: 1033–1044, 2006.

The basic structural unit of chromatin is the nucleosome, which consists of a histone octamer (two copies of each H2A, H2B, H3, and H4) around which is coiled 1.7 turns of 168-bp DNA stabilized by a fifth histone, H1. Controlled nuclease digestion results in a stable subnucleosomal "core particle," which contains 146-bp DNA coiled around the histone octamer. Except for histone H4, each histone is in a family of subtypes. Of particular interest are the histone subtypes called “replacement histones.” Unlike the major histone subtypes, which are synthesized in S phase of the cell cycle, replacement histones are synthesized through the cell cycle and in terminally differentiated cells (1). H2AX is such a replacement histone, which exists in all cell types, and accounts for 2–25% of the total cellular H2A (2, 3). Accumulating evidence has shown that H2AX is a key factor in the repair of DNA double strand breaks (DSBs)\(^{1}\) (4). Recent studies suggest that H2AX may be a major source of genome instability (5). H2AX is rapidly phosphorylated (\(\gamma\)-H2AX) following exposure of cells to IR, which induces DSBs, and forms IR-induced foci at the damage sites (2, 4). It was proposed that \(\gamma\)-H2AX may serve as a docking site for other DNA damage repair/signaling proteins to bind in the vicinity of DNA lesions (6). However, despite major advances achieved primarily by immunofluorescence and immunochemical techniques, important questions regarding the role of H2AX in chromatin in DNA damage/repair remain to be elucidated. For example, what are the factors involved in the formation of H2AX IR-induced foci prior to and after the induction of the DSBs?

Extraction of nuclear proteins from mammalian cells with buffers containing high salt and detergent has become a standard procedure. However, a recent study designed to profile RNA polymerase II-interacting proteins has failed to

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\(^{1}\) The abbreviations used are: DSB, DNA double strand break; CaM, calmodulin; FACT, facilitates chromatin transcription; \(\gamma\)-H2AX, phosphorylated H2AX; IR, ionizing radiation; PARP-1, poly(ADP-ribose) polymerase-1; DMEM, Dulbecco’s modified Eagle’s medium; Gy, gray; BD, binding domain; AD, activation domain; GFP, green fluorescent protein.
identify any known transcription or elongation factors and illustrates the limitations of the salt extraction approach in characterizing chromatin-related protein complexes (7). To obtain H2AX-interacting proteins from insoluble chromatin, we used nuclease to digest chromatin. The advantage of this approach is that soluble intact protein complexes containing histone H2AX can be obtained without using high salt conditions.

Due to the high sensitivity, high specificity, and the capability of high throughput, mass spectrometry has emerged as a powerful tool to efficiently and systematically identify proteins in biological samples (8, 9). To identify the components of H2AX complexes, we used a mass spectrometry based dual-tagging proteomic approach (Fig. 1). The bait protein H2AX is epitope-tagged for affinity isolation of the complex (epitope tagging), and in parallel the whole proteome of the cells expressing the epitope-tagged H2AX at physiologically relevant levels is labeled with deuterium-labeled heavy amino acids (isotope tagging). In mass spectrometric measurements, the heavy amino acids incorporated in the cellular proteins provide “in-spectra” quantitative markers so that proteins in a complex with H2AX can be unambiguously identified after a single step affinity purification (10). In the present study, by using DNA nuclease digestion of nuclei and the dual tagging strategy, we identified the proteins that associate with H2AX in chromatin in mammalian cells both before and after IR to monitor the dynamics of the complex during DNA repair.

EXPERIMENTAL PROCEDURES

Plasmids, Stable Cell Line, and Cell Culture—The coding sequence of H2AX (2) was cloned into the BamHI and Xhol sites of a retroviral vector, pMIR-DFT, with a double FLAG tag at the N terminus. The plasmids containing the tag alone or FLAG-H2AX were transfected into 293T cells with the calcium phosphate method, and the cells were selected in DMEM supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, and 1.2 mM sodium fluoride, and 10 mM EDTA, 0.05% Nonidet P-40, and 0.5 mM β-mercaptoethanol plus the protease and phosphatase inhibitors, and cleared by centrifugation. Completion of the digestion was checked with a 2% agarose gel.

Protein Purification—Following completion of the chromatin digestion, glycerol and NaCl were added to the cleared lysate to the final concentrations of 20% and 150 mM, respectively, and the nuclear extract was incubated with 200 μl of M2 anti-FLAG beads (Sigma) at 4 °C for 2 h. The beads were then washed four times with 4 ml of washing buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 20% glycerol, 1 mM EDTA, 0.05% Nonidet P-40, and 0.5 mM β-mercaptoethanol) plus the protease and phosphatase inhibitors. The bound proteins were eluted with a buffer containing 250 μg/ml 3X FLAG peptide (Sigma). The eluted proteins were concentrated with trichloroacetic acid precipitation and then separated by 4–20% SDS-PAGE. After staining with Coomassie Brilliant Blue, the whole lane of gel was cut into 25 slices for LC-MS/MS analysis.

LC-MS/MS Analysis and Database Searching—In-gel digestion and LC-MS/MS analysis were performed as described previously (12). The MS/MS data for each sample were searched against the NCBI nr database (January 20, 2004) protein sequence database downloaded from the National Center for Biotechnology Information under the species restriction of Homo sapiens using the in-house licensed Mascot searching program (version 2.0). The Leu-d<sub>3</sub> modification was added in the configuration file and selected as variable modifications in the database searching. The parameters for database searching were as follows: (i) 0.2-Da mass error tolerance for both MS and MS/MS, (ii) tryptic enzyme specificity with a maximum of two missed cleavages, and (iii) the following variable modifications: acetylation at peptide N terminus, phosphorylation on tyrosine/serine/threonine, and oxidation on methionine.

Immunoblotting Analysis—293T cells (7 × 10<sup>6</sup> cells/assay) were left untreated or irradiated with 30 Gy, recovered at 37 °C at the times indicated in each specific experiment, and harvested for nuclear isolation/digestion, immunoprecipitation, and Western blotting. The procedures for nuclear isolation/digestion and immunoprecipitation were essentially the same as described under “Irradiation of Cells, Nuclei Isolation, and Nuclease Digestion” and “Protein Purification” except that buffer volumes in each step were reduced ~10-fold.

Mammalian Two-hybrid Analysis—Vectors encoding Gal4 DNA-binding domain (BD) and transcription activation domain (AD) were from a mammalian two-hybrid assay kit (BD Biosciences). The Gal4 GFP reporter plasmid was kindly provided by Dr. Toshi Shioda (Massachusetts General Hospital Cancer Center). The coding sequences of H2AX and CaM were inserted into the BD and AD vectors, respectively, and the two constructs were co-transfected into 293T cells with the Gal4 GFP reporter plasmid (2 μg of each plasmid in a 60-mm plate). The negative control was performed by co-transfection of 293T cells with the Gal4 GFP reporter plasmid and the BD and AD constructs in which the BD and AD were fused with two proteins that do not interact. The expression of GFP was analyzed by both flow cytometry and Western blotting.

Cell Cycle Analysis—The exponentially growing cells in DMEM were mock- or γ-ray-irradiated and immediately replated. For the Ca<sup>2+</sup> treatments, CaCl<sub>2</sub> was added to the culture medium to the indicated concentrations immediately after replating. After allowing cells to recover at 37 °C for the indicated period of time, the cells were harvested, washed, fixed, and stained with propidium iodide, and cellular fluorescence was measured by using a FACSCalibur flow cytometer (BD Biosciences). To detect the phosphorylated histone H3 in mitosis, cells were analyzed as described previously (13), and 10,000 events were recorded for each measurement.

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RESULTS

The Dual Tagging Proteomic Strategy for Protein Complex Identification—The schematic of our quantitative dual tagging proteomic approach for identifying proteins associated with H2AX is illustrated in Fig. 1A, and representative results are shown in Fig. 1B. The stable cells expressing tag alone and the stable cells expressing the FLAG-H2AX are grown in the “light” and “heavy” medium, respectively. After digestion and lysis of the nuclei, the resulting soluble nuclear proteins are affinity-purified with anti-FLAG beads. Proteins eluted from the beads are separated by SDS-PAGE and digested with trypsin, and the resulting peptides are analyzed by mass spectrometry. Whereas the ratios of the labeled:unlabeled peak intensity for the nonspecific binding proteins are around 1, the ratios for the proteins that specifically bind to H2AX are significantly larger than 1. B, representative MS spectra showing the nonspecific and specific binding to H2AX: panel a, nonspecific binding; panel b, specific binding.

The Epitope-tagged H2AX Is Incorporated into Chromatin—To determine whether a tag added to the N terminus of H2AX would affect the correct packing and function of H2AX in the nucleosome, we first determined the extraction profiles with increasing concentrations of NaCl of (i) the FLAG-H2AX from the FLAG-H2AX cells and (ii) the endogenous H2AX from the parental 293T cells (Fig. 2A). The results showed that the FLAG-tagged and endogenous H2AX had similar extraction profiles. Both were resistant to NaCl extraction, suggesting that the tagged H2AX has tightly bound within chromatin. Then we digested nuclei with micrococcal nuclease, immunoprecipitated the digested nuclear extract with anti-FLAG beads, and analyzed the DNA fragment size released by the digestion (Fig. 2B). Whereas, as expected, no immunoprecipitates were found for the parental 293T cells, the immunoprecipitates from the FLAG-H2AX cells produced two DNA bands of approximate 150 and 300 bp, which corresponded to the DNA sizes of core particle and two closely adjacent core particles. This result indicates that the tagged H2AX is correctly packed into the core particle. Lastly we mock- and /H9253-ray-treated (30 Gy) the FLAG-H2AX cells and determined the phosphorylation states of both the tagged and endogenous H2AX using anti-γ-H2AX (Fig. 2C). As for its endogenous counterpart, the tagged H2AX was phosphorylated after IR, suggesting that it responds properly to the induction of DSBs. Furthermore the FLAG-H2AX was expressed at levels similar to those of its endogenous counterpart (Fig. 3), and the cells expressing the FLAG-H2AX showed a growth rate and morphology similar to those of the parental cells, indicating that the expression of the FLAG-tagged H2AX has no effect on the phenotype of the parental cells. Collectively the above results strongly suggest that the FLAG-tagged H2AX, like its endogenous counterpart, is correctly packaged into the nucleosome and functions properly in chromatin.
The Assessments of Chromatin Digestion—We used nuclelease DNase I to digest chromatin to obtain soluble intact H2AX complexes. In such an approach, complete digestion of chromatin is essential for the identification of proteins associated with H2AX directly. For this reason, we tested chromatin digestion conditions extensively. As shown in Fig. 4, when the partially digested chromatin was immunoprecipitated with anti-FLAG beads, Western blotting with anti-H2AX antibody exhibited two bands, one at approximate 14 kDa corresponding to the endogenous H2AX (Fig. 4B, compare lane 1 with lane 3) and another at around 23 kDa corresponding to the FLAG-tagged H2AX (Fig. 4B, compare lane 1 with lane 4). However, when the immunoprecipitation was performed on the completely digested chromatin, only one band around 23 kDa was shown (Fig. 4B, lane 2), corresponding to the FLAG-tagged H2AX (Fig. 4B, compare lane 2 with lane 4). We assume that the endogenous H2AX in the partially digested chromatin was not from the same nucleosome as the tagged H2AX because the two copies of H2A do not interact directly in the same nucleosome (14). Further analysis showed that when the completely digested chromatin was used for immunoprecipitation, H2B could be detected (Fig. 4C) but not H3 or H4 (data not shown). Collectively these results strongly suggest that under the complete digestion conditions established in this study, no intact nucleosomes are present in the digested nuclear extract, and the H2AX-H2B dimer is the major stable form of H2AX in solution. Therefore in this study we refer to the H2AX-interacting proteins as those associated with H2AX directly or indirectly through H2B or other closely associated proteins.

Identification of H2AX-interacting Proteins in the Non-irradiated Control Cells—We first characterized the H2AX complex isolated from the cells without IR exposure. Of the 79 proteins identified, 13 proteins were selectively enriched with the anti-FLAG beads by a factor of at least 1.5 (Table I). Reproducibility was assessed primarily according to the method described by Blagoev et al. (15), that is by (i) deter-
same protein appeared in different gel bands; see Supplemental Table I), and (iv) performing independent runs for some of the fractions. The results from the above analyses led to a cutoff value of enrichment-fold of 1.5 for Table I and the first part (30 min section) of Table II and 1.3 for the second part (2.5 h section) of Table II. A smaller cutoff value was set for the second part of Table II (2.5 h section) due to these data being more uniform, which was reflected by smaller standard deviations (compare the standard deviations in the 2.5 h section in Table II with those in Table I and those in the 30 min section in Table II). H2B and H4 were among the histones identified. Because no intact nucleosomes were found in the digested nuclear extract (Fig. 4), the H4 in the H2AX complex probably resulted from free H4. The crystal structure of the nucleosome core particle shows that the histone octamer is composed of two copies of each H2A-H2B dimer and the (H3-H4)2 tetramer formed by H3-H3 “histone fold” interactions (14). Each H2A-H2B dimer interacts with the tetramer through the H2B-H4 histone fold. Consistent with the direct interactions of H2A and H2B in the structure, the enrichment ratio for H2B was clearly different from the ratio for H4 (Table I) whose interaction is mediated through H2B (14).

CaM is a Ca2+-dependent regulatory protein, and its target enzymes are among important regulators such as protein kinases and phosphatases. Calreticulin is also a Ca2+-binding protein. Although calreticulin is mainly located in the endoplasmic reticulum, it is also found in the nucleus and can bind to histones (16). BiP is a chaperone protein localized in the endoplasmic reticulum, and its function is highly regulated by Ca2+. It has been reported that BiP binds to denatured histones in vitro (16).

The structure-specific recognition protein 1 and the chromatin-specific transcription elongation factor are the two components of the transcription elongation factor FACT. The two proteins form a stable heterodimer, which is required for transcription elongation when chromatin is used as a template (17). In vitro biochemical studies with purified proteins have shown that FACT interacts with the H2A-H2B dimer of the nucleosome and forms a FACT-H2A-H2B complex. It facilitates transcription elongation by displacing the H2A-H2B dimer from the nucleosome (18). In this study, both subunits of the FACT were identified, suggesting that FACT may play a crucial role in DNA damage repair or transcription in vivo.

Nucleophosmin/B23 is a nuclear phosphoprotein and is more abundant in cancer cells than in normal resting cells. UV radiation triggers an immediate up-regulation of nucleophosmin/B23 expression in mammalian cells, suggesting that nucleophosmin/B23 may be involved in the acute response of cells to environmental stress (19). It has been reported that nucleophosmin/B23 binds to histone proteins in HeLa cells (20).

Identification of H2AX-interacting Proteins in the Irradiated Cells—Previous reports have shown that ~30 min after the exposure of cells to IR, H2AX was strongly phosphorylated
and formed IR-induced foci around the DSB site (4). We analyzed the H2AX protein complex to identify the factors associating with H2AX in this dynamic phase. Thirty minutes after IR, four proteins that were found to interact with H2AX from the non-irradiated cells, H2B, calreticulin precursor, nucleophosmin/B23, and BiP (Table I), were again identified to

| NCBI accession no. | Protein namea | Enrichment-foldb | S.D.c | No. of peptides |
|--------------------|---------------|------------------|-------|----------------|
| gi|4504253 | Histone H2AX (bait) | 99.4 | 9.29 | 4 |
| gi|28173554 | Histone H2B | 2.26 | 0.39 | 2 |
| gi|4504301 | Histone H4 | 1.60 | 0.33 | 4 |
| gi|49037474 | Calmodulin | 7.2 | 1.72 | 7 |
| gi|4757900 | Calreticulin precursor | 1.76 | 0.23 | 6 |
| gi|825671 | Nucleophosmin/B23 | 1.54 | 0.09 | 3 |
| gi|6470150 | BiP protein | 1.72 | 0.21 | 16 |
| gi|4507241 | Structure-specific recognition protein 1 | 2.2 | 0.32 | 6 |
| gi|8005757 | Chromatin-specific transcription elongation factor | 1.79 | 0.26 | 4 |
| gi|5031755 | Heterogeneous nuclear ribonucleoprotein R | 1.61 | 0.03 | 4 |
| gi|337457 | Ribonucleoprotein La | 1.58 | 0.18 | 3 |
| gi|7705433 | HSPC021 | 1.83 | 0.38 | 3 |
| gi|3183544 | Polyadenylate-binding protein 1 | 1.64 | 0.29 | 13 |

* Only the proteins with two or more peptides matched are listed.

* Fold of enrichment was calculated as the ratio of Leu-δ2-labeled peptide to unlabeled peptide.

* Standard deviation was determined from multiple peptides.

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| NCBI accession no. | Protein namea | Enrichment-foldb | S.D.c | No. of peptides |
|--------------------|---------------|------------------|-------|----------------|
| gi|4504253 | Histone H2AX (bait) | 97.4 | 29.6 | 4 |
| gi|28173554 | Histone H2B | 2.18 | 0.39 | 2 |
| gi|4504301 | Histone H4 | 1.60 | 0.33 | 4 |
| gi|49037474 | Calmodulin | 7.2 | 1.72 | 7 |
| gi|4757900 | Calreticulin precursor | 1.76 | 0.23 | 6 |
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complex with H2AX (Table II). The repeated identification of these four proteins on one hand suggests that these proteins form stable complexes with H2AX and on the other hand confirms the reproducibility of our experimental approach. In addition to the four proteins, two proteins involved in DNA damage and repair, PARP-1 and the DEAH box polypeptide 30 isoform 1, were identified (Table II). Interestingly CaM and FACT, which were shown to be associated with H2AX in the non-irradiated cells (Table I), had dissociated from H2AX after IR. PARP-1 is the enzyme that uses NAD$^+$ as a substrate to catalyze the transfer of ADP-ribose to a variety of nuclear protein acceptors. The most poly(ADP-ribosyl)ated proteins in vivo are PARP-1 itself and histones H1 and H2B (21). It has been shown that PARP-1 plays important roles in DNA damage repair and in transcription (22, 23). Although histones are the preferred targets in vivo and in vitro (21), the stable histone-PARP-1 complex has not been reported. DEAH box polypeptide 30 isoform 1 is a member of the DEAH helicase family, which plays important roles in basal transcription, DNA repair, and chromosome transmission (24). A member of the DEAH helicase family has been shown to directly interact with the highly conserved C-terminal BRCT repeat of the tumor suppressor BRCA1, and the disruption of this interaction leads to defects in DNA repair and progression of breast and ovarian cancer (25). BRCA1 was reported to co-localize with H2AX at DSB sites (4, 26). However, to our knowledge no physical interaction between BRCA1 and H2AX has been observed. The identified association between H2AX and DEAH box polypeptide 30 isoform 1 suggests that the DNA damage-induced co-localization of BRCA1 and H2AX may be mediated by members of the DEAH helicase family.

The next step was to profile the H2AX complex when cells were irradiated and allowed to recover at 37 °C for a longer period of time, 2.5 h. As shown in Table II, histone H2B, calreticulin precursor, and nucleophosmin/B23 were reproducibly identified. At this time point, DNA repair proteins, PARP-1 and the DEAH box polypeptide 30 isoform 1, were found to be dissociated from H2AX. One interesting phenomenon was that two histone H2B isoforms, gi/4504263 and gi/184086, were identified to join the H2AX complex. The two H2B isoforms (gi/4504263 and gi/184086) are distinguished from the bulk H2B (gi/28173554) by peptide EIQTAVRLLLPGELAK. The two H2B isoforms (gi/4504263 and gi/184086) are distinguished from one another by two unique peptides, KESYSVYVYK for gi/4504263 and KESYSIYVYK for gi/184086 (Supplemental Table I).
Immunochemical and Mammalian Two-hybrid Analyses Validate the Proteomic Data—In agreement with the proteomic data (Tables I and II), Western blotting showed that the two H2AX partners, nucleophosmin/B23 and calreticulin, were co-precipitated with H2AX under all three conditions (Fig. 5A). However, there were some inconsistencies between the proteomic and the Western data. The Western data showed that when cells were irradiated and allowed to recover for 2.5 h more nucleophosmin/B23 and less calreticulin were associated with H2AX than those under the other two conditions (Fig. 5A, compare lane 6 with lanes 2 and 4). Proteomic data showed that the two proteins had similar isotopic enrichment ratios under three different treatment conditions (Tables I and II). A possible explanation is that for the mass spectrometric analysis we had mixed the labeled and unlabeled protein lysates together before affinity purification, and some in vitro binding or exchange might have occurred between the unlabeled proteins and the labeled FLAG-H2AX protein complexes. There were two possible mechanisms: (i) the components of the H2AX complex, which were Leu-d3-labeled, might exchange with the unlabeled counterparts in the mixed protein lysate or (ii) the labeled H2AX protein complex might bind additional unlabeled components in addition to its existing ones (i.e. increasing the copy numbers of the components). Both reactions would lead to lower labeled-to-unlabeled ratios (i.e. the “Enrichment-fold” in Tables I and II) in the mass spectrometric analysis, which have been observed in this experiment (Tables I and II) and another report that used similar experimental strategy (15). The fact that the proteins that specifically associate with bait protein have significantly higher labeled-to-unlabeled ratios than those for nonspecific bound proteins, which were observed in this study and the other report (15), suggests that the unlabeled proteins cannot reach a copy number similar to the existing labeled components of the protein complex under defined experimental conditions. In short, the reason for the inconsistency between proteomic data and Western data is that the isotopic ratios in the mass spectrometric analysis may not reflect the stoichiometry profile of the protein complex as Western blotting indicates. We also tested for PARP-1, which the proteomic data had shown to be associated with H2AX when the cells were irradiated and allowed to recover for 30 min but not

Fig. 6. Ca\textsuperscript{2+} partially relieves the IR-induced G\textsubscript{2} arrest. A, Ca\textsuperscript{2+} partially relieves the IR-induced G\textsubscript{2} arrest. The cells in the exponential growth phase in DMEM were mock- or γ-ray-irradiated and immediately replated. For the Ca\textsuperscript{2+} treatments, CaCl\textsubscript{2} was added to the culture medium to a final concentration of 15 mM immediately after replating. After allowing cells to recover for the indicated period of time, the cells were harvested for analysis by flow cytometry. Panel a, non-irradiated cells in normal medium (1.8 mM Ca\textsuperscript{2+}); panel b, the irradiated cells in normal medium (1.8 mM Ca\textsuperscript{2+}); panel c, the irradiated cells in the medium with elevated Ca\textsuperscript{2+} (15 mM); panel d, non-irradiated cells in the medium with elevated Ca\textsuperscript{2+} (15 mM). The effects of elevated extracellular Ca\textsuperscript{2+} concentration on restoring IR-induced G\textsubscript{2}/M arrest are indicated (compare panel c with panel b). B, Ca\textsuperscript{2+} enhances the G\textsubscript{2} to M transition of the irradiated cells. The cells were grown, irradiated, Ca\textsuperscript{2+}-treated as in A, and allowed to recover for 30 min. The cells were then harvested and costained with propidium iodide and antibody to phospho-histone H3 (Ser-10) as described (13). Panel a, non-irradiated cells in normal medium (1.8 mM Ca\textsuperscript{2+}); panel b, the irradiated cells in normal medium (1.8 mM Ca\textsuperscript{2+}); panel c, the irradiated cells in the medium with elevated Ca\textsuperscript{2+} (15 mM); panel d, non-irradiated cells in the medium with elevated Ca\textsuperscript{2+} (15 mM). It is shown that the IR-induced reduction in G\textsubscript{2} to M transition (compare panel b with panel a) was restored by the elevated extracellular Ca\textsuperscript{2+} concentration (compare panel c with panel b).
under the other two conditions (Tables I and II). The immuno-precipitation and Western blot analysis results (Fig. 5A) were consistent with the proteomic data. The H2AX-H2B interaction in the non-irradiated control cells has been demonstrated in Fig. 4C. To ensure that similar amounts of target protein H2AX were loaded for each treatment, the Western blot filter was also probed with anti-H2AX. As expected, the tagged H2AX was present in similar amounts for all three treatments (Fig. 5A, compare lanes 2, 4, and 6).

We analyzed the interaction between H2AX and CaM with a mammalian two-hybrid system with the expression of the reporter GFP detected by flow cytometry and Western blotting (Fig. 5, B and C). There was no GFP expression in the non-transfected 293T cells (Fig. 5, B, lane 1, and C, lane 1). Co-transfection of 293T cells with the Gal4 reporter plasmid and the constructs encoding fusion proteins Gal4 BD-H2AX and AD-CaM resulted in the expression of GFP in 13.6% of the total cells (Fig. 5, B, lane 3, and C, lane 3) relative to the negative control of 3.9% (Fig. 5, B, lane 2, and C, lane 2). These results suggest that H2AX interacts with CaM in mammalian cells in situ in agreement with our proteomic data (Table I).

Cell Cycle Is Arrested at the G2 Phase after IR, and the Elevated Extracellular Ca2+ Partially Relieves the Arrest—After observing the dynamic alterations of the components of the H2AX complex upon IR, we investigated how chromatin structure and cell cycle checkpoint responded to IR. The results from both 4',6-diamidino-2-phenylindole staining of the cellular DNA and the salt extraction analysis of nuclei showed that chromatin was condensed after cells were exposed to IR (Supplemental Fig. 1); this has also been observed in HeLa cells (27). We then examined the effects of IR on cell growth and division. Whereas the non-irradiated cells continued to grow from about 50% confluency to almost full confluency in 24 h, the irradiated cells stopped dividing and appeared larger than the non-irradiated cells, suggesting that the IR had induced cell cycle arrest (Supplemental Fig. 2). We then analyzed the cell cycle by flow cytometry. As shown in Fig. 6A, panel a, the non-irradiated cells exhibited a similar profile of heterogeneous log phase distribution in the 12-h period. When irradiated with 8 Gy of γ-ray, cells gradually accumulated in the G2/M phase (Fig. 6A, compare panel b with panel a), indicating IR-induced G2/M arrest. Because multiple Ca2+-binding/signaling proteins were identified to be involved in the dynamics of the H2AX complex during DNA repair (Tables I and II), we asked whether Ca2+ binding/signaling was involved in the IR-induced G2/M arrest. To test this speculation, we examined the effects of Ca2+ on cell cycle progression of the irradiated cells. As shown in Fig. 6A, panel c, when CaCl2 was added to the culture medium of the irradiated cells immediately after IR, to increase the Ca2+ concentration from 1.8 mM (the original Ca2+ concentration in culture medium) to 15 mM, the IR-induced accumulation of cells in the G2/M phase was significantly reduced (Fig. 6A, compare panel c with panel b). For control purposes, we also tested the effects of elevated Ca2+ concentration (15 mM) on non-irradiated cells. As shown in Fig. 6A, panel d, no obvious effects of Ca2+ on the cell phase distribution of the untreated cells were observed (Fig. 6A, compare panel d with panel a).

To further examine the effects of Ca2+ on cell cycle progression, we evaluated the transition of cells from the G2 to M phase (Fig. 6B). In Fig. 6B, propidium iodide was used to monitor the cellular DNA content (y axis), and the antibody to histone H3, which is phosphorylated exclusively during mitosis, was used to identify the mitotic cells from the G2 cells (x axis). Compared with the non-irradiated cells, 30 min after IR the transition from the G2 to M phase was reduced significantly in the irradiated cells (Fig. 6B, compare panel b with panel a). When CaCl2 was added to the culture medium of the irradiated cells, the IR-induced reduction in the G2-to-M transition was restored (Fig. 6B, compare panel c with panel b). We did not observe any significant effects of elevated extracellular Ca2+ concentration on the G2-to-M transition of the non-irradiated cells (Fig. 6B, compare panel d with panel a). Collectively these results suggest that Ca2+ is involved in the G2-to-M transition in mammalian cells, and IR-induced G2 arrest may involve Ca2+ binding/signaling.

Ca2+ Promotes the Proliferation of the Irradiated Cells—We also examined the effects of Ca2+ on cell growth and division. The exponentially growing cells were irradiated (8 or 30 Gy), and equal numbers of cells were plated on 60-mm plates. CaCl2 was added to the culture medium to the indicated concentrations, and the cells were allowed to grow at 37 °C. After 2 days, viable cells were counted using trypan blue exclusion assay (Sigma). Fig. 7 shows that in contrast to the somewhat inhibitory effects of elevated extracellular Ca2+ concentration (over ~5 mM) on non-irradiated cell numbers, increasing extracellular Ca2+ concentrations resulted in increased viable cell numbers for the irradiated cells, and the responses were dose-dependent.

DISCUSSION

By combining DNA nuclease digestion of chromatin, which releases intact protein complexes from the insoluble chromatin, and a quantitative dual tagging proteomic strategy, which identifies real time specific protein-protein interactions, we observed dynamic changes in the H2AX protein complex in response to the induction of DSBs. One noteworthy observation is that multiple Ca2+-binding/signaling proteins were found in the H2AX protein complexes (Table I). This result led us to examine whether Ca2+/CaM is involved in the IR-induced G2 arrest and cell proliferation. The results demonstrate that the elevated extracellular Ca2+ partially relieves the IR-induced G2 arrest (Fig. 6A), enhances the IR-affected G2-to-M transition (Fig. 6B), and promotes cell division of the irradiated cells (Fig. 7). It has been established that entry into mitosis requires the activation of the mitotic cyclin-dependent kinase Cdc2 through the dephosphorylation of phosphory-
resulted in increased viable cell numbers. Cells in the exponential growth phase were mock- or mammalian cells.

CaM-dependent regulation of the cell cycle progression in phosphatase (34). The results in Fig. 6 suggest that IR induced dramatically promotes the dephosphorylation of histones by kinase II plays a major role in controlling the G2 checkpoint, it against this hypothesis because if CaM-dependent protein Ser-216 (30, 31). However, the results in this study argue proposed that CaM-dependent protein kinase II controls the phosphorylation of Cdc25c on Ser-216, leading to the G2 arrest (29). The results observed in this study (Fig. 6) suggest that Ca2+-dependent factors may also be involved in the IR-induced G2 checkpoint in addition to Chk1 and Chk2. CaM-dependent protein kinase II is a CaM-dependent protein kinase and can phosphorylate Cdc25c (30, 31). It was proposed that CaM-dependent protein kinase II controls the G2 checkpoint through the phosphorylation of Cdc25c on Ser-216 (30, 31). However, the results in this study argue against this hypothesis because if CaM-dependent protein kinase II plays a major role in controlling the G2 checkpoint, it is difficult to explain the observation that the elevated extra-cellular Ca2+ promotes the G2-to-M transition in the irradiated cells (Fig. 6). Therefore, a mechanism other than CaM-dependent protein kinase II must be responsible for the Ca2+-/CaM-dependent regulation of the cell cycle progression in mammalian cells.

CaM is the primary intracellular Ca2+ receptor and is universally required for cell proliferation in eukaryotes (32). In this study, we found that CaM associated with H2AX in the non-irradiated cells but dissociated in the irradiated cells. In vitro biochemical studies have shown that CaM interacts with histones and forms a CaM-histone complex (33, 34). The interaction between CaM and histones is both Ca2+- and charge density-dependent, and the removal of Ca2+ and phosphorylation of histones cause dissociation of the complex (33). It has also been shown that the binding of CaM to histones dramatically promotes the dephosphorylation of histones by phosphatase (34). The results in Fig. 6 suggest that IR induced the decreased level of intracellular Ca2+ or CaM. Consistent with this finding, it was reported that IR resulted in dramatic reductions in CaM expression in multiple cell lines (35). Perhaps the IR-induced rapid phosphorylation of H2AX (2, 4) and the IR-repressed expression of CaM (35) contributed to the dissociation of the CaM-H2AX complex in vivo (Tables I and II). Because H2AX is a structural component of chromatin, it is possible that changes in H2AX complex structure and H2AX phosphorylation after IR result in chromatin condensation (Supplemental Fig. 1) (27). On the other hand, it was reported that H2AX- cells exposed to low doses of irradiation exhibited mild G2 arrest (36, 37). Furthermore most of the factors in the checkpoint signaling pathway were reported to be localized at the DNA damage sites in chromatin (38). Several lines of recent evidence support the hypothesis that chromatin structure plays crucial roles in DNA repair and checkpoint signaling (39–43). We propose that in mammalian cells the repression of CaM expression and the phosphorylation of H2AX are important cellular responses to the induction of DSBs. These changes, along with other histone modifications and chromatin remodeling, may trigger the reconfiguration of chromatin structure and hence regulate cell cycle progression and DNA repair. According to this model, the elevated extra-cellular Ca2+ is required to compensate the IR-repressed level of intracellular CaM for the maintenance of chromatin structure (32).

Multiple repair/signaling proteins are recruited to DSB sites (6). Among those factors, NBS1, 53BP1, and MDC1 have been shown to physically interact with γ-H2AX (26, 44, 45). In yeast, an ATP-dependent chromatin-remodeling complex, INO80, was shown to interact with γ-H2AX (42). The absence of those proteins in the list of proteins we identified here suggests that the use of more cells for complex purification and employment of higher sensitivity mass spectrometry techniques for protein detection may lead to identification of additional H2AX-interacting partners. It should be pointed out that previous relevant studies usually used many more cells than used in this study. For example, 4.25 × 1010 cells were used to purify a histone deacetylase-dependent corepressor complex (46) in comparison with 8 × 108 cells used in the present study.

In the present study, to obtain soluble intact H2AX protein complexes, we used nuclease DNase I to cleave chromatin every 10 bp of DNA under mild conditions. To identify those proteins associated with intact nucleosomes, micrococcal nuclease can be used for the digestion. For the identification of the protein-protein interactions, many relevant studies used overexpression of the proteins of interest, which may affect the cell phenotypes. The tandem affinity purification approach was developed to isolate complexes in high purity, but it requires multiple steps of affinity purification to reduce contaminating proteins (47). The repetitive washings often result in loss of weak or transient protein interactions of biological relevance. In our dual tagging approach for complex compo-

Fig. 7. Ca2+ promotes the division of the irradiated cells. The cells in the exponential growth phase were mock- or γ-ray-irradiated (8 or 30 Gy), and immediately equal numbers of cells were replated in 60-mm plates. CaCl2 was added to the culture medium to the indicated concentrations, and the cells were grown at 37 °C for 2 days. Viable cells were counted using trypan blue exclusion assay. Note that increasing extracellular Ca2+ concentration of the irradiated cells resulted in increased viable cell numbers.
Dynamics of H2AX Complex during DNA Repair

In summary, using DNA nuclease digestion of chromatin and a quantitative dual tagging proteomic system we identified several proteins that have been shown to interact with members of the histone family, including H2B, CaM, two components of FACT, BIP, calreticulin, and nucleophosmin/B23. We also identified several novel binding partners of H2AX under the defined conditions. That is, we showed that DNA repair proteins PARP-1 and DEAH box polypeptide 30 isoform 1 interacted with H2AX when cells were exposed to γ-ray and allowed to recover for a relatively short period of time (e.g. 30 min). More importantly, we demonstrated that the H2AX protein complex undergoes dynamic changes upon induction of DNA damage and during DNA repair. Finally we characterized the biological functions for part of the identified proteins, i.e. the Ca2+/CaM-binding/signaling proteins. The results demonstrated that Ca2+/CaM played important roles in regulating IR-induced cell cycle arrest. Our data support the hypothesis that chromatin structure may play crucial roles in DNA repair and checkpoint signaling (39–43).

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