Valosin-Containing Protein Phosphorylation at Ser\textsuperscript{784} in Response to DNA Damage

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Abstract

The response of eukaryotic cells to DNA damage includes the activation of phosphatidylinositol-3 kinase–related kinases (PIKK), such as ATM, ATR, and DNA-dependent protein kinase (DNA-PK). These three kinases have very similar substrate specificities \textit{in vitro}, but \textit{in vivo}, their substrates overlap only partially. Several \textit{in vivo} substrates of ATM and ATR have been identified and almost all of them are involved in DNA damage–induced cell cycle arrest and/or apoptosis. In contrast, few \textit{in vivo} substrates of DNA-PK have been identified. These include histone H2AX and DNA-PK itself. We identify here valosin-containing protein (VCP) as a novel substrate of DNA-PK and other PIKK family members. VCP is phosphorylated at Ser\textsuperscript{784} within its COOH terminus, a region previously shown to target VCP to specific intracellular compartments. Furthermore, VCP phosphorylated at Ser\textsuperscript{784} accumulated at sites of DNA double-strand breaks (DSBs). VCP is a protein chaperone that unfolds and translocates proteins. Its phosphorylation in response to DNA damage and its recruitment to sites of DNA DSBs could indicate a role of VCP in DNA repair. (Cancer Res 2005; 65(17): 7533-40)

Introduction

The DNA damage checkpoint is an evolutionarily conserved signaling pathway that in response to DNA damage triggers cell cycle arrest and/or apoptosis (1). The key transducers of the DNA damage signal are the protein kinases ATM and ATR, which are activated primarily by DNA double-strand breaks (DSBs) and replication blocks, respectively (2, 3). ATM and ATR are members of the phosphatidylinositol-3 kinase–related kinase (PIKK) family and phosphorylate serine or threonine residues that are followed by an S/T-Q motif in response to DNA damage. Several \textit{in vivo} substrates of ATM and ATR have been identified, including Chk2, Chk1, p53, SMC1, histone H2AX, NBS1, BRCA1, and FANCD2 (1–4). Most of these proteins are phosphorylated by ATM and ATR at multiple sites that typically cluster near each other. For example, Chk2 has 7 S/T-Q sites within its NH\textsubscript{2} terminus. Of these seven sites, Thr\textsuperscript{68} is the predominant site phosphorylated by ATM, whereas other sites, such as Ser\textsuperscript{33}, are phosphorylated by ATM only when cells are exposed to high doses of ionizing radiation (5, 6).

DNA-dependent protein kinase (DNA-PK) is a third member of the PIKK family (4). Unlike ATM and ATR, inactivation of DNA-PK does not lead to major cell cycle checkpoint defects but instead compromises nonhomologous end joining (NHEJ)–mediated repair of DNA DSBs (7). DNA-PK phosphorylates many proteins \textit{in vitro}, but few \textit{in vivo} substrates have been identified. These include histone H2AX, the Werner syndrome helicase, Artemis, XRCC4, and DNA-PK itself (8–11). DNA-PK autophosphorylation, especially at Thr\textsuperscript{2056}, is important for DNA-PK to stimulate NHEJ (7, 8). A protein recently proposed to play a role in repair of DNA damage is valosin-containing protein (VCP), a 97-kDa homologue of yeast Cdc48p (12–14). VCP is a ubiquitous and highly abundant ATPase that belongs to the AAA (ATPase associated with a variety of cellular activities) family and assembles as a hexamer forming a ring with a channel at its center (15–17). The VCP homologues associate with a number of protein cofactors forming distinct macromolecular complexes, which act as chaperones unfolding target proteins and translocating them to specific cellular compartments or to the proteasome (13). Because VCP can participate in several macromolecular complexes and can act as a chaperone of many proteins, it is involved in many unrelated cellular activities, such as membrane fusion, cell cycle regulation, stress response, programmed cell death, B- and T-cell activation, transcriptional regulation, endoplasmic reticulum (ER)–associated degradation, and protein degradation (13). VCP has also been proposed to play a role in the DNA damage response, because it can associate with the Werner syndrome protein, a member of the RecQ helicase family (18, 19), as well as with BRCA1 (20). However, its precise role in the response of cells to DNA damage is still obscure.

In an effort to identify novel proteins that become phosphorylated at S/T-Q motifs in response to DNA damage, we isolated proteins that cross-react with an antibody raised against a synthetic Chk2 peptide, in which Thr\textsuperscript{26} and Ser\textsuperscript{28} of Chk2 were phosphorylated. Surprisingly, the predominant protein recognized by this antibody in cells exposed to DNA-damaging agents was VCP. We mapped the site of VCP phosphorylation at Ser\textsuperscript{784} and showed that VCP was phosphorylated by multiple PIKK family members. These results suggest that VCP is a direct target of DNA damage signaling pathways in mammalian cells.

Materials and Methods

Antibodies. The phospho-Chk2 (Thr\textsuperscript{26}/Ser\textsuperscript{28}) antibody was produced as previously described (21) using as antigen a synthetic keyhole limpet

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hemocyanin–coupled Chk2 phosphopeptide of the sequence QPHGSV-tQsQGSS, where t and s mark the phosphorylated threonine and serine, respectively. Immunoglobulins from the immunized rabbits were precipitated by Protein A-Sepharose; phospho-Chk2-reactive antibodies were then affinity purified first by removing antibodies that recognize nonphosphorylated Chk2 and then by selecting for antibodies that recognize phosphorylated Chk2 using columns carrying immobilized nonphosphorylated and phosphorylated Chk2 peptides, respectively. The specificity of the affinity-purified antibodies was confirmed using extracts prepared from untreated and DNA damage–treated cells expressing FLAG-tagged wild-type Chk2 or Chk2 mutated at Thr26/Ser28 (22). All other antibodies used in this study were commercially available or were previously described: VCP monoclonal, Maine Biotechnology Services, Inc., Portland, ME (MAB696S); DNA-PKcs rabbit polyclonal, Abcam, Cambridge, United Kingdom (ab230); 53BP1 monoclonal (23); and histone H3 rabbit polyclonal, Abcam (ab1791).

Cell lines. The following human cell lines were obtained from the American Type Culture Collection (Manassas, VA): HeLa cervical carcinoma, U2OS osteosarcoma, 293 embryonic kidney cells, HCT15 colon carcinoma, M059K (wild-type DNA-PK), and M059J (DNA-PK deficient) glioblastomas. AG1522 normal human primary fibroblasts and AT5BI carcinoma, M059K (wild-type DNAPK), and M059J (DNA-PK deficient) glioblastomas. AG1522 normal human primary fibroblasts and AT5BI primary fibroblasts from a patient with ataxia-telangiectasia have been described previously (24).

Induction of DNA damage. DNA damage was induced by exposing cells to ionizing radiation (137Cs source), UV light (254 nm; Stratalinker, Cambridge, United Kingdom) or hydroxyurea (1 mmol/L for 8 hours; Sigma, #H8627).

Cell extracts, immunoprecipitation, and immunoblotting. Whole cell protein extracts were prepared from HeLa, 293, M059K, and M059J cells by lysis in buffer containing 50 mmol/L HEPES (pH 7.55), 150 mmol/L NaCl, 1 mmol/L EDTA, 5% glycerol, 1% NP40, 1 mmol/L DTT, 1 mmol/L sodium vanadate, and a protease inhibitor cocktail for 10 minutes on ice followed by centrifugation to remove the particulate material. To prepare chromatin-enriched fractions and matched whole cell extracts, the cells were lysed in buffer consisting of 100 mmol/L HEPES, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT, 1.5 mmol/L phenylmethylsulfonyl fluoride, and 0.25 N HCl for 1 hour at 4°C. After being solubilized, the chromatin-enriched fraction was clarified by centrifugation and neutralized by adding one-fifth volume 1.5 mol/L Tris-HCl (pH 8.8). Nuclear extracts were prepared from U2OS cells as previously described (25). Immunoprecipitations were done using 800 μg whole cell extract, 0.4 μg phospho-Chk2 (Thr26/Ser28) antibody and 50 μL Protein G-Agarose beads. For immunoblotting either half of the immunoprecipitated reaction or whole cell extracts (100 μg) or nuclear extracts (25 μg) or chromatin-enriched fractions (15 μg) and matched whole cell extracts (1.5 μg) were resolved on SDS-polyacrylamide gels.

Mass spectrometry. Mass spectrometry (MS) was done using as starting material 800 μg HeLa whole cell extract immunoprecipitated with the phospho-Chk2 (Thr26/Ser28) antibody, as described above, or 3 mg U2OS nuclear extract immunoprecipitated with 10 μg phospho-Chk2 (Thr26/Ser28) antibody that had been covalently coupled to epoxy-dynabeads (Dynal M-270 Epoxy 143.01) according to the instructions of the manufacturer. The immunoprecipitated proteins were resolved on SDS-polyacrylamide gels, stained with colloidal Coomassie blue, and destained with 1% acetic acid and 30% methanol. The bands corresponding to proteins immunoprecipitated from HeLa cells were excised and cut in half; one half was subjected to
in-gel proteolytic digestion with trypsin and the other half with chymotrypsin to maximize coverage of the protein by recovered peptides. After quenching the digestions by lowering the pH to 2 to 3 with acetic acid, the two portions of each band were mixed back together and analyzed by liquid chromatography-coupled tandem MS (LC/MS/MS). The bands corresponding to proteins immunoprecipitated from U2OS cells were treated similarly, except that they were digested only with trypsin. Data-dependent LC/MS/MS was done using electrospray ionization on a Finnigan LCQ ion trap mass spectrometer. An aliquot of each digest mixture was introduced to the mass spectrometer by reversed-phase chromatographic separation with a 75-μm-inner-diameter capillary column flowing at a rate of 350 nL/min and eluted using a 60-minute acetonitrile/0.1% acetic acid gradient. Chromatographic separation yielded 30-second peak widths and mass spectra were acquired in 9-second cycles. Each cycle was of the form: one full MS scan followed by four MS/MS scans on the most abundant precursor ions, subject to dynamic exclusion for a period of 1.5 minutes. The identity of each peptide sequenced was determined by interpreting the MS/MS spectra using the SpectrumMill software we have developed (Agilent Technologies, Inc., Santa Clara, CA). Phosphorylated peptides were not detected in this data-dependent mode of operation. To establish the VCP phosphorylation site Ser784, a second LC/MS/MS run was done with the instrument operated in a multiple-reaction monitoring mode where MS/MS of the precursor m/z values 1,160.6 and 1,120.6 were repetitively taken throughout the acetonitrile gradient. These masses were selected because they correspond to the unphosphorylated and phosphorylated forms, respectively, of the chymotryptic VCP peptide RFPSGNQGGAGPSQGSGGGTGGSVY, which contains the region of VCP most similar to the peptide used to raise the phospho-Chk2 (Thr26/Ser28) antibody.

Valosin-containing protein cDNA cloning and plasmid construction. Human VCP cDNA was amplified by PCR from a fetal brain library (Clontech, Palo Alto, CA) and sub cloned in the pCMV-Tag2B vector (Stratagene). Ser 784 was mutated to alanine using the Stratagene QuickChange mutagenesis kit.

Cell transfection and in vitro DNA-PK kinase reaction. Plasmids expressing FLAG-tagged wild-type or Ser 784Ala (S784A) VCP were transiently transfected into exponentially growing 293 cells using Effectene (Qiagen, Inc., Chatsworth, CA). Forty-eight hours after transfection, the medium was replaced with fresh growth medium supplemented with 40 μg/mL bleomycin. Cell lysates were immunoprecipitated overnight with anti-FLAG M2 monoclonal antibody coupled to agarose beads (Sigma, #A-1205). After washing, the beads were resuspended in 150 μL whole cell lysis buffer without sodium vanadate. Then, one third of the beads was saved as untreated control; one third was treated with 5 μL calf intestinal phosphatase (CIP; New England Biolabs, Beverly, MA, #M-0290L); and the other third was treated with

Figure 2. VCP is phosphorylated at Ser784 in doxorubicin-treated HeLa cells. A, ion trap LC/MS/MS spectrum of a precursor m/z 1,160.6 VCP chymotryptic peptide. The sequence of the precursor peptide is shown below the LC/MS/MS spectrum (black letters) and potential cleavage points generated during the MS/MS scans. The precursor mass is consistent with one phosphate in the peptide and the b14/y11 and b11/y14 ions enable the conclusive assignment of Ser784 (Ser784 in the full-length sequence) as the phosphorylated residue. Several ions in the spectrum exhibit the neutral loss of phosphoric acid characteristic of phospho-serine and threonine residues. B, the phospho-Chk2 (Thr79/Ser79) antibody recognizes VCP phosphorylated at Ser784. 293 cells were transfected with plasmids expressing FLAG (FL)-tagged wt or Ser784Ala (S784A) VCP and treated with bleomycin. FLAG-tagged proteins were immunoprecipitated with an antibody that recognizes the FLAG tag and either not treated or treated with phosphatase (PPase) or sequentially with phosphatase and then with DNA-PK, as indicated. The immunoprecipitated proteins were then immunoblotted (IB) with the phospho-Chk2 (Thr79/Ser79) antibody (pT26S28) or with the antibody that recognizes the FLAG tag. C, alignment of the sequence of the synthetic peptide used to generate the phospho-Chk2 (Thr79/Ser79) antibody to the sequence of human VCP surrounding Ser784. Phosphorylated serine and threonine residues (lowercase letters).
CIP, washed, and then incubated with 1 µl DNA-PK (Promega, Madison, WI, #V581A). Proteins bound to the beads were resolved by SDS-PAGE and immunoblotted, as described above.

Small interference RNA transfections. U2OS cells were transfected with luciferase small interference RNA (siRNA) oligonucleotides or siRNA specific for 53BP1 (Dharmacon, Lafayette, CO), as previously described (25). The sequence of the siRNA for 53BP1 was GAACGAGGAGACG-GUAUAADdT.

Immunofluorescence. Immunofluorescence was done as described previously (25). All immunofluorescence images were processed using the Imagevision Tools Library of IRIX (Silicon Graphics, Mountain View, CA).

Results

The phospho-Chk2 (Thr²⁶/Ser²⁸) antibody recognizes valosin-containing protein in cells with DNA damage. Immunoblotting of whole cell lysates from doxorubicin-treated HeLa cells, using the phospho-Chk2 (Thr²⁶/Ser²⁸) antibody, revealed at least four proteins, whose phosphorylation was induced by DNA damage (Fig. 1A, left). The major protein recognized by the phosphospecific antibody had a molecular weight of 97 kDa, so it could not be Chk2. This 97-kDa protein was the only protein detected in lysates of doxorubicin-treated cells by sequential immunoprecipitation and immunoblotting using the phospho-Chk2 (Thr²⁶/Ser²⁸) antibody (Fig. 1A, middle) and practically the only protein detected by sequential immunoprecipitation and staining of the gel with colloidal Coomassie blue (Fig. 1A, right). To determine its identity, a gel slice containing this protein was treated with proteases and the generated peptides were subjected to LC/MS/MS. The 97-kDa protein was unambiguously identified as the p97 VCP. In addition, two very faint protein bands from the same gel were identified as filamin (f 280 kDa) and the catalytic subunit of DNA-PK (f 450 kDa; Fig. 1A, right).

The identity of VCP and DNA-PK as proteins recognized by the phospho-Chk2 (Thr²⁶/Ser²⁸) antibody in doxorubicin-treated HeLa cells was further verified by sequential immunoprecipitation with the phospho-Chk2 (Thr²⁶/Ser²⁸) antibody and immunoblotting with antibodies specific for VCP or DNA-PK (Fig. 1B). These experiments further indicated that doxorubicin treatment did not affect the overall protein levels of VCP and DNA-PK in HeLa cells.

Having established that VCP is the major protein recognized by the phospho-Chk2 (Thr²⁶/Ser²⁸) antibody in doxorubicin-treated HeLa cells, we subsequently examined U2OS osteosarcoma cells exposed to ionizing radiation, hydroxyurea, or UV light. Immunoblotting of nuclear extracts from these cells revealed a 97-kDa molecular weight protein that reacted with the phospho-Chk2 (Thr²⁶/Ser²⁸) antibody in response to DNA damage (Fig. 1C, left). We then used this same antibody for protein immunoprecipitation and identification of the immunoprecipitated proteins by MS. A single protein was immunoprecipitated from extracts of U2OS cells exposed to ionizing radiation; this protein was identified as VCP by MS (Fig. 1C, right). From extracts of cells exposed to UV light, several proteins were immunoprecipitated; one of these was VCP. The remaining were cytoskeletal proteins, such as gelsolin, drebrin E, α-actinin 4, and non–muscle myosin heavy chain type A (Fig. 1C, right). These highly abundant cytoskeletal proteins are constitutively phosphorylated and have been previously described as contaminants in immunoprecipitations with phosphospecific antibodies (26). Thus, based on the analysis of HeLa and U2OS cells described above, we conclude that VCP is phosphorylated in response to several DNA-damaging agents and is the predominant protein recognized by the phospho-Chk2 (Thr²⁶/Ser²⁸) antibody. In support of this conclusion, immunoblotting of lysates prepared from multiple other cell lines (HT29, COS7, 293, WI38, MCF7, MCF7).

![Figure 3.](image-url)
HCT116, MCF10A, and HCT15) with the phospho-Chk2 (Thr 26/Ser 28) antibody revealed that the major protein phosphorylated in response to various DNA-damaging agents (doxorubicin, bleomycin, ionizing radiation, UV, and hydroxyurea) had a molecular weight of 97 kDa (data not shown).

Valosin-containing protein is phosphorylated at Ser 784 in response to DNA damage. To establish the site of VCP phosphorylation in response to DNA damage, VCP was immunoprecipitated with the phospho-Chk2 (Thr 26/Ser 28) antibody from doxorubicin-treated HeLa cells, digested with chymotrypsin, and then analyzed by LC/MS/MS. The instrument was operated in a multiple-reaction monitoring mode where MS/MS of the precursor m/z values 1,120.6 and 1,160.6 were repetitively taken throughout the acetonitrile gradient. These masses were selected because they correspond to the unphosphorylated and phosphorylated forms, respectively, of the chymotryptic peptide RFPSGNQQGAGPSQGSGGSGGGTGGSVY, which contains the region of VCP most similar to the peptide used to raise the phospho-Chk2 (Thr 26/Ser 28) antibody. Interpretation of the spectra of the precursor m/z 1,160.6 unambiguously revealed that Ser 784 was phosphorylated (Fig. 2A).

The identification of Ser 784 as a residue phosphorylated in response to DNA damage raises the possibility that the phospho-Chk2 (Thr 26/Ser 28) antibody recognizes VCP phosphorylated at Ser 784. However, it is formally possible that VCP might be phosphorylated at multiple sites in response to DNA damage, in which case a site other than Ser 784 might be recognized by the phospho-Chk2 (Thr 26/Ser 28) antibody. To establish that the phospho-Chk2 (Thr 26/Ser 28) antibody recognizes VCP phosphorylated at Ser 784, we examined whether the phospho-Chk2 (Thr 26/Ser 28) antibody would recognize a VCP mutant that had Ser 784 substituted with alanine (S784A). Human embryonic kidney 293 cells were transfected with wild-type (wt) or S784A mutant FLAG-tagged VCP, the cells were treated with the DNA-damaging agent bleomycin and then the FLAG-tagged VCP proteins were sequentially immunoprecipitated with an antibody that recognizes the FLAG tag and immunoblotted either with the antibody that recognizes the FLAG tag or with the phospho-Chk2 (Thr 26/Ser 28) antibody. The S784A VCP protein was not recognized by the phospho-Chk2 (Thr 26/Ser 28) antibody, whereas wt VCP was recognized (Fig. 2B). Thus, the phospho-Chk2 (Thr 26/Ser 28) antibody recognizes VCP phosphorylated at Ser 784, consistent with the similarity in amino acid sequence of VCP surrounding Ser 784 to the sequence of the Chk2 peptide used to generate the phospho-Chk2 (Thr 26/Ser 28) antibody (Fig. 2C).

**Figure 4.** Phospho-Chk2 (Thr 26/Ser 28) antibody (pT26S28) recognizes phosphorylated VCP at sites of DNA DSBs. A, colocalization of epitopes recognized by the phospho-Chk2 (Thr 26/Ser 28) antibody with 53BP1 foci in irradiated U2OS cells. Blue circles, computer-generated outlines of 4′,6-diamidino-2-phenylindole-stained nuclei. B, Chk2 is not the predominant protein recognized by the phospho-Chk2 (Thr 26/Ser 28) antibody by immunofluorescence. Foci recognized by the phospho-Chk2 (Thr 26/Ser 28) antibody develop normally in irradiated HCT15 colon carcinoma cells, although these cells express a mutant form of Chk2 that is not activated in response to irradiation. C, ATM dependence of phospho-Chk2 (Thr 26/Ser 28) antibody reactivity at early but not late time points after irradiation. Normal diploid fibroblasts (AG1522) and fibroblasts from a patient with ataxia-telangiectasia (AT5BI) were examined 30 minutes or 8 hours after irradiation. D, 53BP1 expression was suppressed by siRNA in U2OS cells. Only the one cell in the image that retains 53BP1 expression shows foci reactive with the phospho-Chk2 (Thr 26/Ser 28) antibody.
Ser\textsuperscript{784} matches the consensus site for phosphorylation by members of the PIKK family (ATM, ATR, and DNA-PK; ref. 4). To examine whether PIKKs can phosphorylate VCP at Ser\textsuperscript{784} in vitro, the immunoprecipitated FLAG-tagged wt and S784A VCP proteins were first treated with a phosphatase to dephosphorylate Ser\textsuperscript{784} and then with DNA-PK. DNA-PK phosphorylated wt VCP in vitro at Ser\textsuperscript{784} as ascertained by immunoblotting with the phospho-Chk2 (Thr\textsuperscript{26}/Ser\textsuperscript{28}) antibody, whereas no phosphorylation at Ser\textsuperscript{784} was evident on the S784A mutant, as expected (Fig. 2B). Thus, DNA-PK can phosphorylate VCP in vitro at Ser\textsuperscript{784}.

Multiple phosphatidylinositol-3 kinase–related kinases phosphorylate valosin-containing protein in vivo. The PIKK family members ATM, ATR, and DNA-PK have overlapping substrate specificities in vitro and often phosphorylate the same substrates in vivo (1–4). Yet, these kinases differ in terms of activation kinetics and the DNA-damaging agents they respond to. ATM and DNA-PK respond primarily to DNA DSBs and are activated rapidly after induction of the damage. ATR responds primarily to replication blocks induced by UV light or various chemical inhibitors and the kinetics of activation are slow, reflecting the time it takes for the replication machinery to encounter the damage (2, 3).

As a first step in examining which PIKK family members phosphorylate VCP in vivo, we studied VCP phosphorylation in M059J glioblastoma cells, which express mutant DNA-PK and ATM, and in M059K cells, which are derived from the same patient, but have wt DNA-PK and ATM (27). Both cell lines contained similar levels of VCP. After exposure to bleomycin, which causes DNA DSBs, VCP was phosphorylated at Ser\textsuperscript{784} in both cell lines. However, at the early time points (15 and 30 minutes) after bleomycin treatment, VCP phosphorylation was compromised in the M059J cells (Fig. 3A and B). The robust phosphorylation of VCP in M059K cells could be inhibited by wortmannin (Fig. 3A), a known inhibitor of PIKK family members (28). Taken together, these results suggest that DNA-PK and/or ATM phosphorylate VCP in vivo at early time points after DNA damage, whereas at later time points, another kinase, probably ATR, phosphorylates VCP.

A potential role of ATR in phosphorylating VCP at Ser\textsuperscript{784} was examined by exposing M059J and M059K cells to UV light. In both cell lines, VCP was phosphorylated at Ser\textsuperscript{784} in response to UV light (Fig. 3C). Based on the kinetics of VCP phosphorylation and the presence of mutant DNA-PK and ATM in M059J cells, we conclude that ATR is the likely kinase phosphorylating VCP in cells exposed to UV light.

Valosin-containing protein phosphorylated at Ser\textsuperscript{784} localizes at sites of DNA double-strand breaks. In cell extracts, the major protein recognized by the phospho-Chk2 (Thr\textsuperscript{26}/Ser\textsuperscript{28}) antibody after induction of DNA damage was VCP (Fig. 1). We used this same antibody to study by immunofluorescence the intracellular localization of VCP phosphorylated at Ser\textsuperscript{784}. In nonirradiated U2OS osteosarcoma cells, the immunofluorescence signal generated by the phospho-Chk2 (Thr\textsuperscript{26}/Ser\textsuperscript{28}) antibody was very weak, but after irradiation, the signal was intense and corresponded to foci that colocalized with the ionizing radiation–induced 53BP1 foci (Fig. 4A). These results suggested that VCP phosphorylated at Ser\textsuperscript{784} localizes at sites of DNA DSBs. Several additional observations support this conclusion.

First, we showed that the immunofluorescence signal was not due to phosphorylated Chk2. In HCT15 colon carcinoma cells, which express a mutant Chk2 protein that does not become phosphorylated in response to DNA damage (25, 29), the immunofluorescence signal was as robust as in U2OS cells (Fig. 4B), consistent with the phospho-Chk2 (Thr\textsuperscript{26}/Ser\textsuperscript{28}) antibody recognizing primarily phosphorylated VCP.

Second, under a variety of conditions, the immunofluorescence signal correlated well with VCP phosphorylation at Ser\textsuperscript{784} as monitored by immunoblotting. For example, by immunoblotting, VCP phosphorylation at Ser\textsuperscript{784} was dependent on ATM at early but not late time points after irradiation (Figs. 1 and 3; data not shown). Immunofluorescence analysis of irradiated and nonirradiated primary fibroblasts from a normal individual (AG1522) and from a patient with ataxia-telangiectasia (AT5BI) indicated reactivity with the phospho-Chk2 (Thr\textsuperscript{26}/Ser\textsuperscript{28}) antibody at both early (30 minutes) and late (8 hours) time points after irradiation in the normal fibroblasts but only at the late time point in ataxia-telangiectasia cells (Fig. 4C).

Third, phosphorylation of VCP, as phosphorylation of many ATM substrates at sites of DNA DSBs (6, 25), was dependent on 53BP1 (6, 25). After suppression of 53BP1 expression by siRNA, immunofluorescence analysis indicated that VCP phosphorylation was also suppressed (Fig. 4D).

The strongest evidence that VCP phosphorylated at Ser\textsuperscript{784} was present at sites of DNA DSBs came from immunoblotting analysis of chromatin-enriched fractions from untreated and irradiated U2OS cells (Fig. 5). Proteins that localize at sites of DNA DSBs are typically present in the nucleoplasm in untreated cells; but after DNA damage, a certain fraction of the protein is associated with chromatin. We therefore prepared chromatin-enriched fractions and matched whole cell extracts from nonirradiated and irradiated U2OS cells and monitored VCP subcellular localization and phosphorylation at Ser\textsuperscript{784} by immunoblotting (Fig. 5). VCP was present in the chromatin-enriched fraction after irradiation. Furthermore, the chromatin-enriched

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Figure 5. VCP phosphorylated at Ser\textsuperscript{784} is present in association with chromatin in irradiated cells. Whole cell extracts (WCE) and chromatin-enriched fractions (ChromF) were prepared from nonirradiated (0 Gy) or irradiated (10 or 20 Gy) U2OS cells 15 minutes after irradiation and immunoblotted with the phospho-Chk2 (Thr\textsuperscript{26}/Ser\textsuperscript{28}) antibody or antibodies that recognize VCP or histone H3. Unlike the experiments shown in Figs. 1 and 3, phosphorylated VCP was not detected in the whole cell extracts in this experiment, because 70-fold less extract was used per lane.
fraction, whose quality was validated by immunoblotting for histone 
H3, contained the majority of VCP phosphorylated at Ser784. Taken 
together with the immunofluorescence analysis, these data 
suggest that VCP phosphorylated at Ser784 is present at sites of 
DNA DSBs.

Discussion

We have identified VCP as a new substrate of PIKK family 
members in cells exposed to DNA-damaging agents. Furthermore, 
we have mapped the site of phosphorylation as Ser784. These 
findings suggest a role of VCP in the cellular response to DNA 
damage and are consistent with previously published interactions 
of VCP with BRCA1 and the WRN helicase (18–20).

The precise role of VCP in the DNA damage response remains 
to be elucidated. VCP has multiple activities in eukaryotic cells (13). 
The common underlying theme for all these activities is the 
biochemical function of VCP as a chaperone that can unfold and 
refold proteins. Many of the activities of VCP involve interactions 
with polyubiquitinated proteins that VCP unfolds and delivers to 
the proteasome for degradation (30–33). Other activities of VCP 
involve unfolding of mono- or nonubiquitinated proteins, which are 
than delivered to specific subcellular compartments, such as the 
ER. By analogy, the role of VCP in the DNA damage response may 
involve unfolding and removing ubiquitinated proteins from sites 
of DNA damage. Indeed, the response of cells to DNA damage 
Involves several ubiquitination events. Post-replication repair 
is mediated by the ubiquitin ligases Rad5 and Rad18, which 
ubiquitinate proliferating cell nuclear antigen (34–36), whereas 
the response of cells to DNA inter-strand-cross-links involves 
ubiquitination of FANCD2 (37). Furthermore, the DNA damage 
checkpoint protein BRCA1, which has been reported to interact 
with VCP (20), is a ubiquitin ligase, although its physiologic 
substrates have not been identified (38, 39). Thus, it is possible 
that VCP functions together with ubiquitin ligases at sites of DNA 
damage. The ubiquitin ligases might modify proteins that VCP 
would subsequently unfold and channel away from the site of DNA 
damage.

According to this model, the significance of VCP phosphorylation 
at Ser784 might be to target VCP at sites of DNA breaks. The 
specific cellular activity in which a specific VCP molecule 
participates depends in part on its intracellular localization, which 
in turn is regulated by COOH-terminal posttranslational 
modifications (40, 41). For example, phosphorylation of Tyr405 
targets VCP to the ER (40). By analogy, phosphorylation of Ser 
784 may target VCP to sites of DNA damage.

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analysis of the proteins that were immunoprecipitated from U2OS cell extracts.

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