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Coregulated Ataxia Telangiectasia-mutated and Casein Kinase Sites Modulate cAMP-response Element-binding Protein-Coactivator Interactions in Response to DNA Damage*

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Naval P. Shanware, Anthony T. Trinh, Leah M. Williams, and Randal S. Tibbetts[1]

From the Molecular and Cellular Pharmacology Program, Department of Pharmacology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin 53706

The cyclic AMP-response element-binding protein (CREB) is a bZIP family transcription factor implicated as an oncoprotein and neuron survival factor. CREB is activated in response to cellular stimuli, including cAMP and Ca^{2+}, via phosphorylation of Ser-133, which promotes interaction between the kinase-inducible domain (KID) of CREB and the KID-interacting domain of CREB-binding protein (CBP). We previously demonstrated that the interaction between CREB and CBP is inhibited by DNA-damaging stimuli through a mechanism whereby CREB is phosphorylated by the ataxia telangiectasia-mutated (ATM) protein kinase. We now show that the ATM phosphorylation sites in CREB are functionally intertwined with a cluster of coregulated casein kinase (CK) sites. We demonstrate that DNA damage-induced phosphorylation of CREB occurs in three steps. The initial event in the CREB phosphorylation cascade is the phosphorylation of Ser-111, which is carried out by CK1 and CK2 under basal conditions and by ATM in response to ionizing radiation. The phosphorylation of Ser-111 triggers the CK2-dependent phosphorylation of Ser-108 and the CK1-dependent phosphorylation of Ser-114 and Ser-117. The phosphorylation of Ser-114 and Ser-117 by CK1 then renders CREB permissive for ATM-dependent phosphorylation on Ser-121. Mutation of Ser-121 alone abrogates ionizing radiation-dependent repression of CREB-CBP complexes, which can be recapitulated using a CK1 inhibitor. Our findings outline a complex mechanism of CREB phosphorylation in which coregulated ATM and CK sites control CREB transactivation potential by modulating its CBP-binding affinity. The coregulated ATM and CK sites identified in CREB may constitute a signaling motif that is common to other DNA damage-regulated substrates.

The cAMP response element-binding protein (CREB)[2] is a phosphorylation-dependent transcription factor that plays key roles in cell proliferation, homeostasis, and survival (1). Consistent with an important role of this protein in transcriptional control, a recent whole-genome chromatin immunoprecipitation procedure identified ~6000 different genomic loci that are occupied by CREB *in vivo* (2). *In silico* approaches have similarly identified thousands of candidate CREB target genes (3). These include a large number of genes with annotated functions in the nervous system, which is consistent with critical roles for CREB in memory, circadian rhythm entrainment, and neuron survival (4). The current compendium of CREB target genes also includes a significant number of anti-apoptosis factors, cell cycle regulators, and DNA repair enzymes, suggestive of a role for CREB in the response to DNA damage (2, 3). This role, however, remains uncharacterized.

The transactivation potential of CREB is enhanced by stimuli, including cAMP and Ca^{2+} that induce its phosphorylation on Ser-133, which lies within a phosphorylation site-rich region spanning amino acids 100–160 called the kinase-inducible domain (KID) (1). Phosphorylation of Ser-133 promotes an interaction between the KID and the KIX domain of the transcriptional coactivator CREB-binding protein (CBP) (5, 6). Relevant CREB Ser-133 kinases include the cAMP-dependent protein kinase A, calmodulin kinases (CaMKs), AKT, and the stress- and mitogen-activated kinases RSK1, MSK1, and MSK2 (7–14). The fact that several distinct signaling pathways converge at the point of Ser-133 phosphorylation suggests that modulation of CREB-dependent transcriptional programs is a common homeostatic response to changes in the extracellular environment. Although Ser-133 phosphorylation status is a key determinant of CREB transcriptional activity, in some instances phosphorylation of this residue does not correlate with CBP binding activity or transactivation potential (15). This phenomenon may reflect modulatory effects of other post-translational modifications on the CREB-CBP interaction. In addition, CREB can be activated through CBP-independent mechanisms. For

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[1] To whom correspondence should be addressed: Dept. of Pharmacology, University of Wisconsin School of Medicine and Public Health, 1300 University Ave., Madison, WI 53706. Tel.: 608-262-0027; E-mail: rstibbetts@wisc.edu.

[2] The abbreviations used are: CREB, cyclic AMP-response element-binding protein; ATM, ataxia telangiectasia-mutated; CK, casein kinase; RAX, coregulated ATM and casein kinase sites; IR, ionizing radiation; Gy, gray; CBP, CREB-binding protein; KID, kinase-inducible domain; ATR, ATM and Rad3-related protein; CaMK, calmodulin kinase; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; TBB, 4,5,6-tetrabromobenzotriazole.

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example, a family of proteins designated TORCs (transducers of regulated CREB activity) bind to the carboxyl terminus of CREB and strongly activate its transcriptional activity in response to glucogenic stimuli (16–19). Other phosphorylation-independent CREB activators have also been identified, including Oct-1 and members of the 4½ LIM domain family of proteins (20, 21).

The KID of CREB contains consensus phosphorylation sites for several additional protein kinases (22). CaMKII and CaMKIV phosphorylate CREB on Ser-142, which is implicated as a negative regulatory site that modulates CREB-dependent circadian rhythm entrainment *in vivo* (11, 12, 23, 24). The KID also contains consensus casein kinase 1 (CK1) and casein kinase 2 (CK2) sites at Ser-108, Ser-111, Ser-114, and Ser-117 (22). CK1 and CK2 are two unrelated, constitutively active protein kinases that participate in a wide variety of cellular processes, including DNA repair, cell cycle control, and circadian rhythm entrainment (25–27). To date, hundreds of *in vitro* substrates for these kinases have been identified (25, 26). CK1 and CK2 preferentially phosphorylate Ser residues flanked by Asp, Glu, or phosphorylated Ser residues in the +3 or −3 position, respectively (25, 26). This property of the enzymes often leads to the processive phosphorylation of substrates harboring clusters of S*XX*S motifs. In mammalian CREB, the CK1/CK2 sites are spaced such that phosphorylation of Ser-108, Ser-111, Ser-114, or Ser-117 could trigger the processive phosphorylation of the remaining sites. The CK1/CK2 sites are conserved in *Drosophila* CREB, where they have been reported to negatively regulate DNA binding activity (28). However, although CK1/CK2 site mutations alter the electrophoretic mobility of CREB, suggestive of phosphorylation (29), definitive evidence that mammalian CREB is phosphorylated on the CK1/CK2 sites *in vivo* is still lacking, and the precise biochemical functions of these residues remain unknown.

Previous work from our laboratory demonstrated that CREB is a phosphorylation target of the ataxia telangiectasia-mutated (ATM) protein kinase. ATM is a DNA damage-activated protein kinase that plays critical roles in cell cycle checkpoint activation, DNA repair, and regulation of apoptosis in response to genotoxic stimuli (30). CREB contains consensus ATM sites at Thr-100, Ser-111, and Ser-121 within the KID, and by using a phospho-specific antibody, we demonstrated that ATM directly phosphorylates CREB on Ser-121 in response to ionizing radiation (IR), H_2O_2, and other genotoxic agents (31, 32). The IR-dependent phosphorylation of CREB inhibited the interaction between CREB and CBP in an ATM phosphorylation site-dependent manner, suggesting that ATM antagonizes CREB transcriptional activity in response to genotoxic stress. These findings are also consistent with the idea that CREB may be a key determinant of the transcriptional response to DNA damage.

Their close proximity suggests that the CK1/CK2 and ATM sites may collaborate to modulate CREB function. In this report we provide definitive evidence that the CK1/CK2 sites are phosphorylated in intact cells and show that CK1 is a critical mediator of DNA damage-dependent CREB phosphorylation and regulation. We outline a complex phosphorylation cascade whereby CK1-mediated phosphorylation events license CREB for ATM-dependent phosphorylation on Ser-121, which switches CREB into a low affinity CBP-binding state. These findings provide new insights into the biochemical mechanisms of CREB regulation and suggest that, under some circumstances, CK1 and CK2 function as amplifiers of ATM substrate phosphorylation in response to DNA damage.

EXPERIMENTAL PROCEDURES

Cell Culture and Antisera—HEK 293T cells were maintained in Eagle's minimum essential medium containing 5% fetal calf serum. The α-pCREB-108/111/114 antibody was generated by immunizing rabbits with a triply phosphorylated CREB peptide (CIAEpSEDpSQEpSVD) (Cocalico Biologicals, Reamstown, PA). The peptide was synthesized in collaboration with Dr. Gary Case at the University of Wisconsin Biotechnology Center. The resulting antisera were affinity-purified using the CREB phosphopeptide coupled to Sulfo-Link beads (Pierce) according to the manufacturer's conditions. After three passages (6 ml of raw serum) over phosphopeptide-conjugated resin and washing with PBS, the bound antibodies were eluted with 200 mM glycine (pH 2.50) in 1000-μl fractions and immediately neutralized with 50 μl of 1 M Tris-HCl (pH 8.00). A pool of antibody-containing fractions (~4 ml) was dialyzed against PBS and stored in PBS containing 50% glycerol at −20 °C. The α-pCREB-108/111/114 antibody was used at a concentration of 2 μg/ml for immunoblotting. Other antibodies used in this study were used at a concentration of 1 μg/ml. These include α-ATM (Genetex), α-pATM-1981 (Rockland), α-CREB (Cell Signaling), α-pCREB-121 (Novus Biologicals), α-pCREB-133 and α-tubulin (Upstate), and α-FLAG (Sigma). The CK1 inhibitor D4476 and the CK2 inhibitor TBB were obtained from EMD Biosciences. D4476 and TBB were added 3 h prior to application of DNA damage and were used at concentrations of 75 and 50 μM, respectively. The ATM inhibitor, KU-55933 (33), was prepared in Me_2SO and added to a final concentration of 10 μM 1 h prior to cell irradiation.

Plasmids and Transfections—Transfections were performed using the calcium phosphate DNA precipitation procedure. Cells were harvested 36 h later and extracts prepared as described previously (34). Seventy five μg of total protein was separated on 10% SDS-polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Membranes were blocked in Tris-buffered saline (TBS) containing 0.2% Tween 20 (TBS-T) and 5% dried milk and incubated overnight at 4 °C with the indicated primary antibodies diluted in blocking solution. After washing, the blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse or goat anti-rabbit secondary antibodies (Jackson ImmunoResearch) and developed using SuperSignal chemiluminescent substrate (Pierce). Where indicated, band pixel intensities were determined using the GelPlot2 function of ScionImage (Scion Corp.). Values were normalized for lane-to-lane differences in total CREB band intensity. The glutathione *S*-transferase (GST)-KIX pulldown assays were performed as described (35). Site-directed mutagenesis was performed using the QuickChange method (Stratagene) and the following oligonucleotide primer pairs: $CREB^{108A}$ (5′-CAGATTTC-AACTATTGCAGAAGCTGAAGATTCACAGG-3′ (forward)

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and 5′-CCTGTGAATCTTCAGCTTCTGCAATAGTTGAAATCTG-3′ (reverse)); CREB^{111A} (5′-CCACTGACTCCTCTGCATCTTCACTTTCTGC-3′ (forward) and 5′-GCAGAAAGTGAAGATGCACAGGAGTCAGTGG-3′ (reverse)); CREB^{114A} (5′-GAAGATTCACAGGAGGCAGTGGATAGTGTAACTGATTCC-3′ (forward) and 5′-GGAATCAGTTACACTATCCACTGCCTCCTGTGAATCTTC-3′ (reverse)); CREB^{117A} (5′-GGAGTCAGTGGATGCTGTAACTGATTCCC-3′ (forward) and 5′-GGGAATCAGTTACAGCATCCACTGACTCC (reverse)); CREB^{121A} (5′-GGATAGTGTAACTGATGCCCAAAAGCGAAGGG-3′ (forward) and 5′-CCCTTCGCTTTTGGGCATCAGTTACACTATCC-3′ (reverse)); and CREB^{112D} (5′-GCAGAAAGTGAAGATTCAGACGAGTCAGTGG-3′ (forward) and 5′-CCACTGACTCGTCTGAATCTTCACTTTCTGC-3′(reverse)). The template for mutagenesis was a full-length CREB cDNA in the vector pCMV-pSPORT6 (31). Where indicated, we also employed a FLAG-CREB expression construct that was generated by PCR cloning the CREB open reading frame, encoding an amino-terminal FLAG tag, into the NheI-NotI sites of pcDNA3.1-Zeo (Invitrogen). Primers used were 5′-GCTAGCCATGGACTACAAAGACGATGACGACAAGACCATGGAATCTGGAGCCGAGAAC-3′ (forward) and 5′-GCGGCCGCTTAATCTGATTTGTGGCAGTAAAG-3′ (reverse). All oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). To observe subtle DNA damage-dependent changes in CREB electrophoretic mobility (Fig. 2*B*), we resolved whole-cell extracts on 24-cm, 10% SDS-polyacrylamide gels. Each gel was electrophoresed at 40 mA for 12 h under cooling conditions.

Kinase Assays—CK1 and CK2 kinase assays were performed using purified, recombinant CK1 δ and CK2 α/β enzymes (New England Biolabs) according to the manufacturer's conditions. Two micrograms of purified, His_6-tagged CREB (His-CREB) were used for each assay (31). His-CREB was incubated with 0.4 μl of CK1, CK2, or both enzymes in the presence of 200 μM ATP in a total reaction volume of 25 μl. Reactions were performed for 1 h at 30 °C in the absence or presence of D4476 or TBB, and terminated by the addition of 4× SDS sample loading buffer. Phosphorylation of CREB was monitored by immunoblotting with α-pCREB-108/111/114 and α-CREB antibodies.

RESULTS

Effects of CK1 and CK2 Inhibitors on DNA Damage-induced CREB Phosphorylation—We previously showed that ATM directly phosphorylates CREB on Ser-121 in response to DNA damage and provided circumstantial evidence that Ser-111 was also a DNA damage-inducible ATM phosphorylation site (31). Interestingly, an S111A mutation strongly suppressed IR-induced phosphorylation of Ser-121, suggesting that CREB is processively phosphorylated in response to DNA damage (31). Ser-111 is also a consensus CK1/CK2 site, and examination of the CREB amino acid sequence reveals the possibility of linked phosphorylation between Ser-111, Ser-108, Ser-114, and Ser-117 (Fig. 1*A*). Specifically, the phosphorylation of Ser-111 creates a consensus CK2 site at Ser-108 and consensus CK1 sites at Ser-114 and Ser-117. A subset of the candidate ATM and CK1/CK2 sites are also present in the KID of *Drosophila* CREB, suggesting potentially conserved functions.

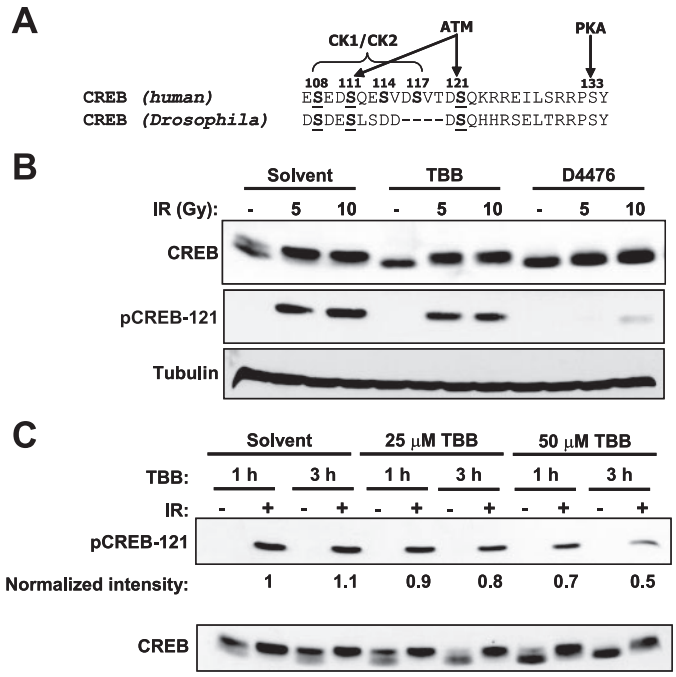

FIGURE 1. **ATM-dependent phosphorylation of CREB on Ser-121 requires CK1.** *A*, CK1/CK2 and ATM phosphorylation sites are positionally conserved between human and *Drosophila* CREB (dCREB). ATM and CK1/CK2 sites in human CREB are shown in *boldface*, and those positionally conserved in dCREB are *underlined* with a *heavy line*. *B*, effects of CK1 and CK2 inhibitors on CREB phosphorylation. HEK 293T cells were exposed to 50 μM TBB (a CK2 inhibitor) or 75 μM D4476 (a CK1 inhibitor) for 3 h prior before exposure to 5 or 10 Gy IR. The irradiated cells were harvested 30 min later and processed for immunoblotting with α-pCREB-121, α-CREB, and α-tubulin antibodies. *C*, TBB dose response. HEK 293T cells were incubated with vehicle or 25 or 50 μM TBB for 1 or 3 h prior to irradiation (10 Gy). Cell extracts were immunoblotted for α-pCREB-121 and α-CREB. Phospho-Ser-121 band intensities were normalized for CREB loading and are presented at the *bottom* of the panel.

The juxtaposition of the ATM and CK1/CK2 sites led us to consider the potential roles of CK1 and CK2 in the DNA damage-induced phosphorylation of CREB. To this end we examined the effects of specific inhibitors of CK1 (D4476) (36) and CK2 (TBB) (37) on the IR-induced phosphorylation of CREB on Ser-121. HEK 293T cells were pretreated with vehicle, TBB, or D4476 for 3 h prior to exposure to 0, 5, or 10 Gy of IR. Under these conditions, TBB strongly suppressed the basal phosphorylation of CREB, as evidenced by a decrease in the relative abundance of the slow electrophoretic mobility form. However, TBB did not inhibit the IR-induced CREB electrophoretic mobility shift (Fig. 1*B*). In contrast, the CK1 inhibitor, D4476, abolished the IR-induced CREB electrophoretic mobility shift in addition to blocking basal CREB phosphorylation. In D4476-treated cells, the suppression of the IR-induced electrophoretic mobility shift was accompanied by a nearly complete defect in Ser-121 phosphorylation (Fig. 1*B*, *middle panel*). TBB also caused a modest but reproducible attenuation of Ser-121 phosphorylation in response to IR, which was further revealed in a dose-response experiment (Fig. 1*C*). These findings strongly suggest that CK1 and CK2 contribute to the IR- and ATM-dependent phosphorylation of CREB on Ser-121 and that CK1 activity is a critical determinant of Ser-121 phosphorylation status in DNA-damaged cells.

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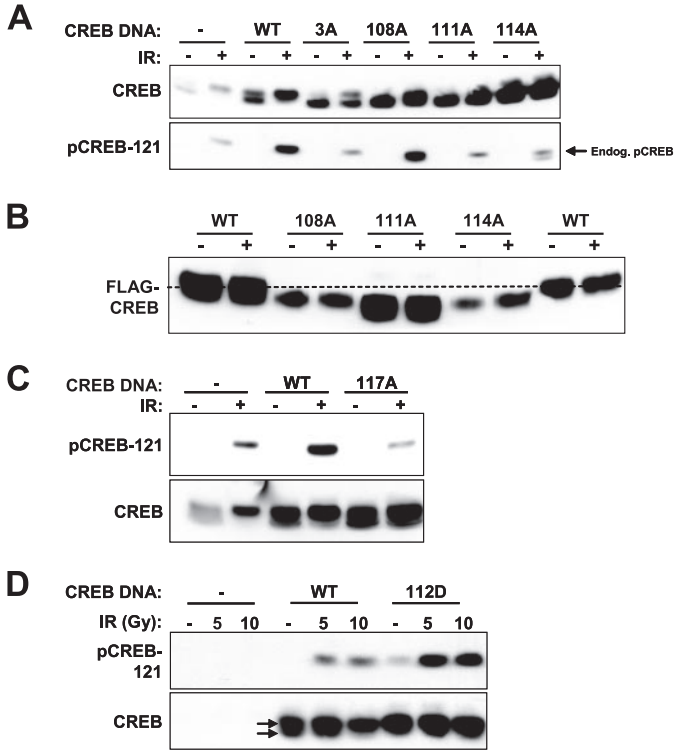

FIGURE 2. **Differential contribution of CK1 and CK2 sites to IR-induced CREB phosphorylation.** *A*, effects of ATM and CK1/CK2 site mutations on IR-induced CREB phosphorylation on Ser-121. HEK 293T cells were transfected with vector DNA (−) or plasmids encoding untagged wild-type CREB (*WT*), or CREB harboring Ser → Ala substitutions at the indicated amino acids. The $CREB^{3A}$ mutant contains Ala substitutions at all three consensus ATM sites (Thr-100, Ser-111, and Ser-121). Cell extracts were prepared from unirradiated or IR-treated cells (30 Gy, 1 h) and analyzed by immunoblotting with α-pCREB-121 and α-CREB antibodies. Low levels of endogenous CREB are also detected in both the α-CREB and α-pCREB-121 blots. The endogenous phospho-CREB (*Endog. pCREB*) band in the *lower panel* is denoted by an *arrow*. *B*, effects of individual Ser → Ala substitutions on CREB electrophoretic mobility shifts. HEK 293T cells were transfected with FLAG epitope-tagged wild-type CREB ($CREB^{WT}$) or FLAG-CREB harboring Ser → Ala substitutions at the indicated amino acids. Cell extracts were prepared from unirradiated or IR-treated cells (30 Gy, 1 h) and analyzed by extended electrophoresis and immunoblotting with α-FLAG. A *dotted line* located at the approximate midpoints of FLAG-$CREB^{WT}$ band densities is included for the purposes of comparison. *C*, phosphorylation of Ser-117 is required for optimal DNA damage-dependent phosphorylation of CREB on Ser-121. HEK 293T cells were transfected with $CREB^{WT}$ or $CREB^{117A}$ expression plasmids. The IR-induced phosphorylation of the CREB proteins was monitored by immunoblotting with α-pCREB-121 and α-CREB antibodies. *D*, Q112D mutation ($CREB^{112D}$) reduces the threshold for ATM-dependent phosphorylation of Ser-121. HEK 293T cells were transfected with empty vector, $CREB^{WT}$, or $CREB^{112D}$ expression plasmids. Basal and IR-induced phosphorylation of the wild-type and mutant proteins were measured by immunoblotting with α-pCREB-121 and α-CREB antibodies. The locations of hyper- and hypo-phosphorylated $CREB^{WT}$ species in unirradiated cells are marked by *arrows*. Note that $CREB^{112D}$ migrates exclusively as a hyperphosphorylated species in the absence or presence of IR.

Contributions of Individual CK1 and CK2 Sites to DNA Damage-induced CREB Phosphorylation—Our next objective was to define the contributions of individual CK1/CK2 phosphorylation sites to basal and IR-induced CREB phosphorylation. As observed previously (31), mutation of Ser-111 ($CREB^{111A}$) or all three ATM consensus sites ($CREB^{3A}$) completely suppressed the IR-induced CREB electrophoretic mobility shift and blocked ATM-dependent phosphorylation of Ser-121 in transiently transfected HEK 293T cells (Fig. 2*A*). Mutation of the CK2 site at Ser-108 ($CREB^{108A}$) or the CK1 site at Ser-114 ($CREB^{114A}$) also inhibited the IR-induced CREB electrophoretic mobility shift and Ser-121 phosphorylation to different degrees. Mutation of Ser-108 had a small inhibitory effect on IR-induced Ser-121 phosphorylation, whereas the S114A mutation exhibited a Ser-121 phosphorylation defect nearly as severe as the $CREB^{111A}$ mutant (Fig. 2*A*). To more carefully examine the effects of individual phosphorylation site mutations on CREB electrophoretic mobility, and to potentially gain insight into the order of phosphorylation, we resolved wild-type and mutant CREB proteins using prolonged electrophoresis conditions (see "Experimental Procedures"). For this experiment we transiently transfected HEK 293T cells with FLAG-tagged CREB constructs so that comparison of electrophoretic mobility shifts was not confounded by endogenous CREB. As expected, the $CREB^{111A}$ mutant exhibited a severe electrophoretic mobility shift defect in comparison to $CREB^{WT}$, either in the absence or presence of IR (Fig. 2*B*). It should be noted that the overexpression of CREB is sufficient to induce its hyperphosphorylation in HEK 293T cells (Fig. 2*B* and Ref. 31) and thus, the majority of $CREB^{WT}$ migrated as the slow mobility form. The electrophoretic mobility of $CREB^{108A}$ was slightly enhanced relative to $CREB^{WT}$, whereas $CREB^{114A}$ demonstrated an electrophoretic mobility intermediate between $CREB^{108A}$ and $CREB^{111A}$ (Fig. 2*B*). These findings suggest that phosphorylation of Ser-108 and Ser-114 contributes to CREB electrophoretic mobility retardation and that the phosphorylation of these sites occurs subsequent to the phosphorylation of Ser-111.

The phosphorylation of Ser-114 is predicted to generate a consensus CK1 site at Ser-117, which we identified as an *in vivo* CREB phosphorylation site through mass spectrometry sequencing (data not shown). Therefore, the effect of an S117A mutation on CREB electrophoretic mobility and IR-induced Ser-121 phosphorylation was investigated. The $CREB^{117A}$ mutant exhibited a subtle electrophoretic mobility shift defect, as well as an ~80% reduction in IR-induced Ser-121 phosphorylation (Fig. 2*C*). From the combined experiments we conclude that the CK1/CK2 sites contribute to IR-dependent CREB electrophoretic mobility shifts and that phosphorylation of the Ser-111, Ser-114, and Ser-117 residues is required for optimal CREB phosphorylation on Ser-121 by ATM. The results also implicate Ser-108 as an *in vivo* phosphorylation site and suggest that the phosphorylation of Ser-111 is the key initiating event in the CREB phosphorylation cascade.

Enhanced CREB Phosphorylation by CK2 Lowers the Threshold for DNA Damage-induced Phosphorylation of Ser-121—In light of the above findings, we reasoned that enhanced CK1/CK2-mediated phosphorylation of Ser-111 would lower the threshold of DNA damage required for ATM-dependent phosphorylation of Ser-121. One way to test this hypothesis is to incorporate specific amino acid mutations that enhance the CK2- and/or CK1-dependent phosphorylation of Ser-111 while not enhancing its phosphorylation by ATM. To this end, we generated a $CREB^{112D}$ mutant harboring a Q112D substitution. The incorporation of an acidic residue in the +1 position is predicted to enhance phosphorylation of Ser-111 by CK2 (26). On the other hand, because glutamine in the +1 position is critical for substrate phosphorylation by ATM and ATR (38),

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the same substitution should compromise Ser-111 phosphorylation by ATM. Therefore, we compared the phosphorylation profiles of $CREB^{WT}$ and $CREB^{112D}$ in untreated and IR-treated HEK 293T cells. The $CREB^{112D}$ mutant exhibited basal Ser-121 phosphorylation, which was not observed for $CREB^{WT}$, and also showed enhanced IR-dependent phosphorylation of this residue (Fig. 2*D*, *top panel*). In addition, although $CREB^{WT}$ migrated as a partially resolved doublet in undamaged cells (Fig. 2*D*, denoted by *arrows*), $CREB^{112D}$ migrated exclusively as the slow electrophoretic mobility, hyperphosphorylated form on SDS-polyacrylamide gels, suggesting that the Q112D mutation stimulated basal CREB hyperphosphorylation (Fig. 2*D*, *bottom panel*). This finding supports the notion that CK1/CK2-mediated phosphorylation of CREB facilitates phosphorylation of Ser-121 by ATM in response to DNA damage.

CREB Is Phosphorylated on Ser-108/111/114 in Intact Cells—Our accumulated results provided strong but indirect evidence that CREB is phosphorylated on Ser-108, Ser-111, Ser-114, and Ser-117 in response to DNA damage. Previous attempts to generate a phospho-Ser-111-specific antibody using a peptide singly phosphorylated on Ser-111 were unsuccessful (31). The present findings provided a plausible explanation why; the phosphorylation of Ser-111 leads to obligatory phosphorylation of Ser-108, Ser-114, and Ser-117, and as a result, CREB rarely exists as a mono-phosphorylated species. As a consequence, phospho-specific antibodies generated against the Ser-111 residue are unlikely to recognize the Ser-108/111/114/117-phosphorylated form of CREB that exists *in vivo*. To test this hypothesis, we raised phospho-specific rabbit antisera against a CREB peptide phosphorylated on Ser-108, Ser-111, and Ser-114 (see "Experimental Procedures"). The antiserum was affinity-purified against the triply phosphorylated peptide, and the resulting antibody (α-pCREB-108/111/114) was used as an immunoblotting reagent. The α-pCREB-108/111/114 antibody displayed basal reactivity with endogenous CREB that was strongly induced upon exposure to IR and abolished upon phosphatase treatment (Fig. 3*A*). The specificity of the antibody was confirmed by assessing the effects of single Ala mutations at Ser-108, Ser-111, and Ser-114 on CREB immunoreactivity. Mutations at Ser-111 or Ser-114 almost completely abolished IR-induced CREB immunoreactivity with the α-pCREB-108/111/114 antibody, whereas the S108A mutation had a more modest inhibitory effect (Fig. 3*B*). In addition, and consistent with our previous observations, the overexpression of $CREB^{WT}$ was sufficient to induce its phosphorylation on Ser-108/111/114 in HEK 293T cells. These findings provide strong evidence that the Ser-108, Ser-111, and Ser-114 residues are phosphorylated in obligatory fashion in mammalian cells.

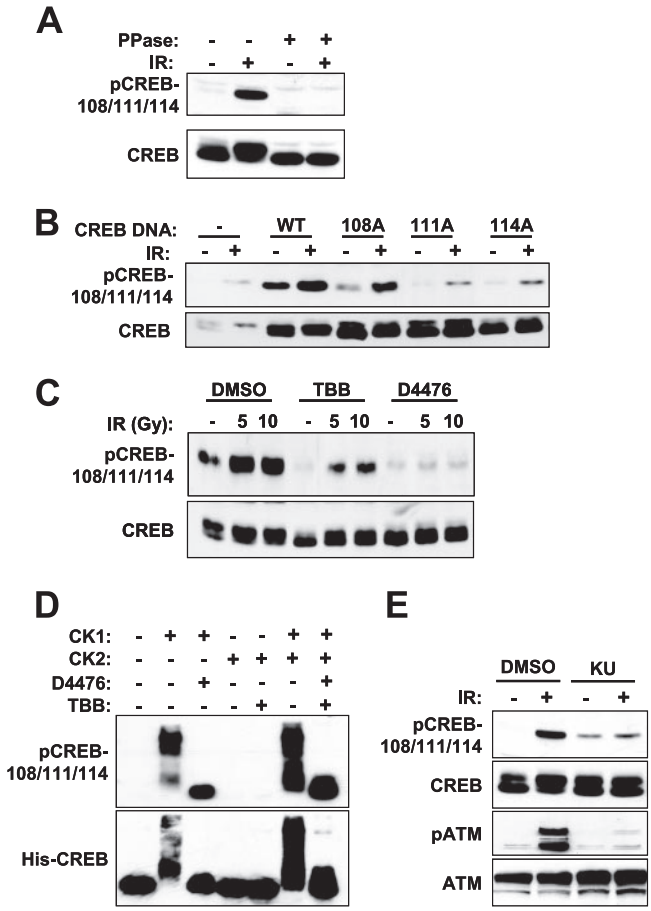

FIGURE 3. **CREB is phosphorylated on Ser-108/111/114 in intact cells.** *A*, IR inducibility and phosphatase sensitivity. HEK 293T cells were exposed to IR (30 Gy) or left untreated, and cell extracts were prepared 30 min later. Where indicated, the extracts were treated with λ phosphatase prior to analysis by SDS-PAGE and immunoblotting with α-pCREB-108/111/114 and α-CREB antibodies. *B*, phosphorylation site requirements. HEK 293T cells were transfected with plasmids encoding $CREB^{WT}$ or the indicated CREB phosphorylation site mutants. *WT*, wild type. Untreated or IR-treated cells were then analyzed by immunoblotting with α-pCREB-108/111/114 and α-CREB antibodies. *C*, inhibition of CREB Ser-108/111/114 phosphorylation by CK1 and CK2 inhibitors. HEK 293T cells were pretreated with TBB or D4476 3 h prior to exposure to 0, 5, or 10 Gy of IR. The cells were harvested 30 min later and analyzed by immunoblotting with α-pCREB-108/111/114 and α-CREB antibodies. *D*, phosphorylation of CREB by CK1 and CK2 *in vitro*. Purified His-CREB was incubated with CK1 δ or CK2 α/β *in vitro* in the presence of 200 μM ATP for 1 h (see "Experimental Procedures"), and phosphorylation products were analyzed by SDS-PAGE and immunoblotting with α-pCREB-108/111/114 and α-CREB antibodies. Where indicated, reactions were performed in the presence of the D4476 or TBB inhibitors. *E*, IR-induced phosphorylation of CREB on Ser-108/111/114 is ATM-dependent. HEK 293T cells were incubated with Me_2SO (*DMSO*) or the ATM inhibitor KU-55933 (*KU*) for 1 h prior to IR exposure (10 Gy) or mock irradiation. Cells were harvested 30 min later and analyzed by SDS-PAGE and immunoblotting with α-pCREB-108/111/114, α-CREB, α-pATM-1981, and α-ATM antibodies.

The α-pCREB-108/111/114 antibody allowed us to directly test whether CK1 and CK2 contributed to the phosphorylation of Ser-108, Ser-111, and Ser-114. In agreement with our earlier results examining the CREB electrophoretic mobility shift, the CK1 inhibitor strongly inhibited basal and IR-induced phosphorylation of Ser-108/111/114 (Fig. 3*C*). The CK2 inhibitor also suppressed Ser-108/111/114 phosphorylation, both under basal and DNA damage conditions, which may reflect contribution of CK2 to the phosphorylation of Ser-111 and Ser-108. The α-pCREB-108/111/114 antibody was also used to demonstrate that CK1 directly phosphorylates CREB *in vitro*. Incubation of purified His_6-tagged CREB (His-CREB) with purified CK1 caused a dramatic CREB electrophoretic mobility shift that correlated with phosphorylation on Ser-108/111/114 (Fig. 3*D*). Fifty micromolar D4476 completely blocked the CREB electrophoretic mobility shift but did not block CK1-induced α-pCREB-108/111/114 immunoreactivity. The partial effects of D4476 in this assay most likely reflect the high concentrations of CK1 and His-CREB substrate used in the kinase reaction. In contrast to CK1, CK2 did not stimulate CREB

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phosphorylation as assessed by α-pCREB-108/111/114 immunoreactivity. However, CK2 appeared to enhance CREB phosphorylation when coincubated with CK1 (Fig. 3*D*). This may reflect the CK2-dependent phosphorylation of Ser-108, which, although not absolutely required for binding to α-pCREB-108/111/114, nevertheless enhances phospho-CREB immunoreactivity toward this antibody (Fig. 3*B*).

Finally, by using the α-pCREB-108/111/114 antibody and the ATM-specific inhibitor, KU-55933 (33), we sought to prove that the IR-induced phosphorylation of Ser-108/111/114 was ATM-dependent. Preincubation of HEK 293T cells with 10 μM KU-55933 completely blocked IR-induced phosphorylation of Ser-108/111/114, establishing that the IR-induced phosphorylation of this motif requires active ATM. Interestingly, KU-55933 induced a low level of basal CREB Ser-108/111/114 phosphorylation that was not further induced upon DNA damage. The mechanism by which KU-55933 stimulates basal CREB phosphorylation is not known, but the result suggests that ATM may play a role in modulating CREB phosphorylation state in unperturbed cells. The combined results establish that CK1 and CK2 phosphorylate CREB on Ser-111, Ser-108, and Ser-114 under basal conditions and that the phosphorylation of the three residues is induced by DNA damage in an ATM-dependent manner. The results also confirm that CK1 plays a major role in the DNA damage-induced phosphorylation of this motif. Although the phosphorylation of Ser-117 was not directly assessed using antibodies, the results of Fig. 2*C* and our mass spectrometry findings suggest that this residue is phosphorylated by CK1 in conjunction with Ser-108, Ser-111, and Ser-114. To differentiate the coordinately regulated CK1/CK2 and ATM phosphorylation sites from the remainder of the KID, we refer to them collectively as the coregulated ATM and casein kinase site (RAX) domain.

Kinetics of CREB RAX Domain Phosphorylation—Our model predicts that the phosphorylation of CREB on Ser-108/111/114 should precede the phosphorylation of Ser-121 in DNA-damaged cells. To test this hypothesis, we performed a time course analysis of cells treated with the radiomimetic drug Zeocin. HEK 293T cells exhibited basal CREB Ser-108/111/114 phosphorylation that was induced after ~1 h of treatment, which was 30 min after detectable ATM activation (Fig. 4*A*). The phosphorylation of Ser-108/111/114 increased only slightly at time points beyond 1.5 h (Fig. 4*A*). By comparison, the Zeocin-induced phosphorylation of Ser-121 was delayed and did not plateau during the 8-h time course of the experiment (Fig. 4*A*). In addition, a Zeocin dose response showed that low concentrations of Zeocin (10–20 μg/ml) induced CREB Ser-108/111/114 phosphorylation in the virtual absence of Ser-121 phosphorylation, whereas higher doses of Zeocin induced phosphorylation on Ser-108/111/114 and Ser-121 (Fig. 4*B*). Together, these findings suggest that phosphorylation of the Ser-108/111/114 sites precedes the phosphorylation of Ser-121 following DNA damage.

CK1-dependent Phosphorylation of Ser-121 Is Required to Inhibit CREB-CBP Interactions—We previously showed that DNA damage-induced phosphorylation of CREB antagonized its association with the KIX domain of CBP (31). Our current results suggested that CK1 activity might be critical for this

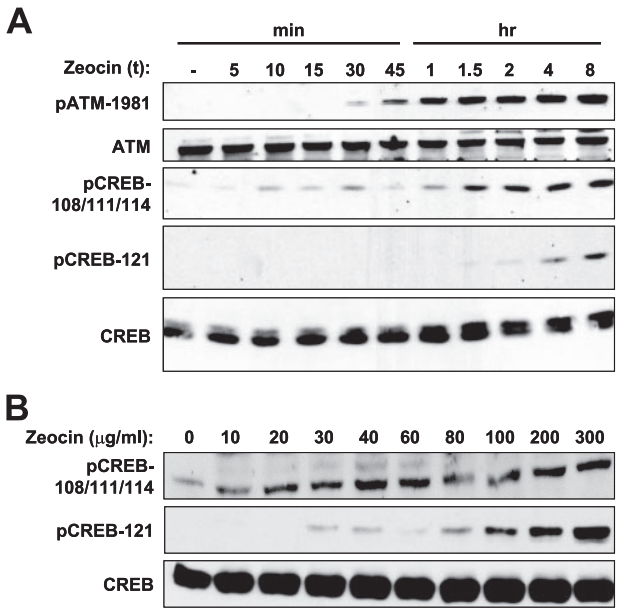

FIGURE 4. **Time courses and dose responses of CREB phosphorylation on Ser-121 and Ser-108/111/114 in response to DNA damage.** *A*, HEK 293T cells were cultured in the presence of the double strand break-inducer Zeocin (0.1 mg/ml) for the indicated lengths of time. Cell extracts were analyzed by immunoblotting with the indicated antibodies. The α-pATM-1981 immunoblot was used as a marker for ATM activation. *B*, Zeocin dose response. HEK 293T cells were cultured in the presence of the indicated doses of Zeocin for 4 h. Cell extracts were prepared and analyzed by immunoblotting with α-pCREB-108/111/114, α-pCREB-121, and α-CREB antibodies.

inhibition. To test this hypothesis, we transfected HEK 293T cells with a $CREB^{WT}$ vector and performed CREB pulldown assays using a GST-KIX domain fusion protein. The KIX domain of CBP is both necessary and sufficient for CREB binding *in vitro* (39). Consistent with our earlier results, the binding of $CREB^{WT}$ to GST-KIX was reduced following IR exposure (Fig. 5*A*). In contrast, pretreatment of HEK 293T cells with D4476 blocked the IR-dependent repression of CBP binding, which correlated with inhibition of CREB Ser-121 phosphorylation. Importantly, the CK1 inhibitor had no effect on the phosphorylation of CREB on Ser-133, which is essential for the CREB-KIX interaction. These data suggest that CK1 activity is required for IR-dependent attenuation of CREB-CBP interactions.

The importance of CK1/CK2 as modulators of CREB-CBP interactions was confirmed through mutagenesis of individual phosphorylation sites. As expected, the IR-dependent inhibition of GST-KIX binding was disrupted by an S111A mutation, either individually or in the context of the $CREB^{3A}$ mutant (Fig. 5*B*). A similar result was obtained using the $CREB^{114A}$ mutant, suggesting that CK1-mediated phosphorylation of this site contributes to CREB-CBP complex dissociation. In contrast, a $CREB^{117A}$ mutant exhibited a partial defect in IR-dependent CREB-KIX dissociation, which mirrors the partial effects of this mutation on IR-induced Ser-121 phosphorylation (Fig. 5*B*, *bottom panel*). Interestingly, the $CREB^{108A}$ mutant also displayed residual GST-KIX binding in the presence of IR, suggesting that CK2-dependent phosphorylation of Ser-108 contributes to destabilization of the CREB-CBP complex (Fig. 5*B*). Together, these findings support the idea that CK1/CK2 sites regulate CREB-CBP interactions *in vitro*.

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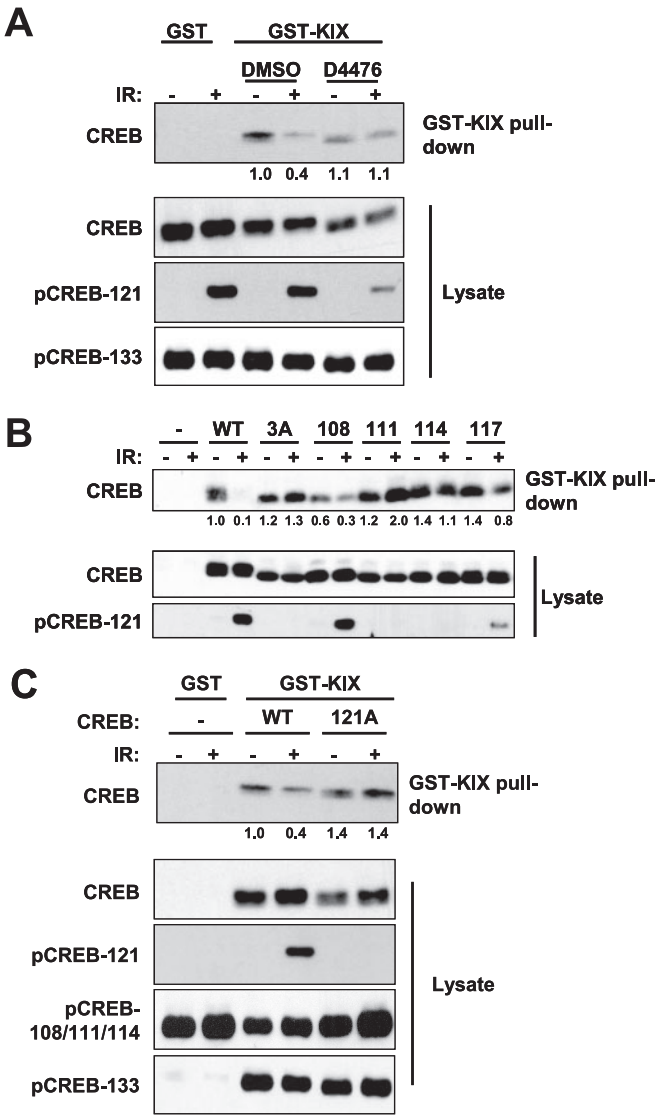

FIGURE 5. **CK1-dependent phosphorylation of Ser-121 is required to inhibit CREB-CBP association in response to DNA damage.** *A*, IR-dependent inhibition of CREB-CBP interaction is antagonized by a CK1 inhibitor. HEK 293T cells were transfected with $CREB^{WT}$, treated with Me_2SO (*DMSO*) or D4476 for 3 h, and then mock-irradiated or exposed to 30 Gy IR. Cell extracts were prepared 1 h later and incubated with GST- or GST-KIX-loaded beads, and the bound CREB proteins were analyzed by immunoblotting with α-CREB (*top panel*). Extract levels of total CREB, Ser-121-phosphorylated CREB, and Ser-133-phosphorylated CREB were compared by immunoblotting with the indicated antibodies. Band intensities normalized for differences in CREB expression are presented below the *top panel*. *B*, effects of ATM and CK1/CK2 phosphorylation site mutations on IR-dependent repression of CREB-CBP interactions. HEK 293T cells were transfected with CREB expression plasmids harboring the indicated Ser → Ala mutations ($CREB^{3A}$ contains mutations at Thr-100, Ser-111, and Ser-121) and left untreated or exposed to IR (30 Gy, 1 h). Cell extracts were incubated with GST-KIX-loaded beads, and the bound CREB proteins were analyzed by immunoblotting with α-CREB (*top panel*). Extract levels of total CREB and Ser-121-phosphorylated CREB were also compared in the *lower two panels*. Normalized band intensities are presented for each mutant. *WT*, wild type. *C*, Ser-121 phosphorylation is required for IR-dependent inhibition of CREB-CBP complex formation. HEK 293T cells were transfected with $CREB^{WT}$ or $CREB^{121A}$ expression plasmids and then left untreated or exposed to IR (30 Gy, 1 h). Cell extracts were then incubated with GST or GST-KIX-loaded beads. The bound CREB proteins and total CREB and phospho-CREB antibodies were analyzed by immunoblotting as described for *B*.

Our data strongly suggest a model of sequential CREB phosphorylation in response to DNA damage where ATM-dependent phosphorylation of Ser-121 is dependent on the processive phosphorylation of CREB on Ser-108, Ser-111, Ser-114, and Ser-117 by CK1 and CK2. In principle, phosphorylation of the CK1/CK2 sites at Ser-108, Ser-111, Ser-114, and Ser-117 could antagonize the CREB-CBP interaction independent of Ser-121. Alternatively, the phosphorylation of Ser-121, the last step in the CREB phosphorylation cascade, could be the critical event that inhibits CBP binding. To distinguish between these possibilities, we compared the GST-KIX-binding properties of $CREB^{WT}$ and the $CREB^{121A}$ mutant before and after DNA damage. In comparison to $CREB^{WT}$, the GST-KIX binding activity of $CREB^{121A}$ was resistant to IR exposure, suggesting that the phosphorylation of Ser-121 is necessary to dissociate CREB-CBP complexes following DNA damage (Fig. 5*C*). We also observed that the S121A mutation partially inhibited IR-induced CREB phosphorylation on Ser-108/111/114. Because of this caveat, we cannot unequivocally conclude that the phosphorylation of Ser-121 is sufficient to inhibit CREB-CBP complex formation. Nevertheless, the results strongly suggest that attenuation of CBP binding is one biochemical function of the Ser-121 residue and that phosphorylation of upstream residues by CK1/CK2 is required to license this event.

DISCUSSION

In this study we have delineated a complex mechanism of CREB phosphorylation in which the coregulated modification of CK1/CK2 and ATM sites attenuates the CBP-binding affinity of CREB in DNA-damaged cells. Our results implicate CK1 as a critical regulator of CREB phosphorylation and suggest that, under certain circumstances, CK1 and CK2 can function as licensing factors for ATM substrate phosphorylation. We propose the term coregulated ATM and casein kinase sites (RACKS; abbreviated as RAX) to describe this CREB phosphorylation motif.

The consensus CK1 and CK2 sites in CREB were originally identified as part of the KID, and evidence suggested that these sites are phosphorylated in intact cells (22, 29). Using the α-pCREB-108/111/114 antibody we provide direct evidence that Ser-108, Ser-111, and Ser-114 are basally phosphorylated in asynchronous cells and that phosphorylation of the sites is strongly induced by IR (Fig. 3*A*). It is also a near certainty that Ser-117 is phosphorylated in conjunction with the Ser-108, Ser-111, and Ser-114 residues. Consistent with this, Ser-117 was unambiguously identified as a phosphorylation site by mass spectrometry using FLAG-CREB purified from IR-treated HEK 293T cells.[3] In addition, mutation of Ser-117 attenuates the IR-dependent CREB electrophoretic mobility shift as well as IR-dependent phosphorylation of CREB on Ser-121 (Fig. 2). Together the findings strongly suggest that Ser-108, Ser-111, Ser-114, and Ser-117 are phosphorylated *en masse* in mammalian cells.

A Model for CREB Phosphorylation in Response to DNA Damage—Our data allow us to construct a detailed model describing the phosphorylation of CREB by ATM and CK1/CK2 in response to DNA damage (Fig. 6). In this model the Ser-111 residue within the RAX domain functions as an initiator site. Ser-111 is basally phosphorylated by CK1 and/or CK2

[3] N. Shanware and R. Tibbetts, unpublished observations.

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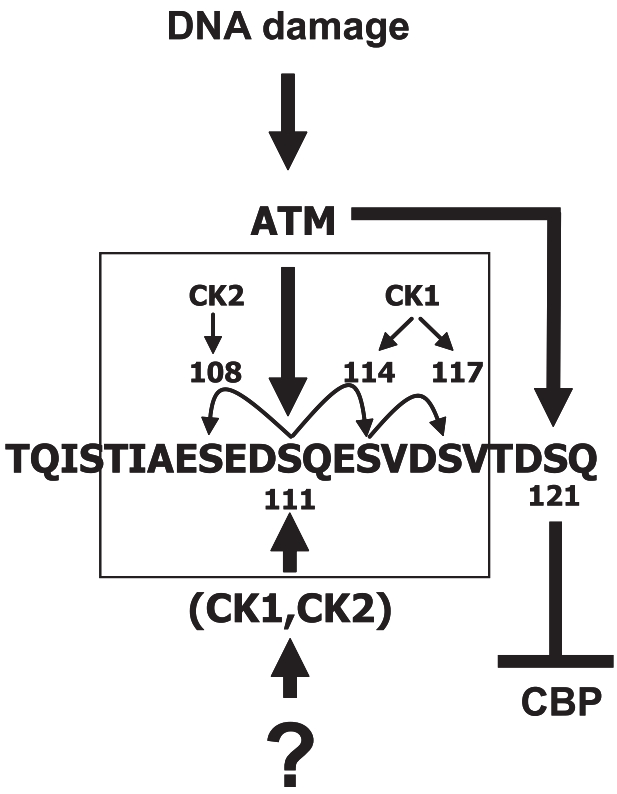

FIGURE 6. **Model for coregulated phosphorylation of CREB by ATM, CK1, and CK2.** The CREB phosphorylation cascade is initiated by the phosphorylation of Ser-111, which is mediated by ATM in response to DNA damage and by CK1 and/or CK2 in undamaged cells. Phosphorylation of Ser-111 triggers CK2- and CK1-dependent phosphorylation of Ser-108 and Ser-114/117, respectively. The priming events (*boxed*) license CREB for its ultimate phosphorylation by ATM on Ser-121, which reduces the affinity of CREB for CBP. The *question mark* denotes that the signals controlling DNA damage-independent phosphorylation of CREB have not been defined.

in the absence of DNA damage and is phosphorylated by ATM in response to DNA damage. The phosphorylation of Ser-111 leads to the processive phosphorylation of Ser-108 by CK2 and Ser-114/117 by CK1. We refer to the Ser-108/111/114/117-phosphorylated form of CREB as the CREB-4P isoform. The CREB-4P isoform is permissive for phosphorylation on Ser-121 by ATM, which switches CREB into a low affinity CBP-binding state (Fig. 5*C*). We refer to the Ser-108/111/114/117/121-phosphorylated form of CREB as the CREB-5P isoform. In response to DNA damage, ATM is also capable of initiating the phosphorylation of a naïve RAX domain on Ser-111, which triggers the CK1/CK2-mediated events and licenses CREB for Ser-121 phosphorylation by ATM. Thus, in this paradigm, ATM both initiates and terminates the CREB phosphorylation cascade. Finally, our results also suggest a feed-forward mechanism whereby phosphorylation of Ser-121 enhances, but is not required for, IR-dependent phosphorylation of Ser-111 (Fig. 5*C*). We speculate that the phosphorylation of Ser-121 may further increase the availability of the Ser-111 site for phosphorylation by ATM and/or CK1/CK2.

It is unclear why such an elaborate phosphorylation mechanism would arise to modulate CREB activity. One possibility is that the CK1/CK2 sites function to prevent the phosphorylation of Ser-121 until a certain threshold of DNA damage has occurred. In this model, activated ATM transiently associates with CREB in response to low levels of DNA damage, catalyzes its phosphorylation on Ser-111, and then dissociates from CREB as processive phosphorylation of Ser-108, Ser-114, and Ser-117 by CK2 and CK1 ensues. The resulting CREB-4P isoform is now competent for phosphorylation on Ser-121 if it encounters another active ATM molecule. However, if the concentration of activated ATM molecules is very low, then the second encounter does not occur, and Ser-121 is not phosphorylated. In this scenario, the CK1/CK2 sites ostensibly act as a Ser-121 phosphorylation buffer. Consistent with this idea, low doses of the DNA-damaging agent Zeocin induce Ser-108/111/114 phosphorylation in the absence of detectable Ser-121 phosphorylation (Fig. 4*B*). In principle, this could prevent undesirable changes in the expression of one or more critically important CREB target genes.

It is also possible that the CREB-4P isoform, without DNA damage and Ser-121 phosphorylation, exhibits altered biochemical activities relative to unphosphorylated CREB. The signals controlling the abundance of the CREB-4P isoform during an unperturbed cell cycle remain to be determined. Ironically, phosphorylation of CREB on Ser-108/111/114 is mildly induced by KU-55933, whereas the IR-induced phosphorylation is completely blocked by this drug (Fig. 3*E*). In addition, overexpression of CREB is sufficient to induce its CK1/CK2-dependent hyperphosphorylation on Ser-108/111/114. Given that CK1 and CK2 are widely regarded as constitutively active enzymes, these findings may point toward an ATM-dependent protein phosphatase as a key determinant of basal CREB phosphorylation status.

Our findings suggest that IR-induced phosphorylation of CREB on Ser-121 reduces the affinity of CREB for the KIX domain of CBP. The solution NMR structure for the CREB-CBP complex reveals that Ser-121 is proximal to negatively charged Glu-655 of CBP (40). Thus, the phosphorylation of Ser-121 may destabilize the CREB-KIX complex through electrostatic repulsion. However, our results do not rule out other biochemical functions for the phosphorylated RAX domain. For example, recent studies suggest that the DNA binding activity of CREB is dynamically regulated in mammalian cells (41–43) and that CK1/CK2-mediated phosphorylation of a positionally conserved RAX domain inhibits the DNA binding activity of *Drosophila* CREB *in vitro* (28). It is therefore plausible that DNA damage-induced phosphorylation of the RAX domain may regulate CREB DNA binding activity in mammalian cells.

The RAX domain may also be a determinant of CREB stability. The phosphorylation of CREB on Ser-121 was reported to target CREB for proteasome-dependent degradation in response to prolonged hypoxia (44). The same site was implicated in CREB degradation in cardiac smooth muscle cells following platelet-derived growth factor treatment (45). However, IR doses up to 30 Gy do not cause changes in steady-state CREB levels in the cell lines that we have examined, although caspase-dependent cleavage of CREB is observed in IR-sensitive leukemia cell lines.[3] These findings would seem to rule out a major role for the RAX domain as a CREB stability determinant in the context of genotoxic stress.

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The RAX Domain as a Functional Motif—Our investigation has uncovered an intricate relationship between ATM and CK1/CK2 phosphorylation sites that negatively regulate the CBP-binding affinity of CREB in response to DNA damage. It is possible that the interplay between ATM and CK1/CK2 in the CREB phosphorylation paradigm reflects a more general collaboration between these proteins in the cellular DNA damage response. Consistent with this idea, a previous study showed that the CK1-dependent phosphorylation of p53 on Ser-18 required the prior phosphorylation of the ATM/ATR site at Ser-15 (46–50). The activities of CK1 and CK2 may be most important in those situations where the incorporation of multiple phosphates is required to elicit a biochemical alteration in substrate activity. Future studies should determine whether the RAX domain is a commonly employed signaling element required for ATM-dependent DNA damage responses.

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Coregulated Ataxia Telangiectasia-mutated and Casein Kinase Sites Modulate cAMP-response Element-binding Protein-Coactivator Interactions in Response to DNA Damage

Naval P. Shanware, Anthony T. Trinh, Leah M. Williams and Randal S. Tibbetts

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