Aurora-A Abrogation of p53 DNA Binding and Transactivation Activity by Phosphorylation of Serine 215*

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The tumor suppressor p53 is important in the decision to either arrest cell cycle progression or induce apoptosis in response to a variety of stimuli. p53 post-translational modifications and association with other proteins have been implicated in the regulation of its stability and transactivation activity. Here we show that p53 is phosphorylated by the mitotic kinase Aurora-A at serine 215. Unlike most identified phosphorylation sites of p53 that positively associate with p53 function (Brooks, C. L., and Gu, W. (2003) Curr. Opin. Cell Biol. 15, 164–171), the phosphorylation of p53 by Aurora-A at Ser-215 abrogates p53 DNA binding and transactivation activity. Downstream target genes of p53, such as p21^{Cip/WAF1} and PTEN, were inhibited by Aurora-A in a Ser-215 phosphorylation-dependent manner (i.e. phosphomimic p53-S215D lost and non-phosphorylatable p53-S215A retained normal p53 function). As a result, Aurora-A overrides the apoptosis and cell cycle arrest induced by cisplatin and γ-irradiation, respectively. However, the effect of Aurora-A on p53 DNA binding and transactivation activity was not affected by phosphorylation of Ser-315, a recently identified Aurora-A phosphorylation site of p53 (Katayama, H., Sasai, K., Kawai, H., Yuan, Z. M., Bondaruk, J., Suzuki, F., Fujii, S., Arlinghaus, R. B., Czerniak, B. A., and Sen, S. (2004) Nat. Genet. 36, 55–62). Our data indicate that phosphorylation of p53 at Ser-215 by Aurora-A is a major mechanism to inactivate p53 and can provide a molecular insight for Aurora-A function.

Aurora is a subfamily of serine/threonine protein kinases. In vertebrates, they comprise Aurora-A, -B, and -C, with one or more highly conserved orthologues being found in the yeasts, flies, worms, and other invertebrates. Saccharomyces cerevisiae cells have a single Aurora gene, IPI1 (3). The Droso phila and Caenorhabditis elegans genomes encode one member in each of the Aurora-A and -B classes (4). The homologs of Aurora-A and -B have also been found in Xenopus (5). Accumulated evidence shows that Aurora kinases play a critical role in G2/M progression of the cell cycle. Their regulatory influence spans from G2 to cytokinesis, encompassing key cell cycle events such as centrosome duplication, chromosome bi-orientation and segregation, and cleavage-furrow positioning and ingress (4, 6).

Aurora-A has attracted intense interest following the discovery that the chromosomal region (20q13.2) where it is located commonly undergoes amplification in human cancers. It has been shown that amplifications of the Aurora-A gene occur in as many as 12−30% of ovarian, breast, colorectal, and gastric cancers (7, 8). Ectopic expression of Aurora-A in murine fibroblasts as well as mammary epithelia induces centrosome amplification, aneuploidy, and oncogenic phenotype (7, 8). Recent studies have also suggested that Aurora-A plays an important role in programmed cell death and chemoresistance (9). However, the underlying molecular mechanism still remains elusive.

A recent report shows that Aurora-A induces p53 degradation by phosphorylation of Ser-315 (2). Here, we demonstrated that DNA binding and transactivation activity of p53 was abrogated by Aurora-A. Aurora-A phosphorylates p53 at Ser-215 in vitro and in vivo. The inhibition of p53 DNA binding and transactivation activity by Aurora-A depends on phosphorylation of Ser-215 but not Ser-315. Further, Aurora-A phosphorylation of Ser-215 of p53 is associated with Aurora-A-regulated cell cycle progression, cell survival, and transformation. These data indicate that p53 is a physiological substrate of Aurora-A and that Aurora-A exerts its function through phosphorylation of Ser-215 of p53. In addition, our study also identifies Ser-215 as a novel phosphorylation site of p53 that might be targeted by other serine/threonine kinases besides Aurora-A.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—Recombinant p53 protein was purchased from Calbiochem. Edman degradation reagents were from Millipore (Bedford, MA). Expression plasmids of p53 and Aurora-A were created by ligation of the cDNA of p53 and Aurora-A into pDeRe2DC1 (EcoRI/ KpnI), pEGFP-C3 (BglII/KpnI), and pOEG-4T1 (BamHI/EcoRI) vectors. Mutant constructs of p53 were prepared with a mutagenesis kit (Stratagene) using pcDNA3-HA-p53 as a template. Immunoblotting, Immunoprecipitation, and In Vitro Kinase—Immunoblotting and immunoprecipitation were performed as described previously (10). In vitro kinase assay was carried out with immunoprecipitated HA-Aurora-A in a reaction buffer (11) using recombinant or glutathione S-transferase-fused p53 as substrate. The reaction was incubated at 30 °C for 20 min and stopped in Laemmli sample buffer. Phosphorylation of p53 was detected by SDS-PAGE and autoradiography. Luciferase Reporter Assay—Cells were seeded in 6-well plates and transfected with p21 or PTEN promoter-driven luciferase reporter, pSV2-β-galactosidase, and plasmids indicated in the legends to Figs. 2 and 3. Following a 48-h culture, cells were lysed, and luciferase and β-galactosidase assays were performed according to the manufacturer’s instructions (Promega, Madison, WI). Transcriptional activity was assessed by reporter luciferase assays.

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The abbreviations used are: HA, hemagglutinin; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; RNAi, RNA interference; CREB, cAMP-response element-binding protein.
measured by normalizing the luciferase activity to the corresponding /H9252-galactosidase activity. Each experiment was repeated three times.

Phosphoamino Acid Analysis, Phosphopeptide Mapping, and Edman Degradation—To prepare the phosphoamino acid and phosphopeptide of p53, Aurora-A-phosphorylated p53 was excised and eluted from the SDS-polyacrylamide gel by incubation in a buffer containing 50 mM ammonium bicarbonate, 1% 2-mercaptoethanol, and 0.2% SDS for 12 h. After chloroform precipitation and oxidation with performic acid, a portion of the sample was subjected to partial hydrolysis by incubation with 6N HCl for 1h at 10° C. The rest was digested with 10 ng of endoproteinase Asp-N, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, or trypsin/Glu-C (Sigma) for 6h at 37° C, followed by an additional 10-h incubation at 37° C in the presence of a fresh 10 ng of the same proteinase.

Phosphoamino acid analysis and phosphopeptide mapping were conducted as described previously (12) using the HTLE-7000 system. Briefly, the phosphoamino acids were resolved by two-dimensional electrophoresis on a thin layer of cellulose. The electrophoresis was carried out for 20 min at 1.5 kV in 88% formic acid/glacial acetic acid/water (1.3:1.35.9, pH 1.9) for the first dimension and for 16 min at 1.3 kV in the second dimension. The phosphopeptides were then excised from the gel and subjected to Edman degradation. The released radioactivity for each cycle was measured by scintillation counting.

FIG. 1. Aurora-A primarily phosphorylates p53 at Ser-215. A, interaction of Aurora-A with p53. A2780S cell lysates were immunoprecipitated with anti-Aurora-A antibody and detected with anti-p53 antibody (left panel) and vice versa (right panel). B, Aurora-A phosphorylation of p53 in vitro and in vivo. HEK293 cells were transfected with HA-Aurora-A. After 36 h of transfection, the cells were immunoprecipitated (IP) with anti-HA antibody. The immunoprecipitates were subjected to in vitro kinase assay using recombinant p53 (r-p53) as a substrate (left panel). The right panel is an in vivo [32P]orthophosphate-labeling experiment. p53-null HCT116 cells were transfected with HA-p53 with or without FLAG-Aurora-A, labeled with [32P]orthophosphate (0.5 mCi/ml) in minimum Eagle’s medium without phosphate for 4 h, and immunoprecipitated with anti-HA antibody. The HA-p53 immunoprecipitates were separated by SDS-PAGE, transferred to membrane, exposed to the film (upper panel) and digested by anti-p53 antibody (middle panel). Expression of Aurora-A was detected with anti-Flag antibody (bottom panel). C. Aurora-A phosphorylation of p53 on serine residue(s). The phosphorylated p53 protein by Aurora-A was recovered from in vitro kinase assay and analyzed by two-dimensional gel electrophoresis as described under “Experimental Procedures.” D and E, phosphopeptide mapping. Aurora-A-phosphorylated p53 was digested with Asp-N (D) or L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (E). Following gel electrophoresis and chromatography (left panels), excised spots from the gel were subjected to Edman degradation. Quantification of radioactivity for each cycle is shown in the right panels. F, trypsin has been shown to cut inefficiently at the R-X-pS/pT sequence (18), and the trypsin-digested p53 produces three fragments that contain a serine residue in cycle 6 of Edman degradation (top). The spot “c” in E from a separate gel was excited and digested with Glu-C. Two-dimensional gel electrophoresis (left panel) showed that the migration pattern differed from E, suggesting that it should be either Glu-C-digested fragment 2 or 3. Edman degradation analysis (right panel) revealed Aurora-A phosphorylation of p53 fragment 3, and thus confirmed the result obtained from D. G and H, in vitro Aurora-A kinase and in vivo [32P]orthophosphate labeling were carried out as described above except using p53-S215A mutant as a control. C.B., Coomassie Blue; WT, wild type; GST, glutathione S-transferase.
pyridine/glacial acetic acid/water (1:10:189, pH 3.5) for the second dimension. Phosphoamino acid standards, which were mixed with each sample, were visualized by staining with 0.25% w/v ninhydrin in acetone.

Phosphopeptides of p53 were resolved by electrophoresis for 30 min at 1.0 kV in formic acid/glacial acetic acid/water (1:3.1:35.9; pH 1.9). The second dimension was accomplished by chromatography in n-butyl alcohol/pyridine/glacial acetic acid/water (5:3.3:1:4). The phosphopeptides were visualized by autoradiography for 12 h at −80 °C and eluted from the thin layer plate with formic acid/glacial acetic acid/water (1:3.1:35.9, pH 1.9). The purified phosphopeptides were subjected to manual Edman degradation as described previously to identify the phosphorylation sites (13). Briefly, phosphopeptides were covalently coupled to Sequelon-AA discs and subjected to consecutive cycles of the Edman degradation. After each cycle, the disc was treated with trifluoroacetic acid to cleave and release the N-terminal amino acid, and the released 32P was determined by Cerenkov counting.

EMSA—EMSA was performed as described previously (14). Briefly, nuclear extract was incubated with 32P-labeled oligonucleotide encoding the consensus p53 DNA binding site (5'-TAC AGA ACA TGT CTA AGC ATG CTG GGG-3') at room temperature for 30 min. Competition for p53 binding activity was carried out in the presence of a 40-fold excess of the cold wild type oligonucleotide or mutant oligonucleotide (5'-TAC AGA AAA TTT CTA AGT CCT CTG GGG-3'), and supershift of p53-oligonucleotide complex was obtained by the addition of DO-1, a specific antibody for p53 (Santa Cruz Biotechnology, Inc.). The protein-DNA complexes were separated by electrophoresis through a 5% native polyacrylamide gel.

Cell Cycle, Apoptosis, and Colony Formation—For cell cycle analysis, cells were fixed in 70% ethanol overnight, stained in 20 μg/ml propidium iodide, and analyzed with flow cytometry. Apoptosis was examined by Annexin-V assay. Briefly, 10^6 cells were double labeled with Annexin-V-allophycocyanin and 7-amino-actinomycin D using a kit according to the manufacturer's instructions (BD Biosciences). The percentage of apoptosis (Annexin-V-positive cells) was determined by flow cytometry. Colony formation was measured as described previously (15). Briefly, cells were transfected with p53 and Aurora-A or their respective empty vectors. The colony formation was assessed after a 3-week culture.

RESULTS

Aurora-A Primarily Phosphorylates p53 at Ser-215—We examined whether Aurora-A associates with tumor suppressor p53 because both Aurora-A and p53 localize to centrosome during mitosis, and the overexpression of Aurora-A and loss of p53 function result in similar phenotypes of centrosome amplification and aneuploidy (16, 17). Coimmunoprecipitation showed Aurora-A interaction with p53 (Fig. 1A). Further ex-
experiments revealed that Aurora-A phosphorylated p53 in vitro and in vivo (Fig. 1B). Phosphoamino acid analysis revealed that Aurora-A phosphorylated p53 on serine residue(s) (Fig. 1C). To map p53 residue(s) phosphorylated by Aurora-A, Aurora-A-phosphorylated recombinant p53 was digested with endoproteinase Asp-N, and the cleaved phosphopeptides were subjected to two-dimensional gel analysis (Fig. 1D). Amino acid analysis by Edman degradation of the phosphopeptides demonstrated that the activity of radiosotope was released after the eighth cycle of the degradation in the higher radioactivity spot, whereas the lower density spot did not reveal radioactivity release after a 10-cycle degradation (Fig. 1D). Judging from the amino acid sequence of p53, the only endoproteinase Asp-N-digested peptide that contains serine or threonine at position 8 in its sequence is \( \text{DRNTFRH5SVVVPYEPVEVS}^{227} \), where bold indicates phosphorylated serine residue. Thus, these results indicate that Ser-215 is a phosphorylation site of p53 for Aurora-A kinase. To confirm this result, Aurora-A-phosphorylated p53 was digested with endoproteinase trypsin (Fig. 1E) and trypsin/Glu-C (Fig. 1F). Edman degradation and amino acid analysis showed that the higher density spot was Ser-215 and the other was Ser-315 (Fig. 1, E and F). Further, the conversion of Ser-215 to alanine markedly decreased Aurora-A phosphorylation of p53 in vitro and in vivo (Fig. 1, G and H). Therefore, Ser-215 of p53 is a major residue targeted by Aurora-A.

**Aurora-A Abrogates p53 DNA Binding and Transactivation Activity**—Because Ser-215 locates within DNA-binding domain of p53, we next examined whether Aurora-A inhibits DNA binding and transactivation activity of p53 using electrophoresis mobility shift assay. Fig. 2A shows that p53 DNA binding activity was significantly inhibited by overexpression of Aurora-A in both A2780S (wild-type p53) and p53-transfected H1299 cells, whereas knockdown of endogenous Aurora-A by RNAi increased the DNA binding activity 3-fold. The effect of Aurora-A on p53 transactivation activity was evaluated with promoter-driven luciferase reporter assay in A2780S cells. Aurora-A inhibited p21 and PTEN promoter activity in a dose-dependent manner. Furthermore, the suppression of Aurora-A expression by RNAi enhanced the promoter activity of p21 and PTEN (Fig. 2B). These results were further supported by cotransfection of p53 and Aurora-A in p53-null H1299 cells (Fig. 2C). Accordingly, protein levels of p21 and PTEN were reduced by ectopic expression of Aurora-A and elevated by knockdown of endogenous Aurora-A (Fig. 2D). These data indicate that Aurora-A directly targets p53 and inhibits its DNA binding and transactivation activity.

**Aurora-A Inhibition of p53 Transactivation and DNA Binding Activity Depends on Phosphorylation of Ser-215 but Not Ser-315**—We further determined whether phosphorylation of Ser-215 is required for Aurora-A abrogation of p53 DNA binding and transactivation activity. Aurora-A phosphomimic p53-S215D and nonphosphorylatable p53-S215A were created by converting Ser-215 to aspartic acid and alanine, respectively.
FIG. 3. Aurora-A inhibition of p53 transactivation and DNA binding activity depends on phosphorylation of Ser-215 but not Ser-315. A, Western blot analysis. H1299 cells were transfected with indicated plasmids, lysed, and immunoblotted with indicated antibodies. B–E, reporter (B and E) and EMSA assays (C and D). H1299 cells were transfected with indicated plasmids. After 24 h of transfection, cells were subjected to reporter and EMSA analyses as described under “Experimental Procedures.” The reaction was incubated with an anti-p53 antibody in the far left lane of C and D for determining the specificity of p53 binding to DNA. C and D, bottom panels, show the expression of transfected plasmids. WT, wild type.
Their functions were evaluated in p53-null H1299 cells. Fig. 3, A and B, shows that nonphosphorylatable p53-S215A induced the expression and promoter activity of p21 and PTEN, which are similar to those of wild-type p53. However, these effects of p53-S215A, unlike wild-type p53, were not inhibited by ectopic expression of Aurora-A. Further, phosphomimic p53-S215D completely lost the ability to induce p21 and PTEN expression and promoter activity. In addition, p53-S215A retained DNA binding activity, which was not inhibited by Aurora-A, whereas p53-S215D failed to bind to DNA (Fig. 3C). Therefore, we conclude that Aurora-A inactivation of p53 depends on phosphorylation of Ser-215.

A recent report shows that Aurora-A phosphorylates p53 at Ser-315, which was also observed in our study (Fig. 1, E and F), leading to MDM2-mediated destabilization of p53 (2). However, its direct effects on p53 DNA binding and transactivation activity are unknown. To this end, we created phosphomimic p53-S315D, nonphosphorylatable p53-S315A, as well as S215A/S315A and S215D/S315D double mutants. EMSA and reporter assays revealed that both phosphomimic p53-S315D and nonphosphorylatable p53-S315A retained p53 DNA binding and transactivation activity and were negatively regulated by Aurora-A (Fig. 3, D and E). However, introducing S215D mutation caused p53-S315A and p53-S315D to completely lose activity.
DNA binding capability (Fig 3D). These data indicate that Aurora-A directly inactivates p53 through phosphorylation of Ser-215 but not Ser-315.

Aurora-A Inhibition of p53 Tumor Suppressor Function Is Dependent on Phosphorylation of Ser-215—Because p53 is a tumor suppressor and a key regulator of cell survival and cell cycle progression, we further examined the effects of Aurora-A phosphorylation of Ser-215 and/or Ser-315 on p53 biological function. We and others have shown previously that A2780CP cells are resistant to cisplatin-induced apoptosis and that reintroducing p53 restores the response of the cell to cisplatin (10, 19). Thus, we evaluated the effect of Aurora-A phosphorylation of p53 on cisplatin-induced programmed cell death in A2780CP cells. The cells were transfected with different forms of p53, treated with cisplatin, and analyzed with Annexin-V labeling and flow cytometry. Fig. 4A shows that ectopic expression of p53-S215A or wild-type p53 restored cisplatin sensitivity and induced apoptosis at ~47%. However, coexpression of Aurora-A inhibited the apoptosis induced by wild-type p53 but not p53-S215A. A similar percentage of apoptosis was detected in the cells expressing p53-S315A and p53-S315D that were abrogated by Aurora-A. In contrast, p53-S215D lost the ability to sensitize A2780CP cells to cisplatin-induced programmed cell death (Fig. 4A). Similarly, wild-type p53, p53-S215A, p53-S315A, and p53-S315D but not p53-S215D inhibited colony formation in both H1299 and SaOS2, the cell lines examined. Aurora-A abrogated wild-type p53-, p53-S315A-, and p53-S315D-inhibited colony formation but had no effect on p53-S215A (Fig. 4B). In addition, flow cytometry analysis revealed that γ-irradiation arrested the cell cycle at G2/M phase in H1299 cells expressing wild-type p53, p53-S315A, and p53-S315D, which were overridden by Aurora-A (Fig. 4, C and D). Expression of Aurora-A, however, failed to rescue the cell cycle arrest in cells transfected with p53-S215A. Further, G2/M arrest was not observed in cells expressing p53-S215D in response to γ-irradiation (Fig. 4D). It is noted that expression of p53-S215D still exhibited some effects on cell survival and cell cycle progression as compared with p53-null cells transfected with pcDNA3 vector alone (Fig. 4, A and D). Nevertheless, these data indicate that phosphorylation of Ser-215 but not Ser-315 of p53 by Aurora-A has a direct inhibitory effect on p53 function.

DISCUSSION

Previous studies have shown that Aurora-A regulates several proteins that are important for mitosis. These proteins include histone H3, a key molecule in conversion of the relaxed interphase chromatin to mitotic condensed chromosomes (20); cytoplasmic polyadenylation element-binding protein, best known for its role in promoting polyadenylation of cyclin B mRNA (21); TACC3, a protein required for stabilization and organization of microtubules (22); Eg5, a kinesin-like protein involved in both centrosome separation and spindle assembly and stability (23); and TPX2, which is required to generate stable bipolar spindle (24). In this study, Ser-215 of p53 has been identified as a phosphorylation site of Aurora-A. Aurora-A phosphorylation of p53 directly inhibits p53 DNA binding and transactivation activity. As a result, p53 target genes, such as p21 and PTEN, were reduced by Aurora-A. Furthermore, p53-dependent proapoptotic function and G2/M cell cycle arrest in response to DNA damage were overridden by Aurora-A. These findings are important for several reasons. First, they provide a mechanistic understanding of the Aurora-A function in cell cycle, cell survival, and malignant transformation. Second, a direct link between Aurora-A and the DNA damage pathway has now been established. Finally, this is the first identification of a phosphorylation site within p53 DNA-binding domain.

p53 is composed of the following four functional domains that regulate its activity as a transcription factor: (i) an N-terminal transactivation domain, (ii) the central conserved core DNA-binding domain, (iii) a tetramerization domain, and (iv) a C-terminal negative regulatory domain. It has been shown that phosphorylation of p53 plays an essential role in activation and/or stabilization of the protein. Phosphorylation of Ser-15 stimulates p300/CBP (CREB-binding protein) to p53 in the N terminus and subsequent acetylation of the C-terminal domain of p53, which leads to its activation (25). Further, phosphorylation of p53 within the N-terminal domain at Thr-18 or Ser-20 can destabilize the p53-MDM2 complex or stabilize p53-p300 protein interactions (26–28). Enhanced phosphorylation of endogenous p53 protein at these sites following DNA damage, quiescence, or senescence can occur through the action of an ATM/ATR/DNA-PK/Chk1/Chk2 kinase-dependent pathway (29, 30). Flanking the tetramerization domain of p53 in the extreme C terminus is a negative regulatory domain whose posttranslational modification also plays an important role in modulating the specific activity of p53 in vivo. Phosphorylation of Ser-392 activates the latent specific DNA binding function of p53 in vitro and in vivo (31, 32).

Ser-315 locates within the nuclear localization signal (amino acids 305–322) of the C-terminal region of p53. Its phosphoryl-
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It has been shown that Ser-315 is a substrate of G2 and S phase cyclin-dependent kinases (33, 34). Phosphorylation of p53 at this site significantly enhances the sequence-specific DNA binding activity of p53 in vitro (34, 35). Conversely, a study has shown that phosphorylation of Ser-315 reverses the stabilizing and activating effects of Ser-392 phosphorylation on tetramer formation (36). Consistent with the latter observation, a recent report demonstrated that Aurora-A phosphorylation of Ser-315 induces MDM2-mediated p53 ubiquitination and degradation (2). Together, these data indicate that the phosphorylation of Ser-315 can have an inhibitory or a stimulatory role in modulating p53-dependent transcription, depending on the context. Nevertheless, no phosphorylation site of p53 has previously been identified within the DNA-binding domain (amino acids 102–292). Our findings indicate that Aurora-A directly phosphorylates Ser-215, as well as Ser-315, within the DNA-binding domain. Aurora-A abrogates p53 DNA binding and transactivation activity by phosphorylation of Ser-215 but not Ser-315.

In summary, we have demonstrated that Ser-215 is a novel phosphorylation site of p53. Aurora-A phosphorylates Ser-215 in vitro and in vivo, leading to direct abrogation of p53 DNA binding and transactivation activity. As a result, the p53 target genes p21 and PTEN are down-regulated, and p53 tumor suppressor activity is inhibited by Aurora-A. Further, nonphosphorylatable p53-S215A retained p53 function whereas phosphomimic p53-S215D lost its tumor suppressor activity. No naturally occurring mutation of Ser215A has been detected in human tumors (37, 38). These data indicate that phosphorylation of p53 at Ser-215 represents a key mechanism for cell cycle progression, cell survival, and malignant transformation induced by Aurora-A. Future investigation will be required to determine whether phosphorylation of Ser-215 occurs in human tumors by developing an anti-phosphoSer-215 p53 antibody and whether the Ser-215 is phosphorylated by other serine/threonine protein kinases.

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