AD_____

Award Number: W81XWH-05-1-0021

TITLE: Aurora-A Oncogene in Human Ovarian Cancer

PRINCIPAL INVESTIGATOR: Jin Q. Cheng, Ph.D., M.D.
Santo V. Nicosia

CONTRACTING ORGANIZATION: University of South Florida
Tampa, FL 33620

REPORT DATE: November 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-11-2005	Annual	20 Oct 2004 – 19 Oct 2005

4. TITLE AND SUBTITLE
Aurora-A Oncogene in Human Ovarian Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0021

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jin Q. Cheng, Ph.D., M.D.
Santo V. Nicosia

E-mail: chengjq@moffitt.usf.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of South Florida
Tampa, FL 33620

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During past year, we have demonstrated that the Aurora-A interacts with and phosphorylates p53 tumor suppressor at serine-215 leading to abrogation of p53 DNA-binding and transactivation activity. Downstream target genes of p53, such as p21 and PTEN, were inhibited by Aurora-A in a Ser-215 phosphorylation-dependent manner. As a result, Aurora-A overrides the apoptosis and cell cycle arrest induced by cisplatin and gamma-irradiation, respectively. Our data indicate that phosphorylation of p53 at Ser-215 by Aurora-A is a major mechanism to inactivate p53. We have also shown that ectopic expression of Aurora-A induces chemoresistance in ovarian cancer cells. Further, Aurora-A inhibits BAX conformation change by inactivation of JNK and activation of Akt. In addition, we have identified 4 potential Aurora-A inhibitors by screening of a Small Focus chemical compound library.

15. SUBJECT TERMS
Aurora-A, p53, BAX, Akt, inhibitor, apoptosis, cisplatin

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	16	

Introduction

Three tasks have been proposed in this project: 1) examine the clinicalpathological significance and the mechanism of Aurora-A overexpression/activation in ovarian cancer; 2) determine the role of overexpression/activation of Aurora-A in OSE cell transformation *in vitro* and *in vivo* and 3) examine the role of overexpression/activation of Aurora-A in chemoresistance and as a target for ovarian cancer intervention.

Body:

During the last budget year, we have demonstrated that Aurora-A interacts with and phosphorylates p53 leading to abrogation of p53 function. We have also shown that Aurora-A inhibits BAX conformational changes resulting in chemoresistance. Further, four potential Aurora-A inhibitors have been identified by screening a Small Focus chemical compound library.

1. Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215.

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 posttranslational modifications and association with other proteins have been implicated in the regulation of its stability and transactivation activity. 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, we examined the possible regulation of p53 by Aurora-A. 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 (1), 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 gamma-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 (2). 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 (3, see appendix).

2. Aurora-A induces chemoresistance and inhibits cisplatin-stimulated Bax conformational change and apoptosis through inactivation of JNK and activation of Akt.

We have previously shown frequent alterations of Aurora-A in human ovarian cancer and recently demonstrated the involvement of Aurora-A in chemoresistance. Ectopic expression of Aurora-A renders A2780S resistant to cisplatin-, Taxol, VP16-induced apoptosis (Figs. 1A and 1B). Further, we show that Aurora-A represses the cisplatin-induced Bax conformational change through inhibition of JNK and activation of Akt. Upon cisplatin treatment, BAX is activated and cytochrome C is released in pcDNA3-A2780S but not Aurora-A-A2780S cells (Figs. 1C and 1D). Meanwhile, we have observed that Aurora-A significantly inhibits cisplatin-induced JNK phosphorylation (Fig. 2A). In addition, elevated level of phospho-Akt and Akt kinase activity were also detected in Aurora-A-A2780S but not in pcDNA3-A2780S cells (Figs. 2B and 2C). Previous studies showed that JNK phosphorylates 14-3-3 protein which leads to activation of BAX (4), whereas Akt directly

phosphorylates BAX and inhibits BAX conformational change (5). Therefore, our data indicate that Aurora-A induces chemoresistance by inhibition of BAX activation and apoptosis through inactivation of JNK and activation of Akt cascade.

3 Identification of Aurora-A inhibitors.

In collaboration with Dr. Nicholas Lawrence at H. Lee Moffitt Cancer Center, we have recently screened a Small Focus chemical compound library using *in vitro* kinase assay. The levels of Aurora-A phosphorylation of Histone H3 were significantly inhibited by compounds RPM215, RPM219, RPM223 and ZK424 (Fig. 3A). Immunocytochemical staining showed that phospho-Ser10 of Histone H3 is inhibited by these compounds during mitosis (Fig. 3B). In addition, the compounds inhibit tumor cell growth in a dose-dependent manner (Fig. 3C). These data suggest that RPM215, RPM219, RPM223 and ZK424 are potential Aurora-A inhibitors.

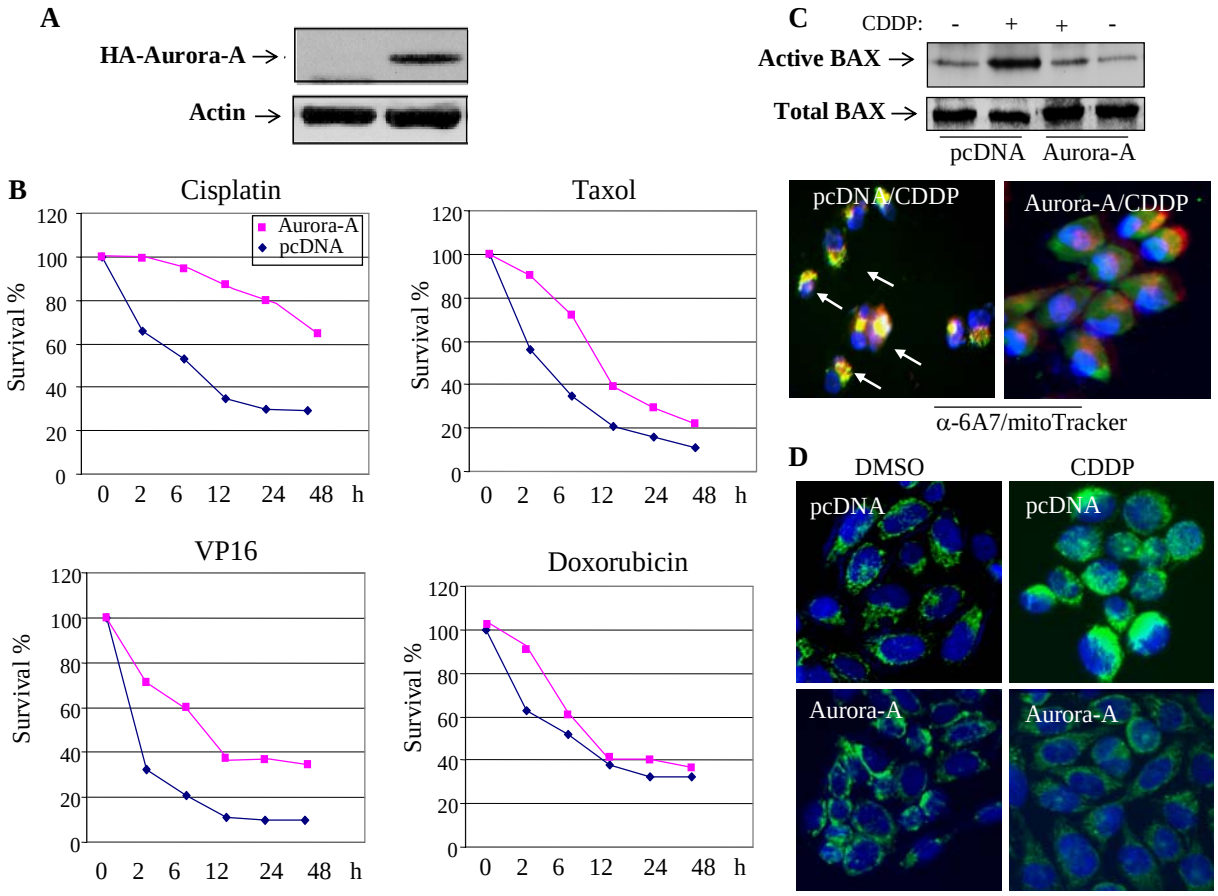

Fig. 1. Aurora-A inhibits apoptosis induced by chemotherapeutic drugs. *(**A**) Immunoblotting analysis of A2780S cells stably transfected with HA-Aurora-A (right lane) and pcDNA (left lane) with anti-HA (top) and actin (bottom) antibodies. . (**B**) Aurora-A-A2780S cells resist to cisplatin (CDDP)-, VP16- and Taxol-induced cell death. (**C**) Aurora-A inhibits BAX activation induced by CDDP. Aurora-A- and pcDNA-A2780S cells were treated with CDDP, immunoprecipitated with anti-active BAX (6A7) and detected with anti total BAX antibody (top). Panel 2 is the total cell lysate immunoblotted with anti-total BAX antibody. Bottom panel shows that pcDNA3- and Aurora-A-A2780S cells were immunostained with anti-active BAX antibody and MitoTracker*

*after CDDP treatment. (**D**) Aurora-A inhibits cytochrome c release. Cells were treated with vehicle DMSO or CDDP and immunostained with anti-cytochrome c antibody.*

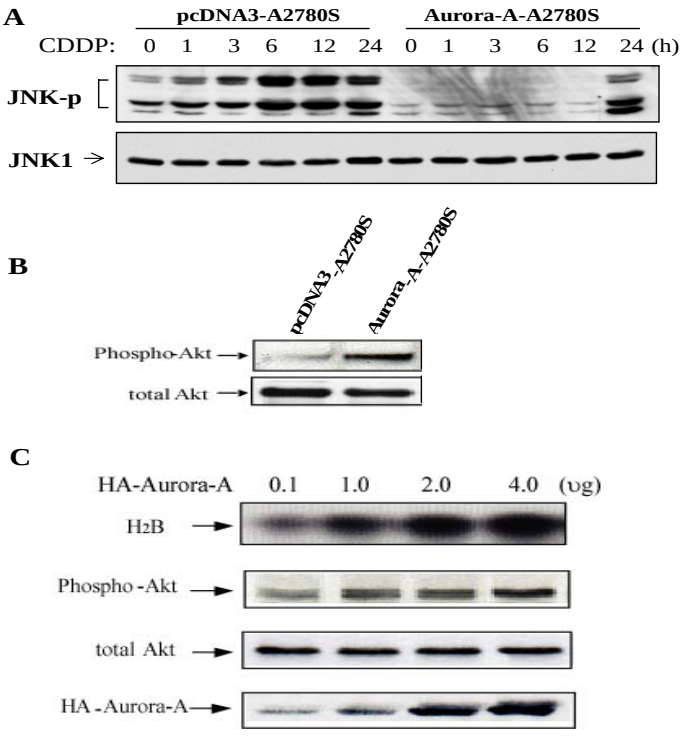

Fig. 2. Aurora-A inhibits JNK and activates Akt. *(**A**) Aurora-A reduces CDDP-induced JNK activation. pcDNA3-A2780S and Aurora-A-A2780S cells were treated with CDDP at indicated times and immuno-blotted with anti-phospho-JNK (top) and total JNK (bottom) antibodies. (**B**) Phospho-Akt is elevated in Aurora-A-A2780S cells. pcDNA3-A2780S and Aurora-A-A2780S cells were serum-starved for 12 h and immuno-blotted with anti-phospho-T308 Akt (top) and total Akt (bottom) antibodies. (**C**) Aurora-A activates Akt. A2780S cells were transiently transfected with increasing amount of HA-Aurora-A and immuno-precipitated with anti-Akt antibody. The immunoprecipitates were subjected to in vitro kinase assay using Histone H2B as substrate (upper). Total cell lysates were immuno-blotted with indicated antibodies (panels 2-4)*

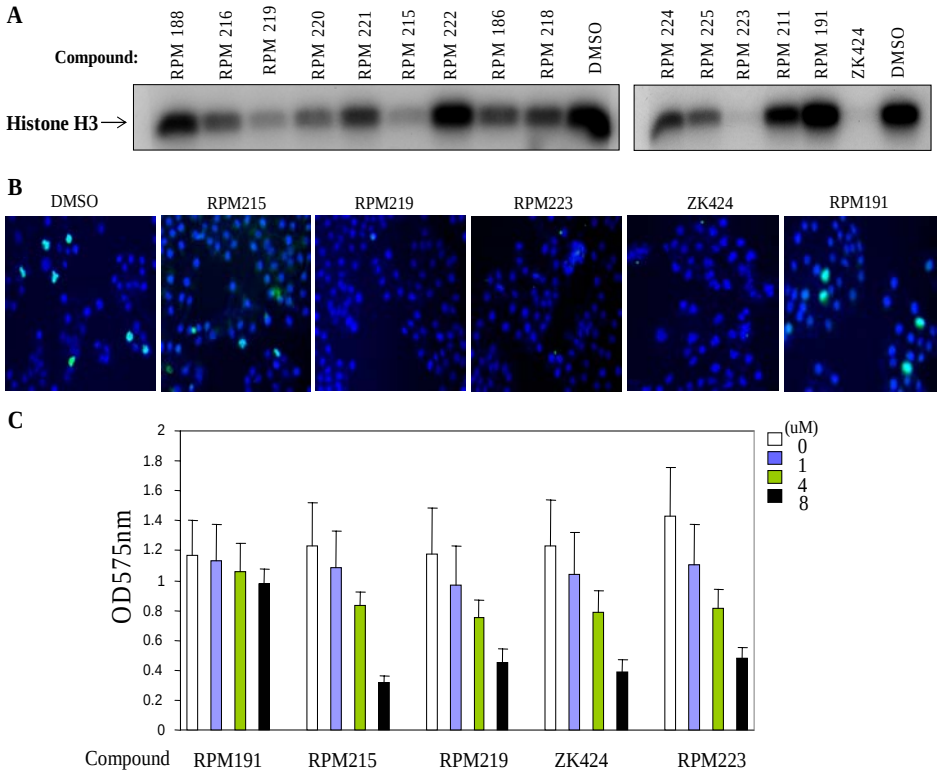

Fig. 3. Identification of Aurora-A inhibitors. *(**A**) In vitro kinase assays were performed by incubation of recombinant Aurora-A protein with individual compound (5 μM) using Histone H3 as substrate. (**B**) Aurora-A-A2780S cells were treated with indicated compound and immuno-stained with anti-phospho-Ser10 Histone H3 antibody. (**C**) OVCAR-3 cells that express elevated level of Aurora-A were treated with indicated compound at different concentrations. After 48 h treatment, cells were subjected to MTT assay. The experiments are triplicate.*

Key Research Accomplishment

1 Aurora-A abrogates p53 DNA-binding and transactivation activity through phosphorylation of serine-215.
2 Aurora-A contributes chemoresistance in human ovarian cancer.
3 Aurora-A inhibits BAX conformational change through inactivation of JNK and activation of Akt.
4 Four potential Aurora-A inhibitors have been identified.

Reportable Outcomes

Publication:

1. Liu, Q., Kaneko, S., Yang, L., Feldman, R.I., Nicosia, S.V., Chen, J.D., and Cheng, J.Q. Aurora-A abrogates p53 DNA-binding and transactivation activity by phosphorylation of serine-215. *J. Biol. Chem.* 279:52175-52182, 2004.

2. Cheng, JQ, Lindsley CW, Cheng GZ, Yang H, Nicosia SV. The Akt/PKB pathway: molecular target for cancer drug discovery. *Oncogene* 24:7482-7492, 2005.

3. Yuan ZQ, Kim D, Kaneko S, Sussman M, Bokoch GM, Kruh GD, Nicosia SV, Testa JR, Cheng JQ. ArgBP2γ interacts with Akt and p21-activated kinase-1 and promotes cell survival. *J Biol Chem.* 280:21483-21490, 2005.

4. Nam S, Buettner R, Turkson J, Kim D, Cheng JQ, Muehlbeyer S, Hippe F, Vatter S, Merz KH, Eisenbrand G, Jove R. Indirubin derivatives inhibit Stat3 signaling and induce apoptosis in human cancer cells. *Proc Natl Acad Sci USA.* 102:5998-6003, 2005.

5. Park S, Kim D, Szewczyk KM, Nicosia SV, Yu H, Jove R, Cheng JQ. Molecular cloning and characterization of the human Akt1 promoter uncovers its upregulation by the Src/Stat3 pathway. *J Biol Chem.* 2005 Sep 20; [Epub ahead of print]

6. Xu Q, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, Gritsko T, Turkson J, Kay H, Semenza GL, Cheng JQ, Jove R, Yu H. Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. *Oncogene.* 24:5552-5560, 2005.

Abstract/presentation

1. Yang H, Liu Q, Cheng GZ, He L, Nicosia SV, Cheng JQ. Crosstalk between Aurora-A and GSK3β/β-catenin pathways induces c-Myc and cyclin D1. 96th American Association for Cancer Research Annual Meeting, 2005.

2. Yuan Z-Q, Kim D, Kaneko S, Nicosia SV, Cheng JQ. Akt/PKB interaction protein APαB mediates Akt and PAK1 cell survival signal. 96th American Association for Cancer Research Annual Meeting, 2005.

3. Yang H, Kruk P, Sun M, Nicosia SV, Cheng JQ. PI3K/AKT2 mediates HER-2/neu-induced telomerase activation through NFκB pathway. 96th American Association for Cancer Research Annual Meeting, 2005.

4. Kim D, Nicosia SV, Cheng JQ. Negative Feedback Regulation of Akt through Autophosphorylation. 96th American Association for Cancer Research Annual Meeting, 2005.

Conclusion

1. Aurora-A inhibits p53 function.
2. Elevated Aurora-A contributes to chemoresistance.
3. Aurora-A inhibits BAX conformational change.
4. Aurora-A activates Akt and inhibits JNK
5. Aurora-A inhibitors could have a great potential for anti-tumor drug discovery.

References

1. Brooks, C. L., and Gu, W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. *Curr. Opin. Cell Biol.* 15, 164–171, 2003.
2. Katayama, H., Sasai, K., Kawai, H., Yuan, Z. M., Bondaruk, J., Suzuki, F., Fujii, S., Arlinghaus, R. B., Czerniak, B. A., and Sen, S. Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53. *Nat. Genet.* 36, 55–62, 2004.
3. Liu, Q., Kaneko, S., Yang, L., Feldman, R.I., Nicosia, S.V., Chen, J.D., and Cheng, J.Q. Aurora-A abrogates p53 DNA-binding and transactivation activity by phosphorylation of serine-215. *J. Biol. Chem.* 279:52175-52182, 2004.
4. Tsuruta F, Sunayama J, Mori Y, Hattori S, Shimizu S, Tsujimoto Y, Yoshioka K, Masuyama N, Gotoh Y. JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. *EMBO J.* 23:1889-1899, 2004.
5. Gardai SJ, Hildeman DA, Frankel SK, Whitlock BB, Frasch SC, Borregaard N, Marrack P, Bratton DL, Henson PM. Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. *J Biol Chem.* 279:21085-21095, 2004.

Appendices

1. Liu, Q., Kaneko, S., Yang, L., Feldman, R.I., Nicosia, S.V., Chen, J.D., and Cheng, J.Q. Aurora-A abrogates p53 DNA-binding and transactivation activity by phosphorylation of serine-215. *J. Biol. Chem.* 279:52175-52182, 2004.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 50, Issue of December 10, pp. 52175–52182, 2004
Printed in U.S.A.

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

Received for publication, June 17, 2004, and in revised form, October 6, 2004
Published, JBC Papers in Press, October 6, 2004, DOI 10.1074/jbc.M406802200

Qiyuan Liu‡, Satoshi Kaneko‡, Lin Yang‡, Richard I. Feldman§, Santo V. Nicosia‡, Jiandong Chen‡, and Jin Q. Cheng‡¶

From the ‡Departments of Pathology and Interdisciplinary Oncology, University of South Florida College of Medicine and H. Lee Moffitt Cancer Center, Tampa, Florida 33612 and the §Cancer Research Department, Berlex Biosciences, Richmond, California 94804

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, *IPL1* (3). The *Drosophila* 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 G_2/M progression of the cell cycle. Their regulatory influence spans from G_2 to cytokinesis, encompassing key cell cycle events such as centrosome duplication, chromosome bi-orientation and segregation, and cleavage-furrow positioning and ingression (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 pDsRed2C1 (EcoRI/KpnI), pEGFPC3 (BglII/KpnI), and pGEX-4T1 (BamHI/EcoRI) vectors. Mutant constructs of *p53* were prepared with a mutagenesis kit (Stratagene) using pcDNA3-HA[1]-*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

* This work was supported by grants from the National Cancer Institute and the Department of Defense (to J. Q. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Pathology, University of South Florida College of Medicine and H. Lee Moffitt Cancer Center, 12901 Bruce B. Downs Blvd., MDC Box 11, Tampa, FL 33612. Tel.: 813-974-8595; Fax: 813-974-5536; E-mail: jcheng@hsc.usf.edu.

[1] The abbreviations used are: HA, hemagglutinin; GFP, green fluorescent protein; EMSA, electrophoresis mobility shift assay; RNAi, RNA interference; CREB, cAMP-response element-binding protein.

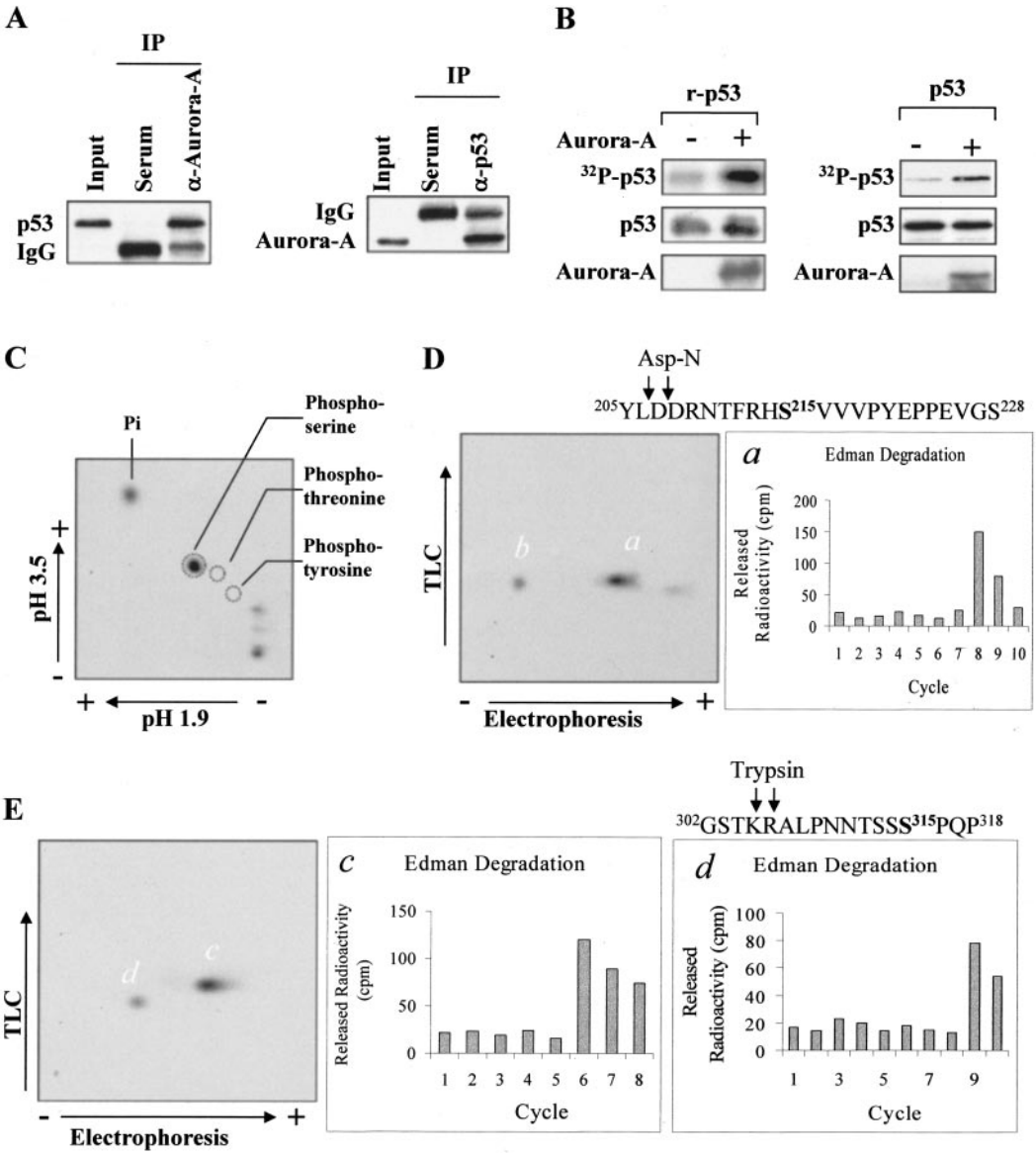

FIG. 1. **Aurora-A primarily phosphorylates p53 at Ser-215.** *A*, interaction of Aurora-A with p53. A2780S cell lysates were immunoprecipitated with anti-Aurora-A antibody and detected with anti-p53 antibody (*left panel*) and vice versa (*right panel*). *B*, Aurora-A phosphorylation of p53 *in vitro* 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* [^{32}P]orthophosphate-labeling experiment. p53-null HCT116 cells were transfected with HA-p53 with or without FLAG-Aurora-A, labeled with [^{32}P]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 detected 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* [^{32}P]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.

measured by normalizing the luciferase activity to the corresponding β-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 6 N HCl for 1 h at 110 °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 6 h 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

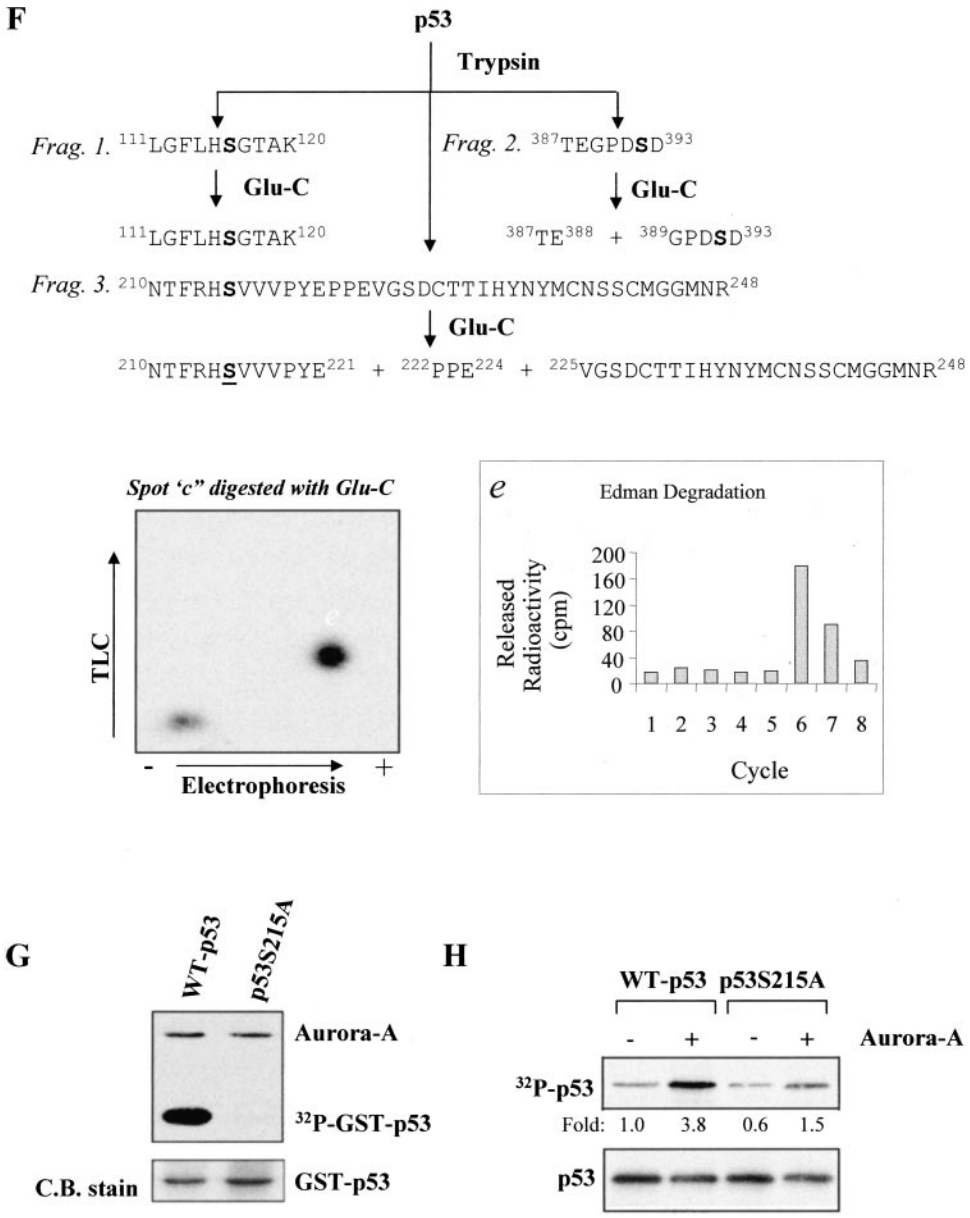

FIG. 1—*continued*

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 ^{32}P was determined by Cerenkov counting.

EMSA—EMSA was performed as described previously (14). Briefly, nuclear extract was incubated with ^{32}P-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. 1*A*). Further ex-

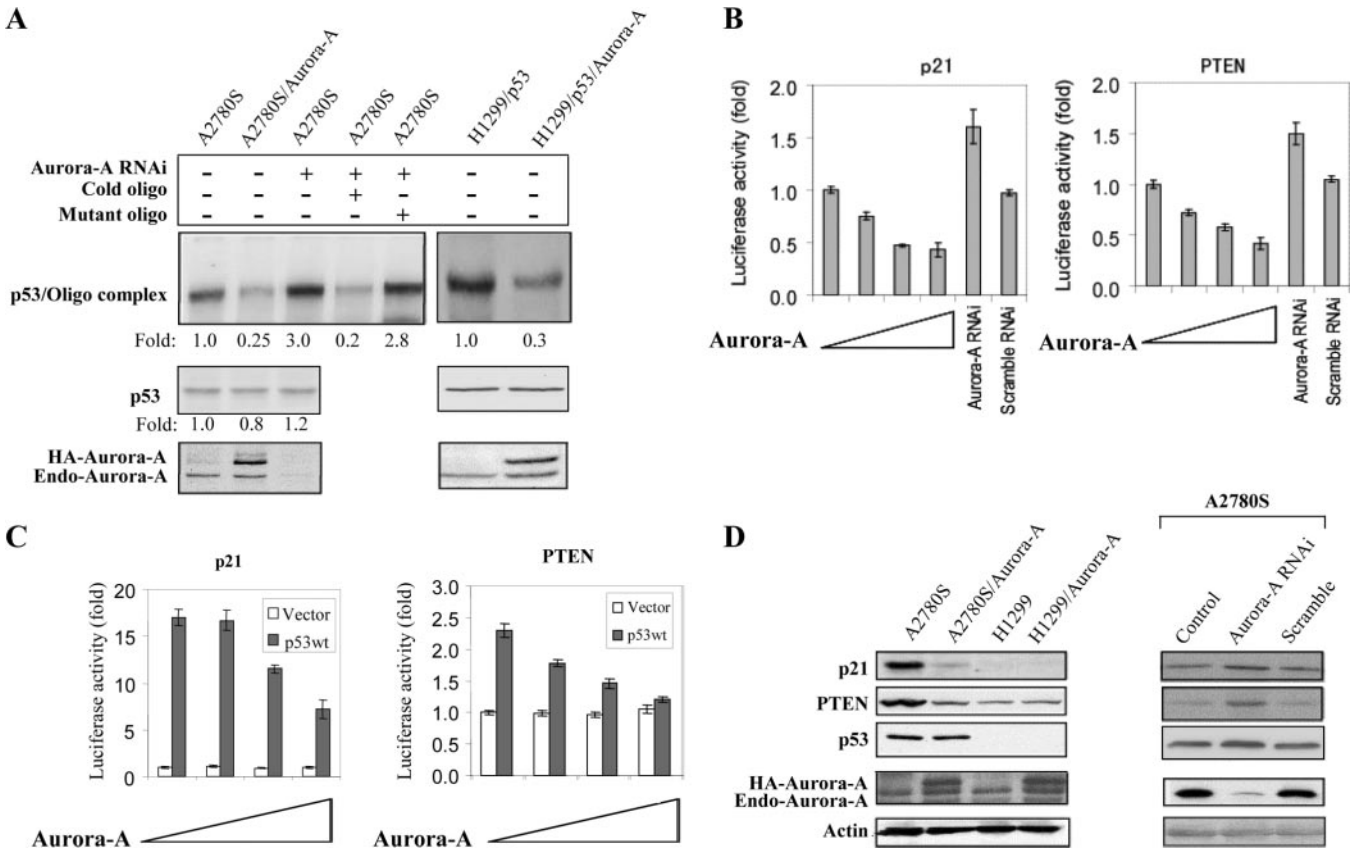

FIG. 2. **Aurora-A inhibits p53 DNA binding and transactivation activity.** *A*, DNA binding activity was abrogated by Aurora-A. EMSA assay was carried out with nuclear extract prepared from A2780S (wild-type p53) and H1299 (p53-null) cells that were transfected and/or treated with indicated plasmids or RNAi. An equal amount of ^{32}P-labeled p53 binding consensus oligonucleotides was incubated with the nuclear extract in the presence or absence of a 40-fold excess of unlabeled wild-type or mutant oligonucleotides (*cold oligo*). The reaction was separated by a native polyacrylamide gel and bands were quantified (*upper panel*). Expression of transfected p53 and HA-Aurora-A is shown in the *middle* and *bottom panels*. *B* and *C*, reporter assay. A2780S (*B*) and H1299 (*C*) cells were transfected with p21- or PTEN promoter-driven luciferase, pCMV-β-galactosidase, increasing amounts of Aurora-A and RNAi, and wild-type p53 (H1299, *p53wt*). Luciferase activity was measured and normalized to β-galactosidase. Results are the mean ± S.E. of three independent experiments performed in triplicate. *D*, Aurora-A inhibits expression of p53-targeting genes *p21* and *PTEN*. A2780S and H1299 cells were transfected, treated with indicated plasmids or RNAi, and immunoblotted with indicated antibodies.

periments revealed that Aurora-A phosphorylated p53 *in vitro* and *in vivo* (Fig. 1*B*). Phosphoamino acid analysis revealed that Aurora-A phosphorylated p53 on serine residue(s) (Fig. 1*C*). 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. 1*D*). Amino acid analysis by Edman degradation of the phosphopeptides demonstrated that the activity of radioisotope 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. 1*D*). 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 ^{208}DRNTFRH**S**VVVPYEPPEVGS^{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. 1*E*) and trypsin/Glu-C (Fig. 1*F*). 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. 2*A* 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 $p21^{Cip/WAF1}$ and *PTEN* 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. 2*B*). These results were further supported by cotransfection of *p53* and *Aurora-A* in p53-null H1299 cells (Fig. 2*C*). Accordingly, protein levels of p21 and PTEN were reduced by ectopic expression of Aurora-A and elevated by knockdown of endogenous Aurora-A (Fig. 2*D*). 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.

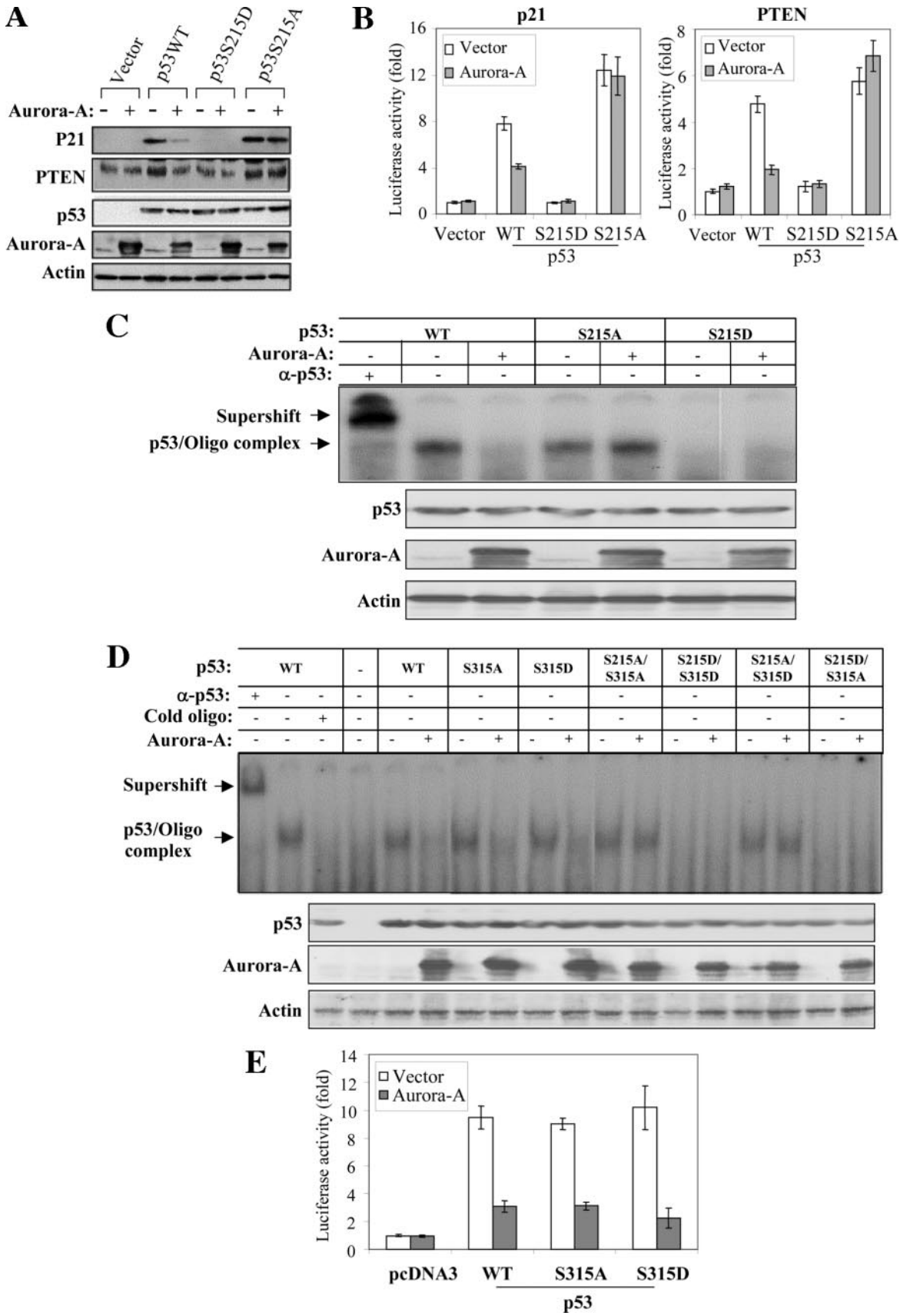

FIG. 3. **Aurora-A inhibition of p53 transactivation and DNA binding activity depends on phosphorylation of Ser-215 but not Ser-315.** *A*, Western blot analysis. H1299 cells were transfected with indicated plasmids, lysed, and immunoblotted with indicated antibodies. *B–E*, reporter (*B* and *E*) and 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.

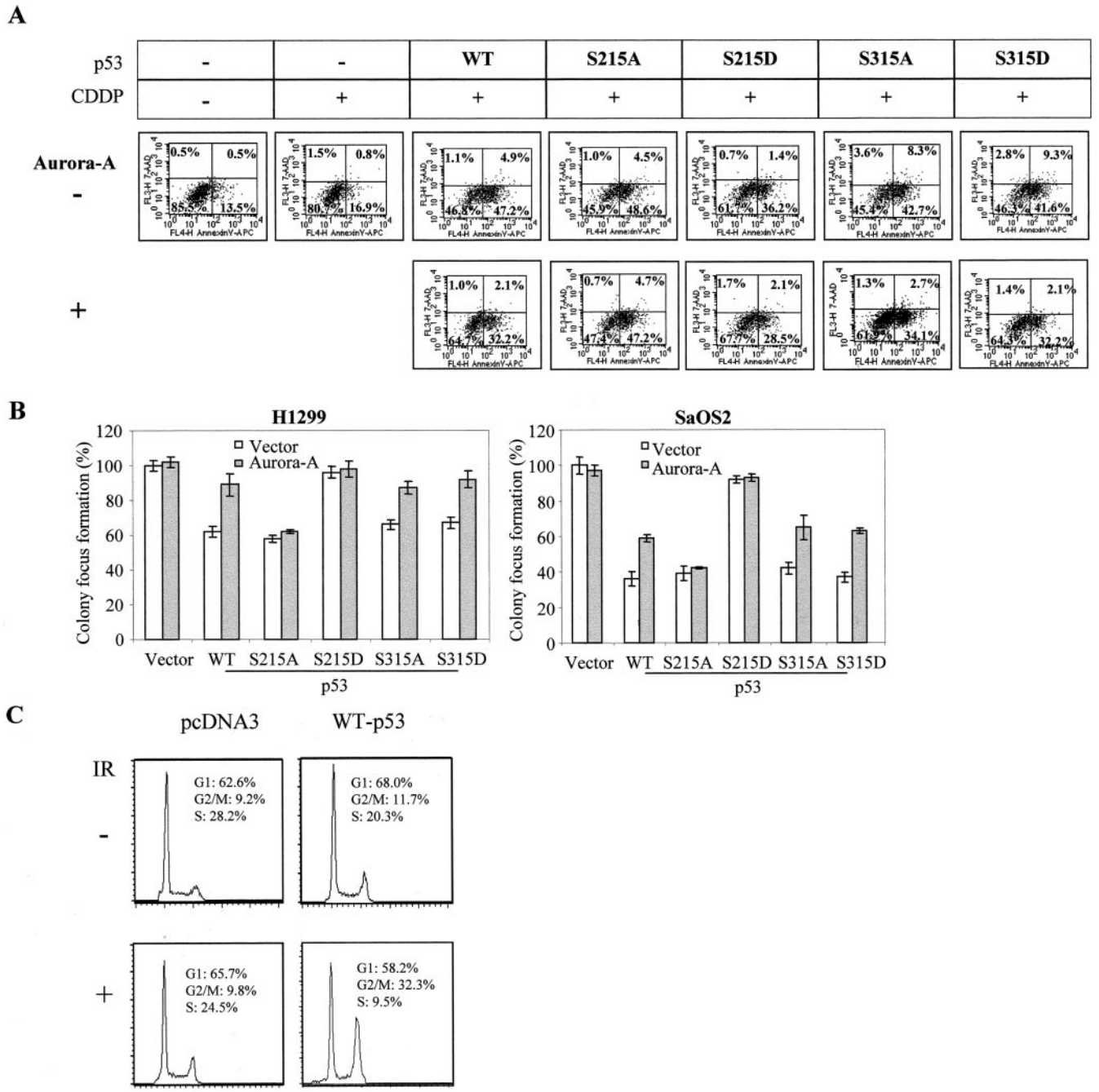

FIG. 4. **Aurora-A inhibition of p53 biological function through phosphorylation of Ser-215.** *A*, apoptosis. Cisplatin-resistant A2780CP cells were transfected with different forms of p53 and GFP with or without Aurora-A. After 36 h of transfection, cells were treated with cisplatin for 12 h. Apoptosis was examined by Annexin-V assay in GFP-positive cells. *B*, colony formation. p53-null H1299 and SaOS2 cells were transfected with indicated plasmids. After 3 weeks, the number of colonies was counted. Each experiment was performed in triplicate. *C* and *D*, cell cycle analysis. After transfection with p53, Aurora-A, and GFP, H1299 cells were treated with γ-irradiation at 12 gray (*C* and *D*, *bottom panels*). Cell cycle was analyzed 12 h postirradiation in GFP-positive cells. *WT*, wild type. *CDDP*, cisplatin.

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. 3*C*). 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, the 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

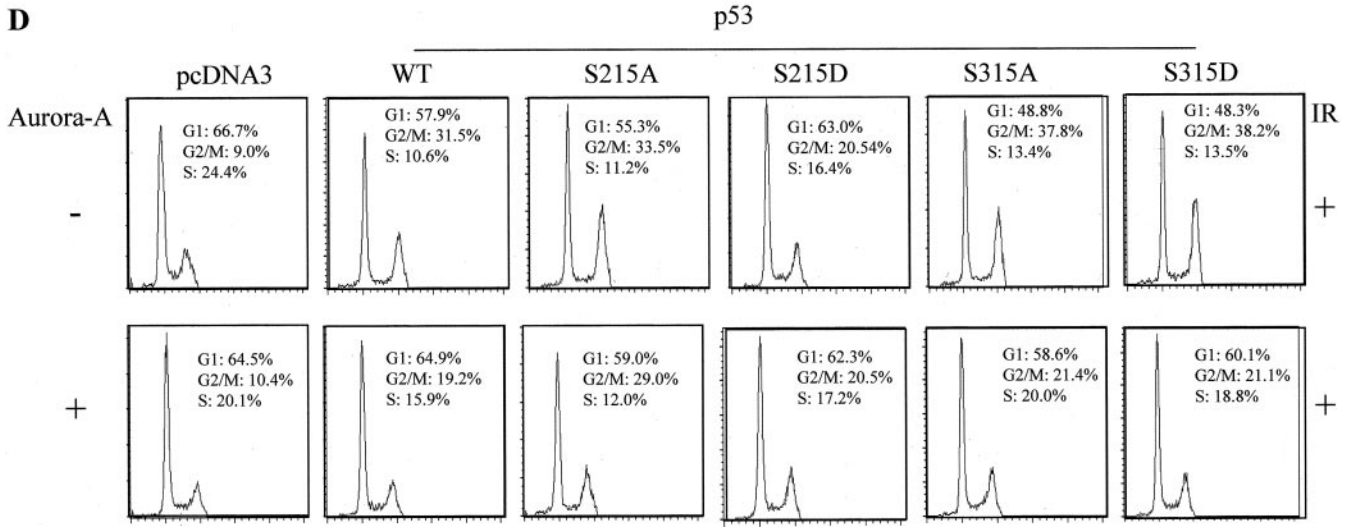

FIG. 4—*continued*

DNA binding capability (Fig 3*D*). 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. 4*A* 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. 4*A*). 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. 4*B*). In addition, flow cytometry analysis revealed that γ-irradiation arrested the cell cycle at G_2/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, G_2/M arrest was not observed in cells expressing p53-S215D in response to γ-irradiation (Fig. 4*D*). 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 G_2/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-

ation, however, does not change subcellular localization of p53. It has been shown that Ser-315 is a substrate of G_2 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 S215A 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.

Acknowledgment—We are grateful to the Molecular Biology DNA Sequence and Flow Cytometry Facilities at H. Lee Moffitt Cancer Center.

REFERENCES

1. Deleted in proof
2. 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
3. Francisco, L., Wang, W., and Chan, C. S. (1994) *Mol. Cell. Biol.* **14,** 4731–4740
4. Meraldi, P., Honda, R., and Nigg, E. A. (2004) *Curr. Opin. Genet. Dev.* **14,** 29–36
5. Mendez, R., Hake, L. E., Andresson, T., Littlepage, L. E., Ruderman, J. V., and Richter, J. D. (2000) *Nature* **404,** 302–307
6. Higuchi, T., and Uhlmann, F. (2003) *Nature* **426,** 780–781
7. Sen, S., Zhou, H., and White, R. A. (1997) *Oncogene* **14,** 2195–2200
8. Goepfert, T. M., Adigun, Y. E., Zhong, L., Gay, J., Medina, D., and Brinkley, W. R. (2002) *Cancer Res.* **62,** 4115–4122
9. Anand, S., Penrhyn-Lowe, S., and Venkitaraman, A. R. (2003) *Cancer Cells* **3,** 51–62
10. Yuan, Z. Q., Feldman, R. I., Sun, M., Olashaw, N. E., Coppola, D., Sussman, G. E., Shelley, S. A., Nicosia, S. V., and Cheng J. Q. (2002) *J. Biol. Chem.* **277,** 29973–29982
11. Gritsko, T. M., Coppola, D., Paciga, J. E., Yang, L., Sun, M., Shelley, S. A., Fiorica, J. V., Nicosia, S. V., and Cheng, J. Q. (2003) *Clin. Cancer Res.* **9,** 1420–1426
12. Le Goff, P. L., Montano, M. M., Schodin, D. J., and Katzenellenbogen, B. S. (1994) *J. Biol. Chem.* **269,** 4458–4466
13. Rowan, B. G., Weigel, N. L., and O'Malley, B. W. (2000) *J. Biol. Chem.* **275,** 4475–4483
14. Kaneko, S., Feldman, R. I., Yu, L., Wu, Z., Gritsko, T., Shelley, S. A., Nicosia, S. V., Nobori, T., and Cheng, J. Q. (2002) *J. Biol. Chem.* **277,** 23230–23235
15. Quartin, R. S., Cole, C.N., Pipas, J.M., and Levine, A.J. (1994) *J. Virol.* **68,** 1334–1341
16. Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and VandeVoude, G. F. (1996) *Science* **271,** 1744–1747
17. Zhou, H., Kuang, J., Zhong, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R., and Sen, S. (1998) *Nat. Genet.* **20,** 189–193
18. Ausubel, F. M., Brent, R., Kinston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1999) *Current Protocols in Molecular Biology*, pp. 18.9.1–18.9.28, 1st Ed., John Wiley & Sons, Inc., New York
19. Leung, B. M., Fraser, M., Yan, X., Dan, H. C., Cheng, J. Q., and Tsang, B. K. (2003) *Cancer Res.* **63,** 7081–7088
20. Scrittori, L., Hans, F., Angelov, D., Charra, M., Prigent, C., and Dimitrov, S. (2001) *J. Biol. Chem.* **276,** 30002–30010
21. Huang, Y. S., Jung, M. Y., Sarkissian, M., and Richter, J. D. (2002) *EMBO J.* **21,** 2139–2148
22. Giet, R., McLean, D., Descamps, S., Lee, M. J., Raff, J. W., Prigent, C., and Glover, D. M. (2002) *J. Cell Biol.* **156,** 437–451
23. Castro, A., Mandart, E., Lorca, T., and Galas, S. (2003) *J. Biol. Chem.* **278,** 2236–2241
24. Kufer, T. A., Sillje, H. H., Korner, R., Gruss, O. J., Meraldi, P., and Nigg, E. A. (2002) *J. Cell Biol.* **158,** 617–623
25. Dumaz, N., and Meek, D. W. (1999) *EMBO J.* **18,** 7002–7010
26. Craig, A. L., Burch, L., Vojtesek, B., Mikutowska, J., Thompson, A., and Hupp, T. R. (1999) *Biochem. J.* **342,** 133–141
27. Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Vogt Sionov, R., Lozano, G., Oren, M., and Haupt, Y. (1999) *EMBO J.* **18,** 1805–1814
28. Sakaguchi, K., Saito, S., Higashimoto, Y., Roy, S., Anderson, C. W., and Appella, E. (2000) *J. Biol. Chem.* **275,** 9278–9283
29. Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999) *Genes Dev.* **13,** 152–157
30. Lakin, N. D., Hann, B. C., and Jackson, S. P. (1999) *Oncogene* **18,** 3989–3995
31. Hupp, T. R., Sparks, A., and Lane, D. P. (1995) *Cell* **83,** 237–245
32. Blaydes, J. P., Craig, A. L., Wallace, M., Ball, M. L., Traynor, N., Gibbs, N., and Hupp, T. R. (2000) *Oncogene* **19,** 3829–3839
33. Bischoff, J. R., Friedman, P. N., Marshak, D. R., Prives, C., and Beach, D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4766–4770
34. Wang, Y., and Prives, C. (1995) *Nature* **376,** 88–91
35. Fuchs, B., Hecker, D., and Scheidtmann, K. H. (1995) *Eur. J. Biochem.* **228,** 625–639
36. Sakaguchi, K., Sakamoto, H., Lewis, M. S., Anderson, C. W., Erickson, J. W., Appella, E., and Xie, D. (1997) *Biochemistry* **36,** 10117–10124
37. Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R., and Harris, C. C. (1994) *Nucleic Acids Res.* **22,** 3551–3555
38. Hollstein, M., Shomer, B., Greenblatt, M., Soussi, T., Hovig, E., Montesano, R., and Harris, C. C. (1996) *Nucleic Acids Res.* **24,** 141–146