
Phosphorylations of DEAD Box p68 RNA Helicase Are Associated with Cancer Development and Cell Proliferation

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Abstract

The nuclear p68 RNA helicase is essential for normal cell growth. The protein plays a very important role in early organ development and maturation. In our previous report, we showed that recombinant p68 RNA helicase was phosphorylated at serine/threonine and tyrosine residue(s). In the present study, we examined the phosphorylation status of p68 in six different cancer cell lines and compared the results with those in cells derived from the corresponding normal tissues. We showed here that p68 was phosphorylated at tyrosine residue(s) in all tested cancer cells but not in the corresponding normal cells/tissues. The tyrosyl phosphorylation of p68 also responded to platelet-derived growth factor. It is thus clear that p68 phosphorylation at tyrosine residue(s) is associated with abnormal cell proliferation and cancer development. The tyrosyl phosphorylation(s) was diminished if the cancer cells were treated with apoptosis agents, such as tumor necrosis factor- α , tumor necrosis factor-related apoptosis-inducer ligand, and STI-571. The tyrosyl phosphorylation of p68, however, was not affected by other anticancer drugs, such as piceatannol, etoposide, and taxol. The close correlation between p68 phosphorylations and cancer may provide a useful diagnostic marker and potential therapeutic target for cancer treatment. (Mol Cancer Res 2005;3(6):355–63)

Introduction

The nuclear p68 RNA helicase is a prototypical member of DEAD box family of RNA helicase (1, 2). As an early example of a cellular RNA helicase, the ATPase and the RNA unwinding activities of p68 RNA helicase were documented with p68 that was purified from human 293 cells (3-5) and recombinant protein expressed in insect cells and *Escherichia coli* (6, 7). However, the biological functions of the protein in cells at a molecular level are not well understood (8). It has been suggested that p68 RNA helicase might be involved in transcription regulation (9-14) and DNA damage-repair

pathways (15). Recently, experiments carried out in our laboratory showed that p68 RNA helicase is an essential human splicing factor *in vitro* that plays a role in unwinding the transient U1:5' splice site duplex (16, 17). Consistently, other research laboratories also suggested that p68 RNA helicase has a functional role in the pre-mRNA splicing process (18-21).

p68 RNA helicase plays a very important role in cell proliferation and development (22, 23). The protein is expressed in all dividing cells of different vertebrates. The expression pattern, however, exhibits significant differences among different tissue types (2, 23), suggesting that the role of p68 RNA helicase in the cell development does not correlate completely with its expression. The protein shows clear cell cycle-related localization in the nucleus, consistent with the regulation of p68 function by cell cycle. Related to the role of p68 RNA helicase in the cell developmental program, the protein was shown to play a critical role in the tumorigenesis process (24). Causevic et al. (25) showed that p68 RNA helicase is overexpressed in colorectal cancer tissues and cell lines compared with the corresponding normal tissues and cells. Moreover, overexpression of p68 in normal cells results in the tumorigenic transformation of the cells (26), indicating the role of p68 in tumor progression.

An interesting question is how the biological function(s) of p68 RNA helicase is linked to cell growth signals, especially the mechanism by which p68 dysfunctions in the cancer development process. By screening specimens from patients with colorectal adenocarcinomas, Causevic and coworkers found that p68 RNA helicase is polyubiquitylated in cancer tissue but not in normal tissues. Thus, it is speculated that polyubiquitylation and overexpression of p68 RNA helicase in cancer cells play a role in tumorigenesis. We observed, however, that recombinant p68 RNA helicase is phosphorylated at multiple sites, including serine/threonine and tyrosine (27). Cellular p68 in HeLa cells is phosphorylated at tyrosine residue(s). We showed that the phosphorylations at tyrosine or threonine respond differently to tumor necrosis factor- α (TNF- α) treatments in HeLa cells.¹ It was also noted earlier that p68 can be phosphorylated *in vitro* by protein kinase C at its COOH terminal (28). Moreover, Akileswaran et al. (29) found that A-kinase anchoring protein AKAP95 is associated with p68 RNA helicase. The AKAP95 functions as an anchor for cyclic AMP-dependent kinase in the cell nuclear matrix. All these observations strongly argue that p68 RNA helicase might be a downstream target for multiple

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¹ Unpublished observation.

protein kinases in the cell signaling pathways. Thus, it is conceivable that protein phosphorylation(s) may provide a link for the role(s) of p68 RNA helicase in the cell development program and tumorigenesis.

In this article, we report our observations that p68 RNA helicase is phosphorylated at tyrosine residue(s) in six different cancer cell lines, but not in cell lines derived from corresponding normal tissues. Furthermore, if cells derived from normal tissue were treated with growth factor that frequently led to cell proliferation, phosphorylation at tyrosine residue(s) was clearly evident, indicating that tyrosine phosphorylation of p68 is likely associated with proliferation status of cells. The tyrosyl phosphorylation(s) was diminished when the cancer cells were treated with some anticancer agents, such as TNF- α , TNF-related apoptosis-inducer ligand (TRAIL), and STI-571. However, the tyrosyl phosphorylation of p68 was not affected by other anticancer drugs, such as piceatannol, etoposide, and taxol. Interestingly, phosphorylation at threonine residue(s) was not observed in five of six cancer cell lines. On treatment of the cancer cells with TNF- α or TRAIL, p68 RNA helicase became transiently phosphorylated at threonine residue(s). The timing of the threonine phosphorylation correlated well with the timing of dephosphorylation at tyrosine residue(s). The close correlation between p68 phosphorylations and cancer may provide a useful diagnostic marker and potential therapeutic target for cancer treatment.

Results

p68 RNA Helicase Is Phosphorylated at Tyrosine Residue(s) in Cancer Cells but not in Corresponding Normal Cells or Tissues

We observed that p68 RNA helicase was phosphorylated at tyrosine residue(s) in HeLa cells. Because the HeLa cell line is derived from human cancer tissue, we reasoned that tyrosine phosphorylation of p68 might correlate with cancer development. To test this possibility, we examined the phosphorylation status of p68 RNA helicase in nuclear extracts made from six different cancer cell lines that were derived from different human cancer tissues (Table 1), including lung carcinoma cells (A549), colonic tumor cells (Caco-2), hepatocellular carcinoma cells (HepG2), leukemia cells (K562), breast cancer cells (MCF-7), and cervix carcinoma cells (HeLa S3). We compared the phosphorylation status of p68 in cancer cells with that of cells derived from each corresponding normal tissue (Table 1). The following seven tissues or cell lines derived from normal

tissues were screened in our experiments: (a) normal human lung tissue, (b) human embryonic lung cells (HEL299), (c) human colon fibroblast cells (CCD-112CoN), (d) normal human liver tissue, (e) human normal breast cells (Hs578Bst), (f) human B lymphocyte cells isolated from peripheral blood (RPMI1788), and (g) human embryonic kidney cells (HEK293). Among the cell lines derived from normal tissue, the HEK293 cells were transformed with adenovirus 5 DNA, and the RPMI1788 cells were immortalized by EBV nuclear antigen. Cellular p68 was first immunoprecipitated from the freshly made nuclear extracts using a polyclonal antibody, PAbp68-rgg, raised against the COOH-terminal of p68 RNA helicase (27). The immunoprecipitated p68 was then probed using a monoclonal antibody against phospho-tyrosine (p-tyr-100; Cell Signaling). It was clear that the p68 RNA helicase that was precipitated from nuclear extracts prepared from all six cancer cell lines was recognized by the antibody p-tyr-100 (Fig. 1A, bottom, lanes 3, 5, 7, 9, 11, and 13). On the other hand, p-tyr-100 did not recognize the p68 RNA helicase that was immunoprecipitated from nuclear extracts prepared from the corresponding normal tissue or cell lines derived from normal tissues (Fig. 1A, bottom, lanes 1, 2, 4, 6, 8, 10, and 12). To verify that the immunoblotting signals were indeed due to tyrosine phosphorylation(s), we carried out tyrosine dephosphorylation of the nuclear extracts made from HeLa cells with protein tyrosine phosphatase 1B. The immunoprecipitation followed by immunoblotting resulted in the inability of the phospho-tyrosine antibody to recognize the precipitated p68 (Fig. 1B, top, lane 2). The tyrosine phosphorylation(s) in cancer cells was further confirmed by other two monoclonal antibodies against phospho-tyrosine, P-tyr-1 (ATCC CRL-1955) and PY20 (data not shown). To further verify the tyrosine phosphorylation of p68 in cells, we carried out metabolism labeling of HeLa cells with ^{32}P . The nuclear extracts were made from the ^{32}P -labeled cells. Immunoprecipitation experiments with antibody against p68 showed that p68 RNA helicase is phosphorylated by ^{32}P (Fig. 1D, lane 1). If the cells were treated with STI-571 before the labeling or the nuclear extracts were treated with protein tyrosine phosphatase 1B before the immunoprecipitation, the phosphorylations of p68 were almost abolished (Fig. 1D, lanes 2 and 4). To rule out the possibility that the phosphorylation detected by the phospho-tyrosine antibodies was due to *in vitro* phosphorylation during the immunoprecipitation procedure, protein tyrosine kinase (BCR-Abl) inhibitor STI-571 was added into lysis buffer during preparation of nuclear extracts. The addition of the

Table 1. Cell Lines Used in This Study

	Human							Mouse		
	Lung	Colon	Liver	Lymphocyte	Breast	Kidney	Cervix			
Cancer	A549	Caco-2	HepG2	K562	MCF-7		HeLa S3			
Normal		CCD-112CoN		RPMI1788*	Hs578Bst					
Embryo	HEL299					HEK293 [†]		NIH3T3	SYF [‡]	Src ^{++§}

*The cells were immortalized by EBV nuclear antigen.

[†]The cells were transformed with adenovirus 5 DNA.

[‡]The cells were deficient in Src, Yes, and Fyn kinases and immortalized by SV40 large T-antigen.

[§]The cells were deficient in Yes and Fyn kinases but expressed Src kinase. The cells were immortalized by SV40 large T-antigen.

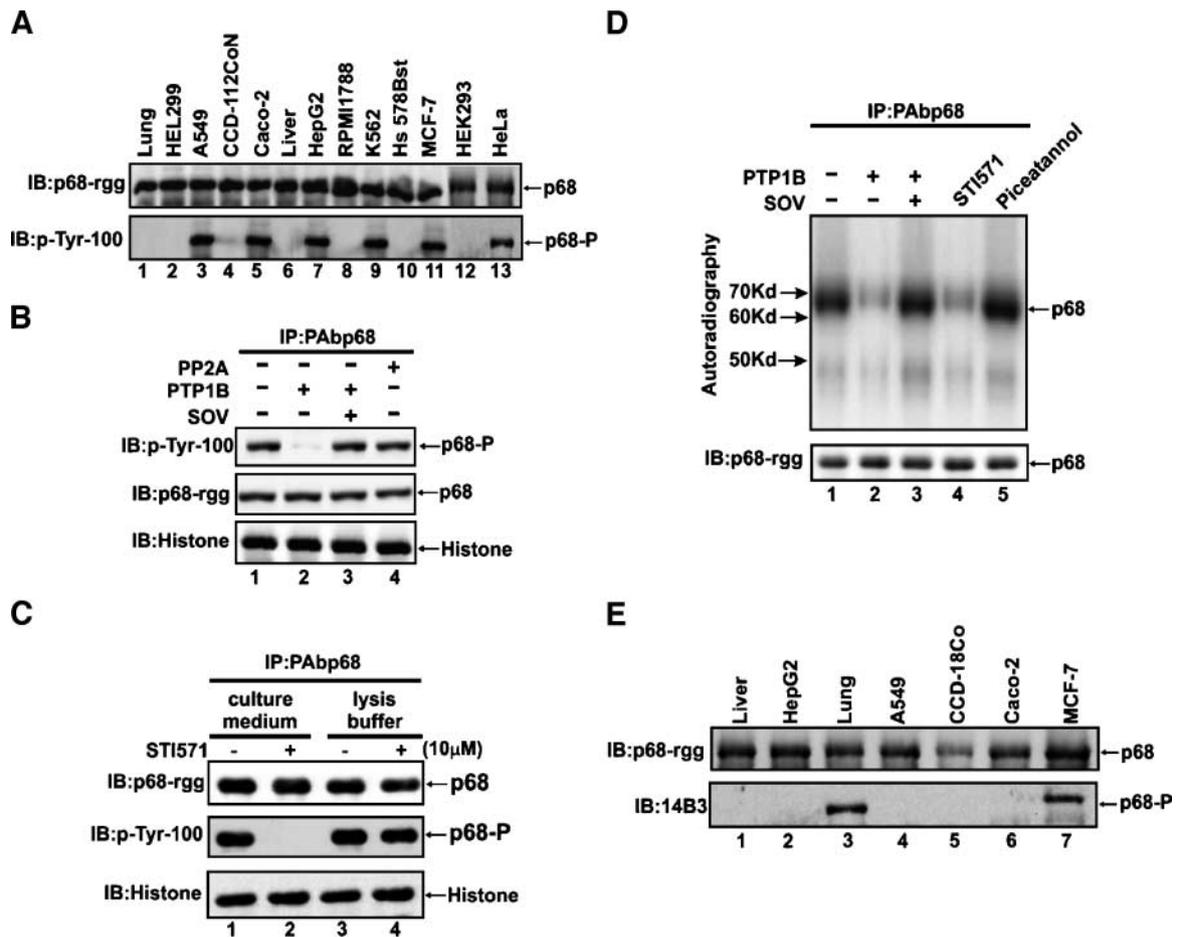


FIGURE 1. Phosphorylation of p68 RNA helicase in cancer cells and normal cells/tissues. The cellular p68 RNA helicase was immunoprecipitated from nuclear extracts made from different cell lines and tissue samples (as indicated). The immunoprecipitated cellular p68 was separated on 10% SDS-PAGE and subjected to immunoblot with monoclonal antibody p68-rgg raised against the COOH-terminal of p68 (A, C, and E, top; B, middle), a commercially available monoclonal antibody against phospho-tyrosine, p-tyr-100 (A, bottom; B, top; C, middle), and a commercially available monoclonal antibody against phospho-threonine, 14B3 (E, bottom). B. The nuclear extracts made from HeLa cells were treated with protein tyrosine phosphatase 1B (PTP1B) or serine/threonine phosphatase protein phosphatase 2A (PP2A) and/or protein tyrosine phosphatase inhibitor (as indicated). p68 RNA helicase was immunoprecipitated from the phosphatase- and/or phosphatase inhibitor-treated extracts. Bottom, immunoblotting of histone in the extracts as controls. C. The nuclear extracts were made from freshly grown HeLa cells with or without (as indicated) addition of the protein tyrosine kinase (BCR-Abl) inhibitor STI-571 (10 μ mol/L) to the lysis buffer. Bottom, immunoblotting of histone in the extracts as controls. D. Metabolism labeling of p68 RNA helicase in HeLa cells. The extracts made from labeled cells were untreated (lane 1) and treated with protein tyrosine phosphatase 1B (lane 2) and with protein tyrosine phosphatase 1B and protein tyrosine phosphatase inhibitor sodium orthovanadate (SOV; lane 3) before the immunoprecipitation. The cells were pretreated with Abl inhibitor STI-571 (lane 4) or piceatannol (lane 5) before the labeling. Bottom, duplicate of top panel, but p68 was detected by immunoblotting with p68-rgg.

tyrosine kinase inhibitor did not affect the tyrosine phosphorylation of p68 (Fig. 1C, lanes 3 and 4). However, addition of the same kinase inhibitor into cell culture media inhibited the tyrosine phosphorylation of p68 (Fig. 1C, lanes 1 and 2).

Because we previously observed that recombinant p68 was phosphorylated at serine/threonine and tyrosine residue(s) and p68 in HeLa cells was not phosphorylated at serine/threonine residues (ref. 27, 30), we questioned whether p68 is phosphorylated at serine/threonine residues in different cancer cells. To this end, we employed monoclonal antibodies against phospho-serine (16B4, Calbiochem) and phospho-threonine (14B3, Calbiochem) to monitor the serine/threonine phosphorylation of p68 that was immunoprecipitated from nuclear extracts made from different tissues or cell lines. It was evident that no serine phosphorylation was detected with any cell line or tissue under our experimental conditions (data not shown).

The precipitated p68 could not be recognized by 16B4, which suggests that p68 is not phosphorylated at the serine residues. However, it is also possible that the antibodies do not recognize all serine phosphorylation sites. We observed threonine phosphorylation by the antibody in nuclear extracts made from human normal lung tissue and breast cancer cells MCF-7 (Fig. 1E, bottom, lanes 3 and 7). Overall, our data indicated that phosphorylation of p68 at tyrosine residue(s) correlated with cancer development.

To further test the correlation between p68 phosphorylation and cancer development, we screened tissue samples from cancer patients. Both cancerous tissue and normal tissue were collected from the same cancer patient. The tissue samples were collected from patients with colon cancer, ovary cancer, and lung cancer. Protein extracts were made from the tissue samples. The phosphorylation status of p68 RNA helicase in

the tissue samples was examined by immunoprecipitation of p68 from the extracts followed by immunoblot with antibodies against specific phospho-amino acids. p68 was phosphorylated at tyrosine residues in all cancerous tissue samples but not in normal tissue samples, as shown by immunoblotting with antibody p-tyr-100 (Fig. 2A, *middle*). No threonine phosphorylation was detected by immunoblotting with antibody 14B3 in both normal and cancerous tissue samples from colon cancer and ovary cancer (Fig. 2A, *bottom*). Strong threonine phosphorylation of p68 was detected in normal lung tissue samples (Fig. 2A, *bottom*). Interestingly, the threonine phosphorylation of p68 disappeared in lung cancerous tissues (Fig. 2A, *bottom*). Thus, our results from tissue screening confirmed that phosphorylation of p68 at tyrosine residue(s) correlated with cancer development.

The Tyrosyl Phosphorylation of p68 RNA Helicase Is Associated with Abnormal Cell Proliferation

The preceding experiments showed a close correlation between p68 tyrosyl phosphorylation and cancer. We asked: "Is the tyrosyl phosphorylation of p68 RNA helicase associated with the proliferation status of cells?" To test the correlation, we stimulated the cells derived from normal tissues with growth factor and then examined the p68 phosphorylations in the growth factor-stimulated cells. The platelet-derived growth factor (PDGF) is a growth factor with receptor tyrosine kinase activity. It has been shown that treatment of cells with growth factor PDGF could lead to cancer development (31, 32). In our experiments, the cells derived from normal embryonic lung tissue (HEL299), normal colon tissue (CCD-112CoN), and normal breast tissue (Hs578Bst) were stimulated by PDGF-BB (PeproTech). We first examined whether stimulation of HEL299 cells with PDGF led to cell proliferation. We used a commercially available cell proliferation assay kit that monitors DNA synthesis rate during cell proliferation (Roche) to monitor cell proliferation under different dosages of PDGF treatment. PDGF stimulated cell proliferation under our experimental conditions (Fig. 3B, *open squares*). To test whether the cell proliferation under PDGF treatment is mediated by p68 RNA helicase, the endogenous p68 was knocked down by RNA interference. Immunoblot showed that p68 was knocked down by over 95% (Fig. 3A, compare *lanes 1* and *2*). The cell proliferation rate was reduced by more than 3-fold (Fig. 3B, *open triangle*). The results suggest that p68 RNA helicase mediated cell proliferation under PDGF stimulation. Tyrosyl phosphorylation of p68 was examined by the same immunoprecipitation and Western blot procedure post PDGF treatment. Our Western blots revealed that, on PDGF growth stimulation, p68 RNA helicase became tyrosyl phosphorylated in HEL299 cells (Fig. 3C, *bottom, lane 2*), CCD-112 cells (*lane 4*), and Hs578Bst cells (*lane 6*). The data suggest that tyrosine phosphorylation(s) of p68 indeed correlated with the cell proliferation status of cells.

To determine whether tyrosine phosphorylation of p68 is associated with cell proliferation or transformation, we probed the phosphorylation of p68 in mouse embryo fibroblast cell lines with or without SV40 large T-antigen transformation. Due to antibody specificity, we used a polyclonal antibody (PABN1) against the NH₂-terminal of p68 to precipitate p68 (16). It was

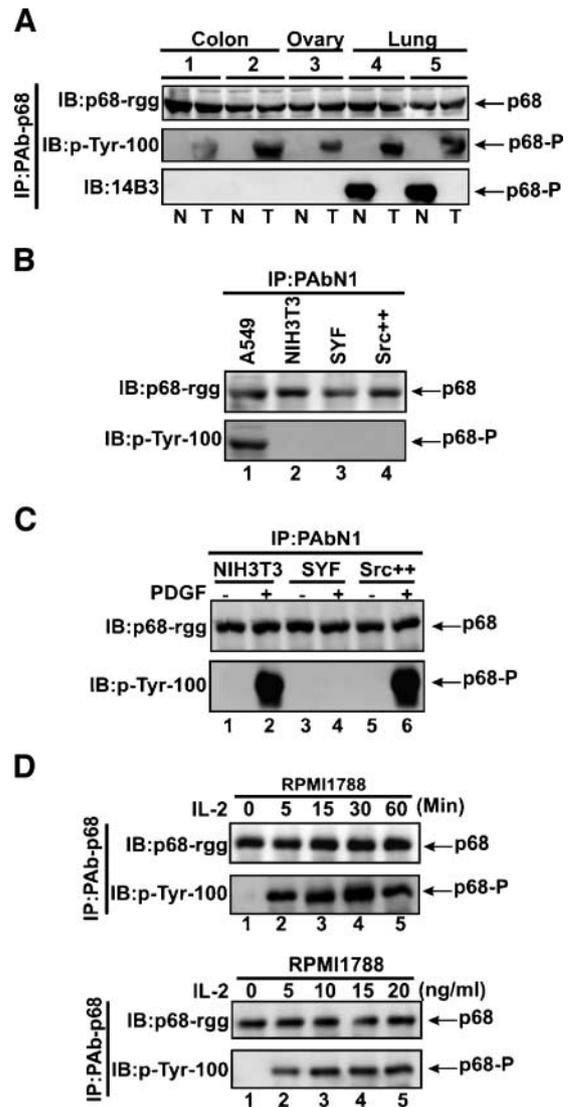


FIGURE 2. Phosphorylation of p68 RNA helicase in the tissue samples from cancer patients (A), mouse embryo cell lines (B), mouse embryo cell lines with or without treatment of PDGF (C), or human B cells RPMI1788 treated with interleukin 2 by different durations (indicated) or doses (indicated; D). The cellular p68 RNA helicase was immunoprecipitated from protein extracts made from different tissue samples (as indicated) or nuclear extracts from different cell lines (indicated). The immunoprecipitated cellular p68 was separated on 10% SDS-PAGE and subjected to immunoblot with monoclonal antibody p68-rgg against the COOH-terminal of p68 (*top*) or a commercially available monoclonal antibody against phospho-tyrosine, p-tyr-100 (*bottom*). N, sample collected from normal tissue. T, sample collected from tumor tissue. A and D. p68 was precipitated by polyclonal antibody PABp68 against the COOH-terminal of p68. B and C. p68 was precipitated by polyclonal antibody PABN1 against the NH₂-terminal of p68.

clear that p68 was not phosphorylated at tyrosine residues in mouse embryo fibroblast cell lines with or without transformation (Fig. 3B, *bottom, lanes 2-4*). However, under PDGF stimulation, p68 RNA helicase became phosphorylated at tyrosine residue in both untransformed cell line (NIH3T3) and transformed cell line (Src⁺⁺; Fig. 2C, *bottom, lanes 2* and *6*). Interestingly, p68 was not tyrosyl phosphorylated in another SV40 large T-antigen transformed cell line (SYF).

The difference between SYF and Src⁺⁺ is the expression of endogenous Src kinase in Src⁺⁺ but not in SYF. The results showed the dependence of p68 tyrosine phosphorylation on Src kinase.

To test whether tyrosine phosphorylation was stimulated by mitogens, we examined the tyrosine phosphorylation of p68 in human B cell RPMI1788. The RPMI1788 cells were derived from normal peripheral blood. The cells were immortalized by EBV nuclear antigen. The RPMI1788 cells were treated with interleukin 2 of different doses (Fig. 2D, *bottom*) or different durations (Fig. 2D, *top*). p68 was not phosphorylated in unstimulated RPMI1788 cells (Fig. 2D, *top* and *bottom*, *lane 1*). However, p68 became phosphorylated at tyrosine residue in RPMI1788 cells on interleukin 2 stimulation (Fig. 2D, *top* and *bottom*, *lanes 2-5*). This observation, along with the observation that PDGF stimulated tyrosyl phosphorylation of p68 in a number of normal cell lines, suggests that phosphorylation of p68 at tyrosine might be a cellular response to mitogenic stimulations.

The Phosphorylations of p68 Respond Differently to Different Anticancer Treatments

The tyrosyl phosphorylation(s) of p68 in HeLa cells could be dephosphorylated with tumor necrosis factor α (TNF- α) treatment.² It is known that TNF- α -induced cell signal triggers cancer cell apoptosis (33). Thus, we questioned whether or not tyrosine dephosphorylation of p68 is an apoptotic marker of cancer cells in response to specific apoptosis-inducing agents. To test this speculation, we examined the responses of tyrosine dephosphorylation of p68 in all six cancer cell lines to TNF- α (PeproTech) and TNF-related apoptosis-inducer ligand (TRAIL; PeproTech) treatments. The phosphorylation at tyrosine residue(s) with the TNF- α and TRAIL treatments was monitored by the same immunoprecipitation and Western blot procedure. It was evident that treatment of all six cancer cell lines with TNF- α or TRAIL led to the dephosphorylation of p68 at tyrosine residue(s) (Fig. 4A and B, *mid*, 2, 4, 6, 8, and 10). Interestingly, treatment of all six different cancer cells with both TNF- α and TRAIL also resulted in phosphorylation of p68 at threonine (Fig. 4A and B, *bottom*, *lanes 2, 4, 6, 8, and 10*). Dephosphorylation at tyrosine residue(s) and phosphorylation at threonine residue(s) occurred at a very early stage of TNF- α or TRAIL treatment (within first 10 minutes; data not shown). The threonine phosphorylation was quickly dephosphorylated again on (30 minutes) treatment with TNF- α or TRAIL for a longer period of time (data not shown). To confirm that the immunoblotting signals were indeed due to threonine phosphorylation(s), we treated the HeLa nuclear extracts with protein phosphatase 2A before immunoprecipitation. The immunoprecipitation followed by immunoblotting resulted in the inability of the phospho-threonine antibody to recognize the precipitated p68 (Fig. 4C, *top*, *lane 3*), indicating that the detection of p68 by antibody 14B3 is indeed due to threonine phosphorylation.

In the light that tyrosyl phosphorylation of p68 in cancer cells was dephosphorylated with TNF- α or TRAIL treatment,

we suspected that tyrosine dephosphorylation might be linked to cancer cell death. To further test this possibility, we examined the effects of several anticancer drugs: STI-571 (Novartis), piceatannol (Sigma), etoposide (Calbiochem), and taxol (U.S. Biochemical). Among them, STI-571 and piceatannol are two different classes of protein tyrosine kinase inhibitors (34, 35). Etoposide is a DNA topoisomerase inhibitor, and taxol is an antitumor drug that interacts with microtubules. Treatment of the six different cancer cell lines with STI-571, a BCR-Abl protein tyrosine kinase inhibitor, led to dephosphorylation of p68 at tyrosine residue(s) (Fig. 5A, *bottom*, *lanes 2, 4, 6, and 8*). The exception was the breast cancer cells MCF-7. We repeatedly observed decreases in the tyrosine phosphorylation level rather than complete dephosphorylation in MCF-7 cells (Fig. 5A, *bottom*, *lane 10*). However, tyrosyl dephosphorylation was not observed when cancer cells were treated with piceatannol, a Syk protein tyrosine kinase inhibitor (Fig. 5B, *bottom*, *lanes 2, 4, 6, 8, and 10*), etoposide (Fig. 5C, *bottom*, *lanes 2, 4, 6, 8, and 10*), and taxol (Fig. 5D, *bottom*, *lanes 2, 4, 6, 8, and 10*). We also did not detect threonine phosphorylation on treatments with piceatannol, etoposide, and taxol as we observed with TNF- α and TRAIL treatments (data not shown). A separate experiment showed that

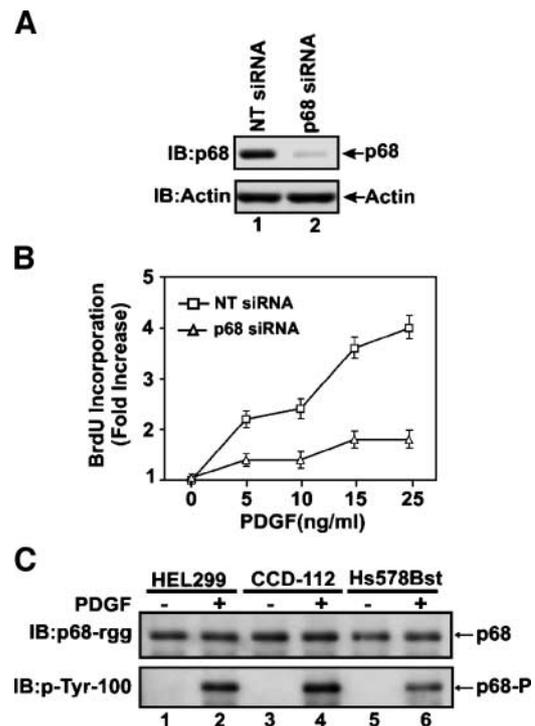


FIGURE 3. Growth factor-stimulated tyrosyl phosphorylation of p68 RNA helicase. **A.** RNA interference of knocked down p68 in HEL299 cells. NT siRNA, cells treated with nontargeting small interfering RNA. Cellular p68 was detected by immunoblotting nuclear extracts with p68-rgg. Blotting for actin served as control. **B.** Cell proliferation under different doses of PDGF stimulation was monitored by a commercially available cell proliferation kit. Cell proliferation was expressed as fold increase in BrdUrd incorporation. **C.** Cells (indicated) were treated with PDGF-BB (20 ng/mL) for 20 minutes. Cellular p68 was immunoprecipitated from nuclear extracts made from cells before and after PDGF-BB treatment. The immunoprecipitated cellular p68 was separated on 10% SDS-PAGE and subjected to immunoblot with monoclonal antibodies p68-rgg (*top*) and p-tyr-100 (*bottom*). p68 is knocked down by RNA interference.

² Unpublished observation.

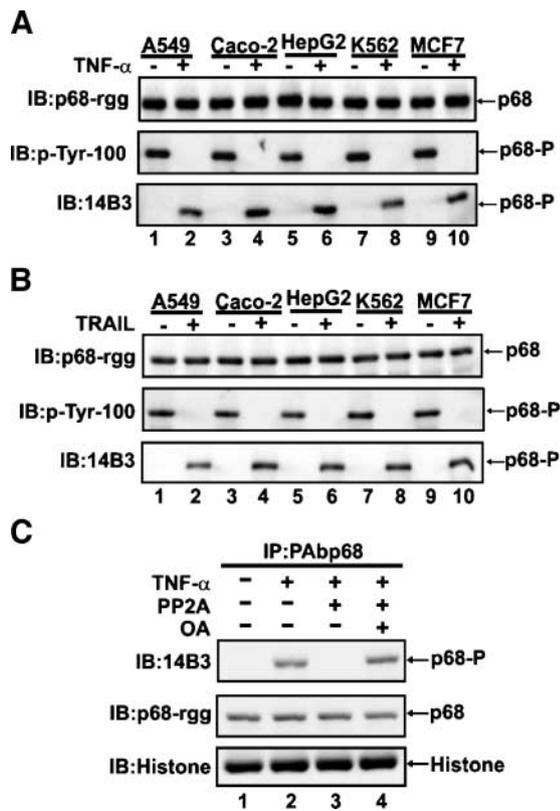


FIGURE 4. Tyrosine dephosphorylation and threonine phosphorylation of p68 in cancer cells on TNF- α (A) or TRAIL (B) treatment. Western blot of immunoprecipitated cellular p68 RNA helicase from different cancer cells (indicated). The immunoblots were carried out with antibody p68-rgg (A and B, top), antibody p-tyr-100 (A and B, middle), and antibody 14B3 (A and B, bottom). + and -, with or without TNF- α or TRAIL treatment. C. The nuclear extracts made from A549 cells with or without TNF- α treatment were treated with serine/threonine phosphatase protein phosphatase 2A and/or serine/threonine phosphatase inhibitor okadaic acid (as indicated). p68 RNA helicase was immunoprecipitated from the phosphatase- and/or phosphatase inhibitor-treated extracts and subjected to immunoblot with 14B3 (top) and p68-rgg (middle). Bottom, immunoblotting of histone in the extracts as controls.

the drugs used in our experiments, piceatannol, etoposide, and taxol, were effective (data not shown). Our data showed that the cancer cell-specific tyrosine phosphorylation(s) of p68 RNA helicase responded differently to different anticancer treatments. However, tyrosine dephosphorylation and threonine phosphorylation may not necessarily be linked to all cancer cell deaths.

Discussion

We reported here the observation that the nuclear p68 RNA helicase is tyrosyl phosphorylated in six different cancer lines and five cancerous tissues but not in the cell lines derived from each corresponding normal tissue. The close correlation between p68 tyrosyl phosphorylation(s) and cancer could be a useful diagnostic marker for cancers, especially the dephosphorylation response at tyrosine residue(s) on treatment with TNF- α , TRAIL, or STI-571 drugs. The dephosphorylation at tyrosine residue(s) resulting from anticancer drug treatment might potentially be used to monitor the effectiveness of anticancer drug treatments. Nevertheless, to use the tyrosine

phosphorylation status of p68 as a diagnostic marker for cancer, additional vigorous tests are needed to further confirm the correlation between phosphorylation and cancer. Currently, we are expanding our screening to a wider range of cancer cell lines and tissue samples from cancer patients.

The functional role of tyrosyl-phosphorylated p68 in cancer cells is an open question. The nuclear p68 RNA helicase is essential for maintaining normal cell growth. The protein is expressed in all tissue types (23). However, p68 was not phosphorylated at tyrosine residue(s) in cells derived from all normal tissues. This suggests that tyrosyl phosphorylation of p68 is not associated with the function of the protein in maintaining normal cell growth ("default function"). p68 RNA helicase plays a very important role in the cell development program, especially during early organ maturation process (22, 23). Consistent with the role of p68 in early organ development, we observed a high level of p68 expression in human embryonic kidney cells (HEK293) and embryonic lung cells (HEL299; data not shown). Interestingly, the protein is not tyrosyl phosphorylated in both of the cell lines (Fig. 1), indicating that tyrosyl-phosphorylated p68 may not be associated with the function of the protein in the early organ maturation. The tyrosyl-phosphorylated p68 most likely represents an aberrant p68 species with altered cellular function(s). This altered cellular function is associated with cell proliferation, such as in cancer cells or in PDGF-stimulated cells. This notion is consistent with our previous observations that the ATPase/helicase activities of p68, as well as the function of the protein in the pre-mRNA splicing process, were affected by tyrosine phosphorylation.³ Although we do not know whether the altered biochemical activities and the function in the spliceosome due to the protein phosphorylation are relevant to cancer development, it is clear that phosphorylation indeed alters the activity and cellular function of p68. A further step in understanding the functional role of p68 phosphorylation in cell proliferation would be to identify the cellular protein(s) that specifically interacts with the phosphorylated p68.

Dephosphorylation at tyrosine residue(s) triggered by TNF- α , TRAIL, and STI-571 is intriguing. It was reported that both TNF- α - and TRAIL-induced cell signals inhibit cell proliferation and trigger apoptosis through activation of a protein tyrosine phosphatase (Src homology phosphatase-1; ref. 36). In our previous report, we showed that the tyrosyl-phosphorylated p68 that was purified from HeLa cells could be dephosphorylated by Src homology phosphatase-1. Thus, it is highly likely that Src homology phosphatase-1, activated by TNF- α and/or TRAIL, is responsible for tyrosine dephosphorylation of p68. The tyrosine dephosphorylation of p68 is possibly associated with the apoptosis pathway induced by TNF- α and TRAIL. It was shown that the TNF- α or TRAIL-induced "extrinsic" apoptosis pathway was eventually linked to caspases 8 and 10 within a death-inducing complex (37). It would be very interesting to find out whether or not the tyrosyl dephosphorylation of p68 is linked to this complex. STI-571 is an effective antileukemia drug. The mechanism of action of the drug is to inhibit the BCR-Abl tyrosine kinase (38, 39). The drug also

³ Unpublished observation.

inhibits other protein tyrosine kinases, including c-Abl kinase (40). Thus, it is possible that the dephosphorylation of p68 at tyrosine residue(s) is due to the inhibition of c-Abl activity. Consistent with this conjecture, we observed that p68 was a cellular target of c-Abl in response to multiple cell signals (data to be published elsewhere). Because STI-571 is not completely specific for Abl protein tyrosine kinases, its inhibitory action extended to c-kit and PDGF receptor protein tyrosine kinases (41). Thus, other possibilities cannot be excluded. Dephosphorylation at tyrosine residue(s) responded differently to different anticancer drug treatments, suggesting that tyrosine dephosphorylation was not an inevitable outcome of cancer cell apoptosis. The tyrosyl dephosphorylation of p68 may only associate with certain apoptosis pathways. It is not clear whether the tyrosyl dephosphorylation of p68 plays any role in triggering cancer cell death.

Materials and Methods

All chemicals, TNF- α , TRAIL, interleukin 2, PDGF-BB, and other anticancer drugs were purchased from Sigma (St. Louis, MO), Cell Signaling (Beverly, MA), Calbiochem (San Diego, CA), PeproTech (Rocky Hill, NJ), and U.S. Biochemical (Cleveland, OH), respectively. The reagents were used according to the suggested conditions of the manufacturer without further treatment. STI-571 was a kind gift from Novartis (Basel, Switzerland).

Cells, Tissues, Cell Cultures, and Nuclear Extracts

All cell lines (Table 1) were purchased from American Type Culture Collections (Rockville, MD) and were grown according to the instructions of the vendor. All cell culture media contained penicillin (100 units/mL) and streptomycin (100 μ g/mL). The cells were grown to 90% confluency before performing any experiment. Nuclear extracts from each cell line were made using a nuclear extract preparation kit (Active-motif, Carlsbad, CA). In the cases of treatment of cells with TNF- α , TRAIL, and PDGF, the cells were serum starved for 24 hours before the

agents (the concentrations are indicated in the figure legend) were added to the cell culture medium. After the treatment for the time indicated, the cells were immediately harvested for nuclear extract preparations. For the treatment of cells with protein tyrosine kinase inhibitors, STI-571 and piceatannol, the appropriate concentrations of drugs (indicated in the figure legend) were added to the cell culture medium and the cells were incubated with the drugs overnight. For the treatment with anticancer drugs taxol or etoposide, the drugs at different concentrations (indicated in the figure legend) were added to the cell culture medium. The cells were incubated in drug-containing medium for 24 hours. Nuclear extracts from normal human lung tissue and normal human liver tissue were purchased from Active-motif. Tissue samples from cancer patients were obtained from Cooperative Human Tissue Network, South Division (University of Alabama at Birmingham, Birmingham, AL). Protein extracts were prepared from tissue samples using tissue extract kits (Active-motif).

RNA Interference for p68 Knockdown

HEL299 cells were grown to 50% confluence and transfected with small interfering RNA (100 pmol). The duplex RNA oligonucleotides for targeting p68 RNA helicase were purchased from Dharmacon siGENOME and SMARTpool (Lafayette, CO). The cells were treated with PDGF 24 hours after transfection with small interfering RNA.

Immunoprecipitation and Western Blot

Immunoprecipitation experiments were done as described in previous studies (17, 42). The cell nuclear extracts (80 μ L) were diluted to 200 μ L with radioimmunoprecipitation assay buffer [160 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L sodium vanadate, and 1 mmol/L phenylmethylsulfonyl fluoride]. The antibody PAbp68-rgg was then added to the mixture. The solution was subsequently incubated at 4°C overnight. Protein

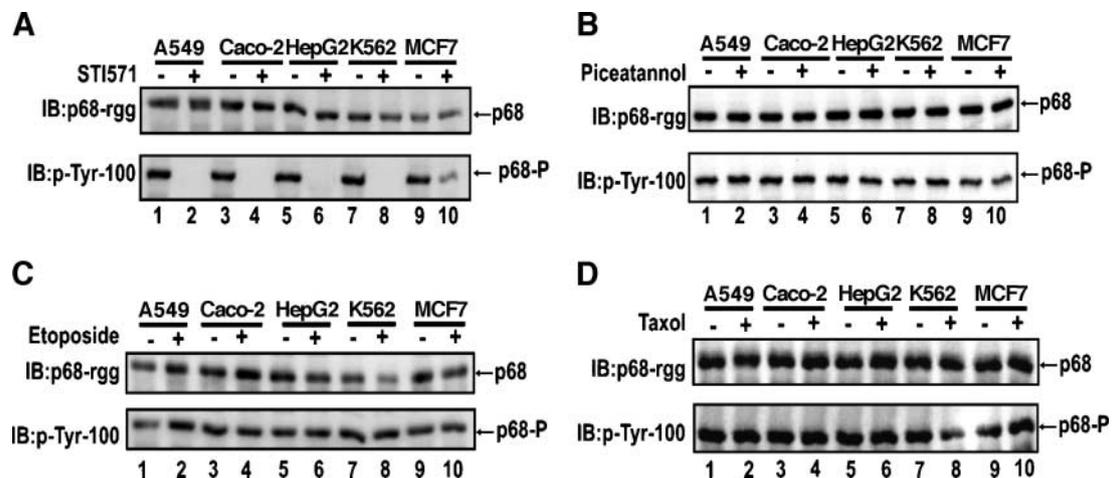


FIGURE 5. The effects of anticancer drug treatments on the tyrosyl phosphorylation of p68 RNA helicase: **A**, STI-571 (10 μ mol/L); **B**, piceatannol (50 μ mol/L); **C**, etoposide (50 μ mol/L); **D**, taxol (100 nmol/L). Western blot of immunoprecipitated cellular p68 RNA helicase from different cancer cells (indicated) before (–) and after (+) treatment with the anticancer drugs. The immunoblots were carried out with antibody p68-rgg (top) and antibody p-Tyr-100 (bottom). The p68 RNA helicase was immunoprecipitated by antibody PAbp68-rgg.

A agarose bead slurry (40 μ L) was then added and the mixture was rotated at 4°C for 2 hours. The beads were recovered and washed with 600 μ L of 5 \times radioimmunoprecipitation assay buffer with 0.08% SDS. Finally, the precipitated proteins were analyzed by SDS-PAGE followed by Western blot or were used in a further reaction described in the text.

The Western blot analyses were done with a commercially available enhanced chemiluminescence Western blot and detection kit (Amersham Biosciences, Piscataway, NJ). The supernatant from the culture medium of hybridoma cells p68-rgg was used in the blotting experiments at 5:1 dilution. The polyclonal antibody PAbp68-rgg was used at 1:3,000 dilution. The antibodies p-tyr-100, 16B4, and 14B3 were used at 1:1,000 dilution.

³²P Metabolism Labeling

HeLa cells were grown to 80% confluence in 100-mm cell culture dishes. The cells were serum starved overnight in media containing 0.5% bovine serum albumin. After removing the media, the cells were washed twice with phosphate-free media. The phosphate-free media (2 mL) containing 0.2 mCi [³²P]orthophosphate (Amersham Biosciences) was added per plate. The cells were gently shaken on a rocker for 4 to 6 hours in 5% CO₂ and 37°C. After labeling, the media were aspirated quickly and the cells were quick frozen by dousing in liquid nitrogen. Excess liquid nitrogen was poured off and 1 mL/plate of radioimmunoprecipitation assay buffer was added for cell lyses. The resulting cell lysates were subjected to immunoprecipitation followed by SDS-PAGE and autoradiography. For kinase inhibitor experiments, the cells were pretreated with indicated protein tyrosine kinase inhibitors at the concentrations indicated in the figures overnight before the addition of [³²P]orthophosphate.

Cell Proliferation Assays

HEL299 cells were distributed into 96-well plates (10⁴ cells/well) and treated as indicated in the figures. Cell Proliferation ELISA Kit (Roche, Indianapolis, IN) was used to measure BrdUrd incorporation. Briefly, cells were incubated for 6 hours in the presence of 10 μ mol/L BrdUrd. The cells were fixed, washed, and further incubated with anti-BrdUrd-peroxidase second antibody. Nuclei incorporations of BrdUrd were measured by chemiluminescence emission.

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