Phosphorylation of p68 RNA Helicase Plays a Role in Platelet-derived Growth Factor-induced Cell Proliferation by Up-regulating Cyclin D1 and c-Myc Expression*

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Phosphorylation of p68 RNA helicase is a prototypical member of DEAD box family RNA helicase. The protein plays an important role in the cell developmental program and organ maturation. We demonstrated previously that, in response to growth factors platelet-derived growth factor (PDGF)-BB stimulation, p68 is phosphorylated at Tyr593, and the phosphorylation of p68 promotes epithelial-mesenchymal transition via promoting β-catenin nuclear translocation (Yang, L., Lin, C., and Liu, Z. R. (2006) Cell 127, 139–155). We show here that the tyrosine phosphorylation of p68 also mediates the effects of PDGF in stimulating cell proliferation. The phosphorylated p68 (referred to as phospho-p68) promotes cell proliferation by activating the transcription of cyclin D1 and c-Myc genes. We show that the ATPase/helicase activities of p68 are required for the activation of cyclin D1 transcription. The phospho-p68 participates in the complex assembled at the cyclin D1 and c-Myc promoters, which strongly suggests a direct role in transcriptional regulation. Furthermore, our data demonstrated that the phosphorylation of p68 at Tyr593 plays a role in mediating the autocrine loop effects of PDGF, suggesting an important role for p68 phosphorylation in cell proliferation.

Platelet-derived growth factor (PDGF)3 plays an important role in cell proliferation (1), which is essential for early organ development, tissue remodeling, and wound healing (2–4). The growth factors act through the cell surface receptor PDGFR (5). The PDGFRs are prototypical receptor tyrosine kinases (6). The receptor dimer interacts with a number of Src homology domain 2-containing proteins that are connected to various cellular signal transduction pathways, such as phospholipase Cγ, Ras GTPase-active protein, and Src family tyrosine kinases (7–10). An increase in PDGF level or PDGFR activity is often observed in a variety of human diseases, including glomerulosclerosis (1, 11) and diabetic proliferation retinopathy (12). Abnormally high levels of PDGF or PDGFR activity are also associated with cancer progression, including invasive gastric carcinomas and gliomas (13, 14). In human gastric carcinoma, a high level of PDGF is an effective prognostic marker (15). It was demonstrated from transgenic studies that high levels of PDGFB promoted oligodendroglialomas and oligoastrocytomas in neural progenitors or glial cells (16–18).

The molecular mechanism by which PDGF stimulates cell proliferation is not well understood. Recent works have demonstrated that c-Abl plays a role in cellular response to PDGF stimulation. PDGF stimulation activates the membrane pool of c-Abl mediated by Src family of kinases in fibroblast cells. The activation of c-Abl consequently leads to c-Myc expression and DNA synthesis in cells (19), which is important for mitogenesis. It has also been suggested that the same signaling cascade promotes cell morphology changes (20, 21). The c-Abl kinase is a proto-oncogene nonreceptor tyrosine kinase (22, 23). The kinase localizes to the plasma membrane, the cytoplasm, as well as the cell nucleus (24). The activated c-Abl kinase targets a variety of cellular proteins, including c-Jun (25), Mdm2 (26), p73 (27–29), and Dok1 (30). In the nucleus, c-Abl expression responds to DNA damage (31, 32). The c-Abl pools in the plasma membrane and the cytoplasm regulate the cell shape and movement (21, 33).

The PDGF autocrine loop is one of the common growth factor autocrine loops (18). The cells co-express the growth factor PDGF and its receptors PDGFRs. Because of its powerful role in stimulating cell proliferation, angiogenesis, and EMT (34–36), the PDGF autocrine loop is often seen in the most aggressive diseases, such as glioblastoma (10), many metastatic cancers and cancer relapses (35–37). The PDGF autocrine loop also plays an important role in cell survival (38, 39). Cancer cells with PDGF autocrine loop activity often acquire strong resistance to multiple anticancer drug treatments (40). Because of the important role in tumor progression, PDGFR and the PDGF autocrine loop are important targets for designing the anticancer therapies. However, efforts are impeded by the limited knowledge about the functions of the PDGF autocrine loop in tumor progression at the molecular level. Although the PDGF autocrine loop was described extensively in gliomas, recent studies demonstrated that the PDGF autocrine loop functions in tumors of many other tissue/cell types (41, 42).

The nuclear p68 RNA helicase is a prototypical member of DEAD box family of RNA helicase (43, 44). As an early example

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3 The abbreviations used are: PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; EMT, epithelial-mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; RNAi, RNA interference; HA, hemagglutinin; wt, wild type; BrdUrd, bromodeoxyuridine; TCF, T-cell factor.
of a cellular RNA helicase, the ATPase and the RNA unwinding activities of p68 RNA helicase were documented with the protein that was purified from human 293 cells (45–47). p68 RNA helicase plays a very important role in cell proliferation and early organ development and maturation (48, 49). The protein shows clear cell cycle-related localization in the nucleus. Related to the role of p68 RNA helicase in cell proliferation, the expression of the protein was shown to correlate with tumor progression and transformation (50–52). We have previously reported that p68 RNA helicase is phosphorylated at multiple amino acid residues, including serine/threonine and tyrosine (53). We also demonstrated that p68 was phosphorylated at tyrosine residue(s) in all seven different cancer cell lines but not in the corresponding normal cells/tissues. In response to growth factor PDGF-BB stimulation, p68 is phosphorylated at Tyr593 by c-Abl in HT-29 cells, a colon cancer cell line. Phosphorylation of p68 at Tyr593 promotes EMT via promoting JUN/hc/HD9252 growth factor PDGF-BB stimulation, p68 is phosphorylated at tyrosine phosphorylation of p68 at Tyr593 also mediates the effects of PDGF in stimulating cell proliferation. The phosphorylated p68 promotes cell proliferation by activating the transcription of cyclin D1 and c-Myc genes. We show that the ATPase/helicase activities of p68 are required for the activation of cyclin D1 transcription. The phospho-p68 participates in the complex assembled at the cyclin D1 and c-Myc promoters, which strongly suggests a direct role in transcriptional regulation. Furthermore, our data demonstrated that the phosphorylation of p68 at Tyr593 is required for the effects of the PDGF autocrine, suggesting an important role for p68 phosphorylation in cell proliferation.

MATERIALS AND METHODS

Reagents and Antibodies—Recombinant PDGF-BB and Wnt-1 were purchased from PeproTech. Both polyclonal antibody PAb68 and monoclonal antibody anti-p68-rGG against human p68 were raised against bacterially expressed His-tagged C-terminal domain of p68 (Invitrogen). Commercial antibodies used in this study were purchased from Cell Signaling Technology (p-Tyr-100, against phosphotyrosine; DCS6, against cyclin D1; c-Myc; Lamin A/C; and 27E8, against His-tag ChIP grade), Calbiochem (7A7 and 9G10, against β-catenin at amino acids 35–50; and core armadillo repeats, respectively), Chemicon (6C5, against GAPDH), Covance Research (HA.11, against HA tag), Roche Applied Science (12CA5, against HA tag ChIP grade), and Upstate Biotechnology, Inc. (8E4, against β-catenin).

Cell Culture, Plasmids, and Transfection—Mouse fibroblast NIH3T3 cells, Human T98G, A549, HCT-116, A549, HEK, and U-2OS cells were purchased from ATCC and grown by following vendor’s instructions. DNA plasmid transfection was performed using FuGENE HD transfection kit (Roche Applied Science). The experiments were performed according to the manufacturer’s instructions. For the siRNA experiment, the cells were grown to 50% confluence and transfected with siRNA (200 pmol of RNA duplex) using X-tremeGENE siRNA transfection reagent (Roche Applied Science). The duplex RNA oligonucleotides for RNAi were purchased from Dharmacon siGENOME™. The siRNA oligonucleotides against p68 RNA helicase (Sense sequence, GCAAGUAGCUGCUAGAAUU; antisense sequence, 5′-pUAUUAGCAGCUACGU-UU; for co-transfection of plasmid DNA and siRNA, the cells were transfected with the indicated plasmid DNA 24 h after the siRNA transfection and harvested an additional 48 h later. The HA-tagged p68 expression plasmid was constructed by subcloning of wt p68 full-length cDNA (75) into pHM6 vector (Roche Applied Science). The various p68 mutants were generated using QuikChange site-directed mutagenesis (Stratagene) and confirmed by DNA sequencing. The plasmid for luciferase reporter of the cyclin D1 promoter (CD1(-962)luc) and its TCF-binding site mutant (TCF1–4mt) were provided by Dr. Osamu Tetsu and Frank McCormick (57).

Subcellular Extracts Preparation, Immunoprecipitation, and Immunoblot Analyses—All of the nuclear extracts, cytoplasm, or whole cell extracts were made freshly after appropriate treatments (indicated in figures) using nuclear extraction, cytoplasm, and cell extract kits (Qiagen). The protein concentration of the extracts was determined using the Bradford assay (BioRad). For immunoprecipitation, lysates or extracts were mixed with antibodies (1 µg) and incubated for 2 h followed by the addition of 30 µl of protein G plus-Sepharose beads (Santa Cruz Biotechnology). After incubation for an additional 2 h at 4 °C, immunoprecipitates were washed five times with radioimmunoprecipitation assay buffer (Upstate) supplemented with protease inhibitor mixture (Roche Applied Science) and protein phosphatase inhibitor mixture (Sigma) and boiled in loading buffer. The samples were separated by SDS-PAGE and transferred to Immob-Blot polyvinylidene difluoride membrane (Bio-Rad). Immunoblottings were performed via standard procedures and visualized with SuperSignal West Dura Extended Duration Substrate detection system (Pierce).

Luciferase Reporter and Cell Proliferation Assays—Before the cells were appropriately treated (indicated in figures), the cells were transfected with 1 µg of the indicated reporter plasmid and 0.01 µg of pRL null, which expresses Renilla luciferase from Renilla reniformis as an internal control. The total amount of plasmid DNA was adjusted with pcDNA3-β-galactosidase. Firefly and Renilla luciferase activities present in cellular lysates were assayed using a dual luciferase reporter system (Promega). The data are represented as firefly luciferase activity normalized by Renilla luciferase activity.

For analyses of cell proliferation, a cell proliferation enzyme-linked immunosorbent assay kit (Roche Applied Science) that measures BrdUrd incorporation was used. Briefly, the cells were incubated for 18 h in the presence of 10 µM BrdUrd under different conditions (indicated in figures). The cells were fixed after incubation and washed three times. The fixed cells were detected by anti-BrdUrd-POD antibody and secondary antibody. The nuclei incorporations of BrdUrd were measured by chemiluminescence emission (Victor 3TM; PerkinElmer Life Sciences).

Chromatin Immunoprecipitation—The ChIP experiments were performed using a ChIP-IT™ kit (ActiveMotif). The cells were treated as indicated in the figures. The precipitation of cyclin D1 promoter by the indicated antibodies was determined by PCR using primers spanning the TCF-binding sites (nucleotides −190 to +57) (sense, 5′-CTATGAAAACCGGACTACGCUAUAUU; antisense sequence, 5′-pUAUUAGCAGCUACGU-UU). For co-transfection of plasmid DNA and siRNA, the cells were transfected with the indicated plasmid DNA 24 h after the siRNA transfection and harvested an additional 48 h later. The HA-tagged p68 expression plasmid was constructed by subcloning of wt p68 full-length cDNA (75) into pHM6 vector (Roche Applied Science). The various p68 mutants were generated using QuikChange site-directed mutagenesis (Stratagene) and confirmed by DNA sequencing. The plasmid for luciferase reporter of the cyclin D1 promoter (CD1(-962)luc) and its TCF-binding site mutant (TCF1–4mt) were provided by Dr. Osamu Tetsu and Frank McCormick (57).
AGG-3'; antisense, 5'-GGCTCTGCAGTAGGGGAG-3') or non-TCF-binding site (nucleotides −550 to −300) (sense, 5'-AATGAAATGAAAGAAGATGCAG-3'; antisense, 5'-GGCTCTGAGTAGGGGAG-3'). Precipitation of the c-Myc promoter was detected using PCR primers spanning the TCF-binding sites (nucleotides −1160 to −580) (sense, 5'-AGTTAACGAGTTTTACGGGCG-3'; antisense, 5'-ATAATCAGCAAGCGATTCG-3'). TFIIB antibody and mouse IgG were included in the kit as positive/negative controls. The primers for the TFIIB-binding site at GAPDH promoter (sense, 5'-TACTAGCGGTTTTACGGGCG-3'; antisense, 5'-TCAAGAAGCGCCAGCAGCGA-3') were provided by the vendor. The control ChIP experiments gave a 166-bp band.

Protein Delivery—The recombinant proteins or antibodies (indicated) were delivered into HT-29 cells using the Chariot protein delivery kit (Active motif). Briefly, 1 μg of protein (in 100 μl of phosphate-buffered saline) was mixed with 6 μl of Chariot (in 100 μl of H2O). The mixture was incubated at room temperature to allow complex to form. The cells were washed with serum-free medium. The Chariot-protein complex was added to the cells with an additional 400 μl of serum-free medium. The cells were then incubated at 37 °C for 2 h to allow for protein internalization.

RESULTS

The Phospho-p68 Up-regulates Cyclin D1 and c-Myc and Promotes Cell Proliferation upon PDGF Stimulation—We previously reported that p68 RNA helicase became phosphorylated at Tyr593 in HT-29 and HCT-116 cells by c-Abl upon PDGF-BB treatment. The phospho-p68 promotes EMT by facilitating β-catenin nuclear translocation (54). We noted in our studies that the cell growth rate appeared to be dramatically increased upon PDGF treatment (data not shown). To test whether the phospho-p68 also mediates the effects of PDGF in stimulating cell proliferation, we carried out cell proliferation assays using a commercially available cell proliferation kit that measures the incorporation of BrdUrd into cellular DNA in the proliferated cells. The endogenous p68 RNA helicase was knocked down by RNAi. The p68 wt or Y593F mutant was transiently expressed in the p68 knockdown cells by mutating four nucleotides within the RNAi target sequence in the p68 expression vector (54). The mutations did not change the amino acid sequence of p68. Cell proliferation assays showed that HT-29 cells proliferated under treatment of PDGF without p68 knockdown. The PDGF-stimulated proliferation of the cells was largely inhibited by p68 knockdown. The inhibitory effects were relieved by transient expression of wild type p68. In fact, the cells proliferated even more quickly under exogenous expression of wt p68 (Fig. 1A). However, the cells demonstrated a much lower level of BrdUrd incorporation if the Y593F mutant was transiently expressed compared with that in the cells where p68 wild type was expressed (Fig. 1A). Thus, it is clear that phosphorylation of p68 at Tyr593 mediates the effects of PDGF in stimulating cell proliferation.

Our previous data demonstrated that the phospho-p68 promoted β-catenin nuclear translocation (54). Two of the immediate downstream targets of nuclear β-catenin in complex with LEF/TCF are cyclin D1 and c-Myc genes. Substantial evidence suggests that elevated cellular levels of cyclin D1 and c-Myc often lead to cell proliferation (55, 56). Thus, we examined whether the nuclear translocation of β-catenin promoted by the phospho-p68 affected cyclin D1 and c-Myc expression. We carried out immunoblotting experiments with cell extracts made from PDGF-treated/untreated HT-29 cells using commercially available antibodies against cyclin D1 and c-Myc. It was evident that the cellular levels of both cyclin D1 and c-Myc were elevated in HT-29 cells upon PDGF treatments (Fig. 1B). Thus, it is clear that phosphorylation of p68 at Tyr593 mediates the effects of PDGF on the up-regulation of cyclin D1 and c-Myc expression. We next examined the effects of Tyr593 phosphorylation of p68 on the expression of cyclin D1 and c-Myc. Knockdown of p68 by RNAi abolished the effects of PDGF on the up-regulation of cyclin D1 and c-Myc expression. We then exogenously

FIGURE 1. Phosphorylation of p68 at Tyr593 mediates the effects of PDGF in induction of cell proliferation and up-regulation of cyclin D1 and c-Myc. A, cell proliferation assays of HT-29 cells with or without p68 knockdown (WT or p68, respectively) by siRNA and expression of HA-p68s, wild type (WT), or Y593F mutant. The cells were either untreated (open bar) or treated (filled bar) with PDGF-BB (20 ng/ml in culture media) for 6 h. The cell proliferation was measured as described under "Materials and Methods" and presented as fold of increases in BrdUrd incorporation. B, expressions of cyclin D1 (upper panel) and c-Myc (lower panel) in HT-29 cells were examined by immunoblot of Lamin A/C and GAPDH in the extracts that were the loading controls. C, expressions of cyclin D1 (IB: DCS6) and c-Myc (IB: c-Myc) in HT-29 cells were examined by immunoblot of Lamin A/C and GAPDH in the extracts that were the loading controls. The cells were treated with nontarget siRNA (NT) or siRNA target p68 (p68). HA-p68s (wild type or Tyr593 mutant) was expressed in the p68 knockdown cells. The cells were treated (−) or treated (+) with PDGF-BB (20 ng/ml, 6 h). Immunoblot by antibodies p68-rgg (IB: p68-rgg) and HA.11 (IB: HA.11) were the loading controls indicating total p68 and exogenously expressed HA-p68. The immunoblot of Lamin A/C was a loading control for loading of total cellular proteins.
expressed HA-p68s (wt or Y593F mutant) in the p68 knockdown cells. Expression of wt p68 after RNAi knockdown restored the effects of PDGF on the up-regulation of cyclin D1 and c-Myc expression. However, expression of Y593F mutant did not restore the effects of PDGF (Fig. 1C). In fact, the cyclin D1 and c-Myc expressions were further suppressed by the expression of Y593F mutant. These results indicated that phosphorylation of p68 at Tyr593 was required for the elevation of cyclin D1 and c-Myc expression under the stimulation of PDGF.

The Phospho-p68 Up-regulates Cyclin D1 and c-Myc Expression by Activating Transcription of Both Genes—To understand the molecular mechanism by which the phospho-p68 mediated the effects of PDGF in up-regulating the cellular levels of cyclin D1 and c-Myc, we examined whether the phospho-p68 played a role in regulating cyclin D1 transcription. We employed luciferase assays using a transcription template that fused a luciferase gene with a cyclin D1 promoter (Fig. 2A) (57). It was clear that transcription activity of the cyclin D1 promoter was over 7-fold increased under PDGF treatment (Fig. 2A). The timing of activation of cyclin D1 transcription correlated with the β-catenin nucleus translocation (data not shown). Knockdown of p68 RNA helicase by RNAi repressed cyclin D1 transcription and completely diminished activation of cyclin D1 transcription by PDGF treatment (Fig. 2A). The transcription activation of cyclin D1 under PDGF stimulation could be restored by transient expression of wild type p68 after RNAi knockdown but not by expression of p68 Y593F mutant (Fig. 2A). In fact, the transcription activity of the cyclin D1 promoter was increased over 15-fold when the wild type p68 was transiently overexpressed after RNAi knockdown (Fig. 2A, comparing with and without HA-p68 expression under condition of p68 knockdown). The activation of the transcription of cyclin D1 promoter.
is β-catenin/TCF-specific because the activation was not observed with the mutated cyclin D1 promoter (TCF1–4mt) (Fig. 2A). Interestingly, the ATPase activity of p68 was also required for the transcription activation, because the lack of ATPase mutants of p68 (HLIGR and LGLD) (58) could not activate the transcription of the cyclin D1 promoter (Fig. 2A).

To investigate whether the phospho-p68 played a direct role in regulating cyclin D1 transcription, we probed the interaction of p68 with the cyclin D1 promoter by ChIP assay. The exogenously expressed wild type HA-p68 was co-immunoprecipitated with the cyclin D1 promoter as demonstrated by amplification of precipitated DNA fragments by polymerase chain reaction (Fig. 2B). However, the cyclin D1 promoter did not co-precipitate with the HA-Y593F mutant (Fig. 2B). We also carried out the same ChIP experiments with c-Myc promoter. Similarly, the exogenously expressed wild type HA-p68 was co-immunoprecipitated with the c-Myc promoter. The c-Myc promoter did not co-precipitate with the HA-Y593F mutant (Fig. 2C). Taken together, our data demonstrated that phosphorylation of p68 at Tyr^{593} up-regulates cyclin D1 and c-Myc transcription. The phospho-p68 directly participated in both the cyclin D1 and c-Myc promoters, indicating a direct role in the transcriptional regulation.

Both Nuclear Phospho-p68 and β-Catenin Are Required for Activation of Cyclin D1 and c-Myc Transcription—We further asked whether the p68 phosphorylation is sufficient to promote cyclin D1 transcription. To this end, we delivered in vitro phosphorylated (via c-Abl) His-p68 to HT-29 cells using the Chariot protein delivery kit. Most of the His-p68 delivered to HT-29 cells localized to the cell nucleus (54). We then measured the activity of the cyclin D1 promoter by luciferase assay. Delivery of unphosphorylated wt p68 to the cells did not result in an increase in the activity of the cyclin D1 promoter. Delivery of the in vitro phosphorylated p68 increased the cyclin D1 promoter activity by over 10-fold (Fig. 3A, comparing β-Gal with WT*). However, delivery of c-Abl-treated Y593F mutant did not lead to an increase in the promoter activity (Fig. 3A). The results suggested that the phospho-p68 is sufficient to promote cyclin D1 transcription.

In the Wnt signaling pathway, nuclear β-catenin in complex with LEF/TCF activates cyclin D1 and c-Myc transcription (59, 60). To test whether nuclear β-catenin is required for the activation of cyclin D1 transcription mediated by the phospho-p68, the in vitro phosphorylated or unphosphorylated His-p68 was co-delivered with the antibody against the N-terminal of β-catenin (54) to HT-29 cells. Immunoblotting of the nuclear extracts with antibody against β-catenin (8E4) demonstrated that the nuclear β-catenin level was decreased when the antibody 7A7 was co-delivered with the phosphorylated His-p68. However, no effects on the nuclear β-catenin level were observed when another antibody 9G10 was co-delivered with to the luciferase activity of the cells in which β-galactosidase and mouse IgG were co-delivery (defined as 1). Nuclear β-catenin levels in each corresponding experiments were examined by immunoblot (IB) of nuclear extracts made from the above treated cells. The immunoblot of Lamin A/C and total β-catenin were loading controls. Lower panel, immunoblotting analyses of nuclear (Nuc) and cytoplasmic (Cyto) GAPDH (IB:6C5) and Lamin A/C (IB:Lamin A/C). This is a control for cross-contaminations of nuclear and cytoplasmic extracts.

**FIGURE 3. Nuclear β-catenin is required for the phospho-p68-mediated transcriptional activation of cyclin D1 gene.** A, luciferase reporter assays of cyclin D1 promoter in HT-29 cells in which the in vitro phosphorylated (*) by recombinant c-Abl or unphosphorylated His-p68 (wild type (WT) or Y593F mutant) were delivered to the cells using a commercially available Chariot protein delivery kit. Treatments of the cells by the Chariot kit and delivery of β-galactosidase (β-Gal) were the negative controls for delivery of His-p68s. The luciferase activity was expressed as relative luciferase activity by comparing to the luciferase activity of the cells treated with Chariot kit (defined as 1). B, upper panel, luciferase reporter assays of the cyclin D1 promoter in HT-29 cells in which the in vitro phosphorylated (*) or unphosphorylated His-p68 were co-delivered with indicated antibodies to the cells. Delivery of mouse IgG was used as negative control for the deliveries of 7A7 and 9G10. The luciferase activity was expressed as relative luciferase activity by comparing.
The phosphorylated His-p68. The observations were consistent with our previous results (54). The transcriptional activity of the cyclin D1 promoter was then examined by the luciferase assay. It was clear that the transcriptional activity of the cyclin D1 promoter was up-regulated when the phosphorylated His-p68 was delivered to the cells. Up-regulation of cyclin D1 transcription was not observed when unphosphorylated His-p68 was delivered to the cells. Interestingly, up-regulation of the cyclin D1 transcription was significantly reduced when the antibody 7A7 and the phosphorylated His-p68 were co-delivered (Fig. 3B). On the other hand, if the antibody (9G10) was co-delivered with the phosphorylated His-p68, the transcriptional activity of the cyclin D1 promoter was up-regulated to the same level of that without antibody delivery (Fig. 3B). These results indicated that nuclear β-catenin was required for the transcriptional activation of the cyclin D1 gene mediated by phospho-p68 in response to PDGF stimulation.

The phospho-p68 promoted β-catenin nuclear translocation by blocking GSK-3β binding and unwinding Axin-1 (54). Nuclear β-catenin in complex with LEF/TCF activates the transcription of cyclin D1 and c-Myc (61). Therefore, one possibility is that the cyclin D1 and c-Myc transcription were activated by the nuclear β-catenin. The phospho-p68 is only required for promoting β-catenin nuclear translocation but not for the transcriptional activation. Nevertheless, direct participation of the phospho-p68 at the cyclin D1 and c-Myc promoters seems to argue against this possibility, suggesting a direct role for the protein in transcriptional regulation. To test whether the phospho-p68 has a role in transcription activation of cyclin D1 in addition to promoting β-catenin nuclear translocation, we stimulated HT-29 cells with Wnt-1. Wnt-1 treatments activated cyclin D1 transcription. The in vitro phosphorylated His-p68s (wt or lack of ATPase mutant, LGLD) were delivered to the Wnt-1-treated cells (Fig. 4A). Delivery of in vitro phosphorylated His-p68 (wt) further increased cyclin D1 transcription. However, the delivery of the p68 mutant (LGLD) that lacks the ATPase activity largely decreased the cyclin D1 transcription stimulated by Wnt-1 (Fig. 4B). ChIP experiments showed that the in vitro phosphorylated His-p68s (both wt and LGLD mutant) interacted with the cyclin D1 promoter (Fig. 4C). The interaction of the phospho-p68 wt and the LGLD mutant was specific to the region of β-catenin/TCF-binding sites because the control ChIP experiments using a PCR primer targeting to a different region (−550 to −300 nt) did not yield ChIP products (Fig. 4C). These experiments suggested that the phospho-p68 played a direct role in the transcription of cyclin D1 and c-Myc in addition to promoting β-catenin nuclear translocation. The ATPase activity of p68 is directly required for the cyclin D1 transcription activation.
The Phospho-p68 Plays a Role in Cell Proliferation Stimulated by PDGF Autocrine Loop—The PDGF autocrine loop activity constitutes an important mechanism for tumor growth/progression and resistance to anti-tumor treatments (37). Our preceding data demonstrated that the phospho-p68 mediated the effects of PDGF in stimulating cell growth and proliferation. Therefore, we asked whether the phospho-p68 mediates the effects of the PDGF autocrine loop in stimulating cancer growth. We examined the phosphorylation status of p68 in cell lines with/without the PDGF autocrine loop (NIH3T3, A549, T98G, HEK, HCT-116, and U-2OS). Among them, T98G is a glioblastoma cell line and the U-2OS is an osteosarcoma cell line derived from bone. These two cell lines are PDGF autocrine loop-positive. T98G and U-2OS cells are commonly used for studying the effects of the PDGF autocrine loop on cell proliferation and survival (39, 62–64). NIH3T3, A549, HEK, and HCT-116 cells are PDGF autocrine loop-negative. It appeared that there was a correlation between the tyrosine phosphorylation of p68 RNA helicase and PDGF autocrine loop by comparing cell lines with the PDGF autocrine loop with the cell lines without (Fig. 5A; also see Ref. 53). To test whether phosphorylation of p68 mediated the effects of the PDGF autocrine loop, the HA-p68s (wt or Y593F mutant) were transiently expressed in six cell lines with/without the PDGF autocrine loop. The cell proliferations were then monitored. Exogenous expression of p68 (wt) had no significant effects on the cell proliferation in the PDGF autocrine loop-negative cells (within 3% for NIH3T3, HEK, and HCT-116), with exception of A549. The cell proliferation rates were significantly increased in the PDGF autocrine loop-positive cells (almost 1.8-fold for T98G and 1.6-fold for U-2OS, respectively) (Fig. 5B). No significant effects on the cell proliferation were observed in the PDGF autocrine loop-negative cells by exogenous expression of Y593F mutant (Fig. 5B). Interestingly, transient expression of Y593F mutant in the PDGF autocrine loop-positive cells drastically reduced the cell proliferation (by ~2-fold for T98G and almost ~2.5-fold for U-2OS) (Fig. 5B). These experiments supported the notion that the phospho-p68 plays a role in cell proliferation stimulated by PDGF autocrine loop. The effects of expression of p68 (wt and mutant) on the cell proliferation of A549 were somewhat interesting. We repeatedly observed that expression of both p68 wild type and Y593F mutant significantly increased the proliferation of A549 cells (Fig. 5B).

**DISCUSSION**

In this article, we report that phosphorylation of p68 RNA helicase at Tyr593 mediates the effects of PDGF in promoting cell proliferation. We demonstrate that the phospho-p68 mediated the effects of PDGF by activation of the transcription of cyclin D1 and c-Myc genes. Our data show that β-catenin...
nuclear translocation promoted by the phospho-p68 is required for the transcriptional activation of cyclin D1 and c-Myc genes. The phospho-p68 has a direct role in the activation of cyclin D1 and c-Myc gene transcription. Importantly, our experiments provide evidence to support the hypothesis that phosphorylation of p68 at Tyr<sup>593</sup> plays a role in mediating the effects of PDGF autocrine loop-induced cancer cell proliferation.

Phosphorylation of p68 at Tyr<sup>593</sup> by c-Abl mediates the effects of PDGF in promoting EMT (54). Here, we describe that the same phosphorylation of p68 RNA helicase also plays a role in mediating the effects of PDGF in stimulating cell proliferation. Similarly, it was demonstrated that Wnt/β-catenin signaling also has a major impact on both EMT and cell proliferation (65–68). Wnt signaling promotes β-catenin nuclear localization, which subsequently activates a number of downstream genes that play a role in cell proliferation and EMT. Similar to the Wnt signaling, the phospho-p68 also promotes β-catenin nuclear translocation by blocking cytoplasmic β-catenin phosphorylation by GSK-3β and displacing the scaffold protein Axin from β-catenin (54). The phospho-p68, in complex with the nuclear β-catenin, activates the EMT program. The phospho-p68 along with the nuclear β-catenin also up-regulates the expression of cyclin D1 and c-Myc genes, which consequently leads to cell proliferation.

The PDGF autocrine loop induces cell proliferation, angiogenesis, and EMT in tumor cells (6, 35, 69). However, the molecular basis for the multiple functions of the autocrine loop is not well understood. We show that phosphorylation of p68 at Tyr<sup>593</sup> plays a role in mediating the effects of PDGF in promoting EMT and cell proliferation. More importantly, we observed a substantially higher level of tyrosine phosphorylation(s) of p68 in metastatic cancer tissue samples than those in primary sites. Given the role of the phosphorylation of p68 in cell proliferation, it is tempting to speculate that phosphorylation of p68 at Tyr<sup>593</sup> is an important and general component of PDGF and the PDGF autocrine loop signaling pathway that promotes cell proliferation and survival.

The molecular mechanism by which the phospho-p68 up-regulates the transcription of cyclin D1 and c-Myc genes is intriguing. Our data clearly demonstrated that only the Tyr<sup>593</sup>-phosphorylated p68 participates in the complex assembled on the cyclin D1 and c-Myc promoters. Association of the phospho-p68 with the promoters requires nuclear β-catenin. Given that only the phospho-p68 interacts with β-catenin, it is reasonable to conclude that the phospho-p68 is brought to the cyclin D1 and c-Myc promoters by interacting with the β-catenin-TCF/LEF complex. This case is different from another observation made in our laboratory that the phospho-p68 regulates Snail gene transcription, where the phosphorylated/unphosphorylated p68 and Y593F mutant can all participate in the complex assembled on the Snail promoter. However, only the phospho-p68 has a role in the up-regulation of Snail transcription. p68 is a DEAD box RNA helicase. We demonstrated that the phospho-p68 gained a strong protein-dependent ATPase activity by interacting with β-catenin (54).

Thus, it is conceivable that the functional role of the phospho-p68 in complex with β-catenin is to remodel the protein-protein interaction(s) at the cyclin D1 and c-Myc promoters. However, the target molecule(s) or protein(s) of the protein-dependent ATPase p68 at the cyclin D1 and c-Myc promoter remains to be identified. Because the phospho-p68 up-regulates transcription, it is expected that a protein factor that suppresses the cyclin D1 and c-Myc transcription is most likely the target of the protein-dependent ATPase activity of p68. p68 RNA helicase is implicated in regulation of transcription of a number of genes. Interestingly, the mechanisms of action of p68 in the transcription regulation are different depending on each individual regulated gene. p68 RNA helicase was suggested to function as a co-activator (adapter) at estrogen receptor-α promoter (70). The protein is also demonstrated to interact with p300/CBP and RNA polymerase II holoenzyme (71), histone acetyltransferase (72), and histone deacetylase 1, suggesting a role for p68 in nucleosome remodeling during transcription (73). More recently, drosophila p68 is suggested to have a role in unwinding RNA transcripts from its DNA template, which facilitates a quick reset of the nucleosome structure (74). Apparently, the phosphorylated or unphosphorylated p68 employs somewhat different mechanisms in transcriptional regulations. The phosphorylated or unphosphorylated p68 also appears to target different genes. The difference in transcriptional regulation by the phosphorylated/unphosphorylated p68 RNA helicase adds yet another dimension for the functions of p68 in gene regulation.

An interesting observation in our studies is the role of the Y593F mutant in the cell proliferation in the PDGF autocrine loop-positive cells. Exogenous expression of the mutant inhibits cell proliferation in the presence of the endogenous wild type protein, indicating that the mutant possesses dominant-negative effects. This observation is consistent with our previous observation that expression of the Y593F mutant inhibits endogenous p68 phosphorylation at Tyr<sup>593</sup>. We believe that the mutant competes with the wild type p68 for binding to the upstream molecule(s), most likely a protein-tyrosine kinase (c-Abl). The mutant binds to the kinase, forming a nondissociable complex (because of the mutation at the phosphorylation site). Thus, this competition leads to the inhibition of the kinase activity. One very promising aspect of the inhibitory effects of the mutant is the potential application of this property in a therapeutic strategy for growth inhibition.

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