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Attenuation of DNA damage checkpoint by PBK, a novel mitotic kinase, involves protein–protein interaction with tumor suppressor p53

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Abstract

Pathways adopted by developing cancer cells for evasion of cellular surveillance mechanism deserve attention for therapeutic exploitation as well as for better prognosis. A novel mitotic kinase, PDZ-binding kinase or PBK, which is upregulated in a variety of neoplasms including hematological malignancies, has been the focus of our attention with a goal to understand its role in malignant conversion and to examine as a possible new therapeutic target in disparate types of cancer. Earlier, we reported that PBK expression was downregulated during macrophage differentiation of HL60 promyelocytic leukemia cells, during doxorubicin-induced growth arrest in G2/M phase and that PBK was regulated by cell cycle-specific transcription factors E2F and CREB/ATF. Here, we demonstrate that HT1080 fibrosarcoma cells become adapted to doxorubicin-induced DNA damage checkpoint upon ectopic expression of a phosphomimetic mutant of PBK as indicated by the accumulation of polyploid cells. Aberrant entry into the mitotic phase by these cells is suggested by the appearance of a mitotic phase-specific marker, MPM-2. We propose that the effect is due to downregulation of p53 caused by direct physical interaction with PBK as detected by both a biochemical means as well as by yeast two-hybrid analysis. Together, our studies provide a plausible explanation for the role of PBK augmenting tumor cell growth following transient appearance in different types of progenitor cells in vivo as reported.

Keywords: DNA damage checkpoint; PDZ-binding kinase; Tumor suppressor p53; Mitotic kinase; Protein–protein interaction

Studying upregulated kinases in cancer provides important clue as to the mechanism of malignant conversion [1–3]. Thus, c-abl, a member of the tyrosine kinase family undergoes structural changes following chromosomal translocation leading to chronic myelogenous leukemia (CML) [4]. Raf-1 kinase, a downstream intermediate of Ras signaling pathway functions as an oncogene [5]. AKT or protein kinase B which is a Ser/Thr kinase, when stimulated, acts as a survival factor for cancer cells [6]. Mitotic kinases such as Polo- like kinases (PLK), Aurora kinases, NIMA, Bub1, and BubR1 have important roles in mitotic segregation process [7–10]. Aneuploidy or abnormal chromosomal make-up, a common characteristic of tumor cells is a manifestation of chromosomal instability (CIN) [11–15]. Aberrant mitoses following polyploidization resulting from a variety of mechanisms including defects in the centrosomal duplication, maturation or segregation are considered as an early step toward stable aneuploidy that may prove advantageous to tumor cell growth. Inactivation of tumor suppressor p53, RB (retinoblastoma protein) and BRCA1 confer CIN leading to polyploidization and the development of aneuploid cells [16–18].

We have been examining the role of a mitotic protein kinase called PDZ-binding kinase or PBK [19–25]. It was reported that knockdown expression of PBK led to cytokinetic dysfunction [26,27]. Recent studies have suggested a role for PBK in DNA damage sensing and repair through phosphorylation of γ-H2AX [28,29]. We earlier reported that PBK expression was regulated by cell cycle-specific transcription factors E2F and CREB/ATF [30].

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PBK is phosphorylated by Cdc2-cyclin B at a site in the amino terminus (Thr 9) which is implicated in the binding of alpha-tubulin in vitro and its localization to mitotic spindles and spindle poles during metaphase [24,27]. Here, we have ectopically expressed a phosphomimetic mutant of PBK (T9E) in HT1080 human fibrosarcoma cells that express the PBK gene endogenously at a relatively low level. Although HT1080 cells possess activating mutations in the N-ras gene at codon 61, no mutation in the p53 gene was detected [31]. Therefore we intended to study the effect of PBK on p53 in fibrosarcoma cells. Studying the effects of ectopic expression of Src oncogene in fibrosarcoma cells unveiled important growth regulatory mechanisms involving p53 function [32]. We now demonstrate that PBK forms a complex with p53 when ectopically expressed in fibrosarcoma cells, destabilizes p53 and attenuates G2/M checkpoint during doxorubicin induced DNA damage.

Materials and methods

Plasmids and cell line. HT 1080 fibrosarcoma cell line was obtained from American Type Culture Collection, Manassas, VA (#CCL-121). Yeast vectors pGBKT7 and pGADT7 were utilized for expressing PBK and p53 as fusion proteins with Gal4 DNA-binding domain and activation domain, respectively.

Western immunoblotting and immunoprecipitations. Western immunoblotting of cell lysates derived from PBK expressing clones were carried out as described previously [25]. Monoclonal PBK antibody and FSE-conjugated MPM-2 antibody were obtained from BD Biosciences Inc., p53, phospho-Thr58/Ser62-c-Myc, phospho-Tyr15-Cdc2, Cdc25C antibodies were obtained from Cell Signaling Technology Inc., Immunoprecipitations of PBK and p53 were carried out utilizing the immunoprecipitation system from Upstate Inc., according to manufacturer's protocol.

Flow cytometry. Cell cycle analysis of propidium iodide stained clones of fibrosarcoma cells were carried out as described previously [30]. MPM-2 staining of fibrosarcoma cells was carried out by harvesting the cells following doxorubicin treatment at a concentration of 100 nM or nocodazole at a concentration of 300 nM for specified periods of time. Harvested cells were fixed, permeabilized and were incubated with FSE (Fluorescein-5-EX Succinimidyl ester)-conjugated MPM-2 antibody (BD Biosciences Inc.) for 1 h at 4 °C in 1× PBS containing 1% BSA and 0.5% Tween-20. Following this period, excess antibodies were washed off and cells were incubated with propidium iodide in the presence of RNase in the same way as that for cell cycle analysis. Bi-variate analysis of cells with orange (propidium iodide) and green (MPM-2) fluorescence were carried out in BD LSRI flow cytometer (BD Biosciences Inc.). Areas of orange fluorescence corresponding to the horizontal bar as shown in Fig. 4 and overlapping to the green fluorescence peak were integrated in order to estimate percent G2 cells that were MPM-2 positive.

Yeast two-hybrid analysis. Both PBK and p53 entire protein coding regions were cloned in pGBKT7 and pGADT7 vectors to express as fusion proteins with Gal4 DNA-binding domain or activation domain, respectively (Clontech Inc.). Isolated yeast colonies from opposite mating type hosts (AH109, a-type and Y187, alpha-type) were mated to generate diploid colonies on -Leu-Trp plates and isolated single colonies were grown in broth culture and finally plated on triple drop-out (-His-Leu-Trp) medium or double drop-out (-Leu-Trp) plates in the presence of X-alpha-gal to screen blue colonies as a positive test for protein–protein interactions.

Results

Upon examination by immunoblot analysis, fibrosarcoma cells (HT1080) showed low levels of expression of endogenous PBK gene as compared to HL60 promyelocytic leukemia cell line (Fig. 1A). However, PBK expression was negatively regulated during growth arrest predominantly in the G2/M phase by the presence of low doses of an antineoplastic compound doxorubicin, similar to leukemia cells (Fig. 1B) [30]. Thus in the presence of doxorubicin, PBK protein level diminished slowly until it was barely detectable at 96-h posttreatment. Based on these properties, we decided to transfect an engineered PBK gene driven by CMV promoter in fibrosarcoma cells and monitored expression from the transfected gene which we were able to distinguish from the endogenous PBK expression. Stable clones expressing constitutively activated PBK were isolated.

The fibrosarcoma clones carrying integrated copies of constitutive PBK (HT1080-PBK) or an empty vector (HT1080-Vector) were treated with doxorubicin. Growth arrest predominantly in the G2/M phase of the cell cycle was apparent in both cell lines with or without the transfected PBK gene, although G2 cells were relatively more abundant in the presence of PBK (Fig. 2A). PBK expression resulted in the accumulation of polyploid cell population (peak designated as M4) during later time points of

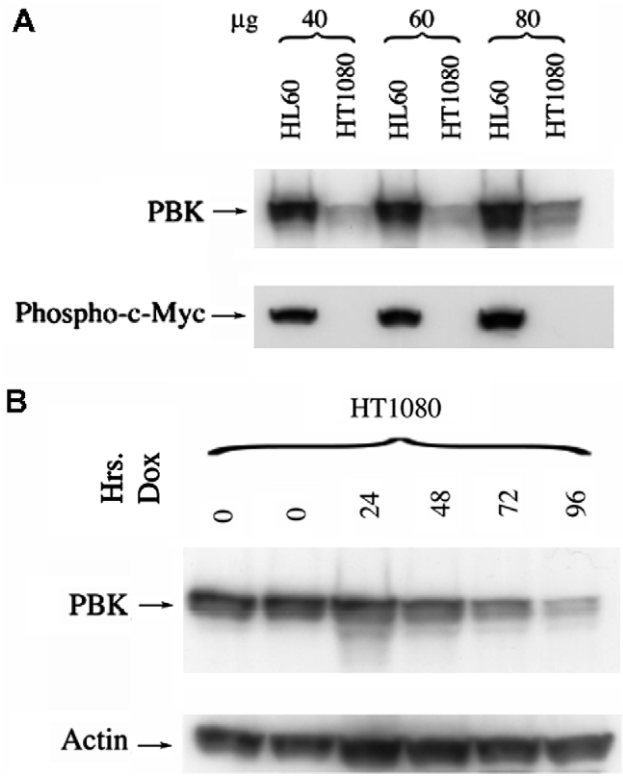


Fig. 1. A low level regulated expression of PBK in HT1080 human fibrosarcoma cells. (A) PBK expression was measured in the designated amounts of lysates from HL60 and HT1080 cells by Western immunoblotting utilizing monoclonal PBK antibody recognizing total PBK protein. (B) PBK expression was measured in HT1080 fibrosarcoma cells utilizing approximately 100 μg of total protein following doxorubicin treatment at a concentration of 100 nM after specified periods of time. Actin levels were monitored as a loading control.

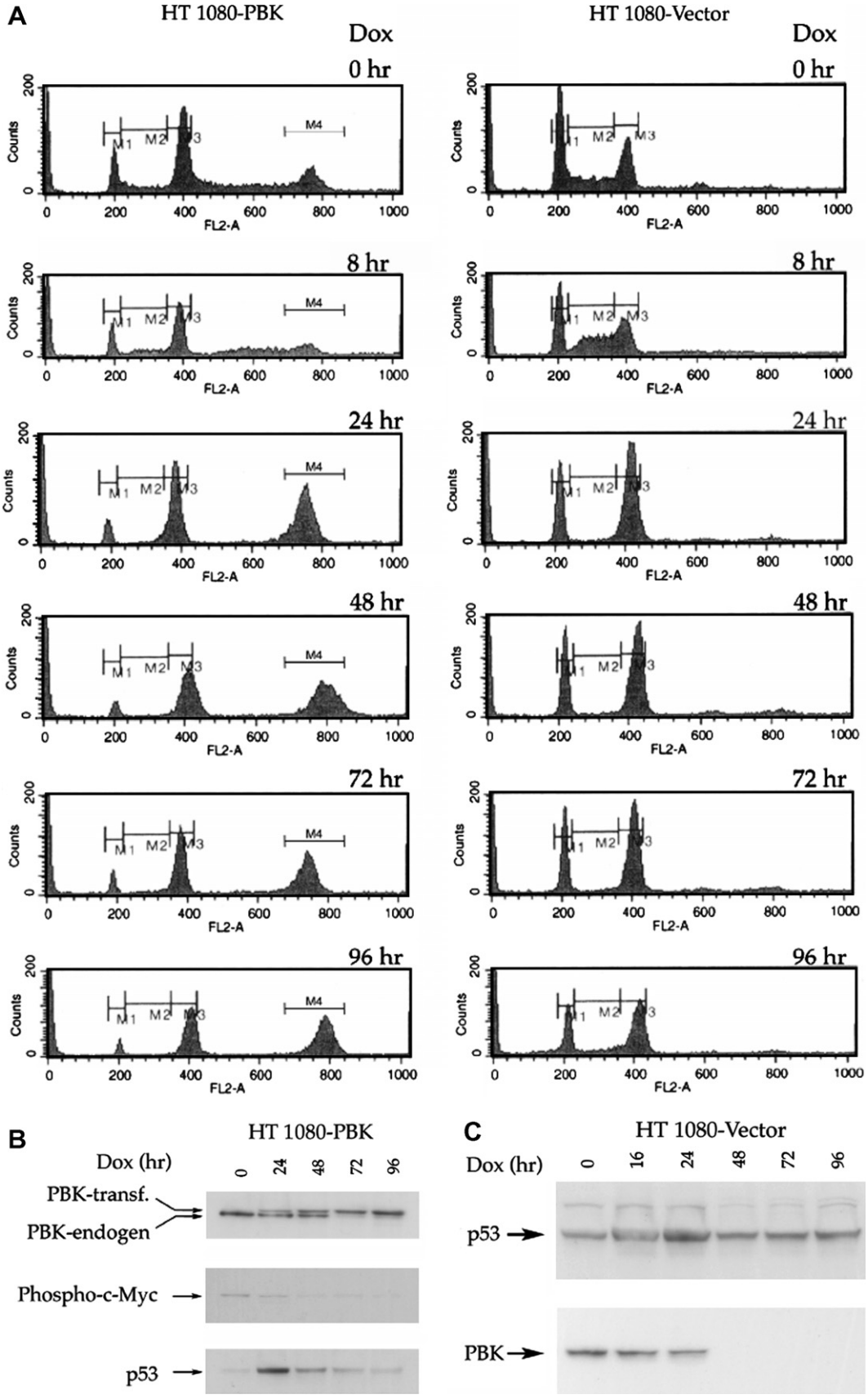
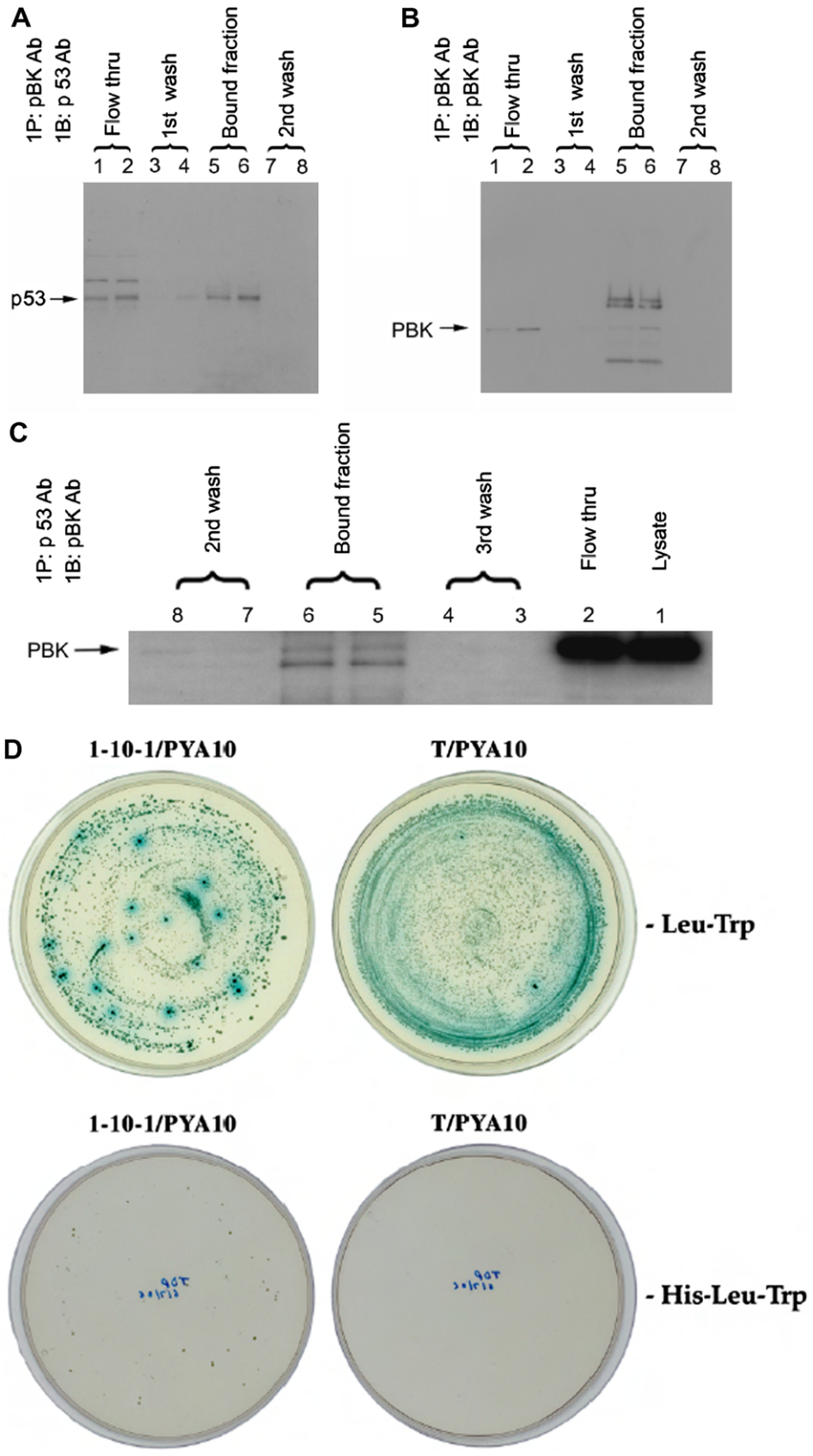


Fig. 2. Attenuation of DNA damage checkpoint by ectopic PBK expression and p53 destabilization. Fibrosarcoma cells expressing constitutive PBK or the empty vector were treated with 100 nM doxorubicin for varying lengths of time as indicated. Following these periods, cells were either used (A) for cell cycle analysis by propidium iodide staining or (B) and (C) for Western immunoblotting.



doxorubicin treatment. Upon analysis, p53 was found to be downregulated selectively in the PBK-overexpressing cells following an initial induction at 24 h in the presence of doxorubicin (Fig. 2B). Phospho-c-Myc was downregulated concomitant to endogenous PBK downregulation. The p53 levels remained almost constant in control cells during later

time points overlapping to the downregulation of endogenous PBK (Fig. 2C). These results indicated that PBK may have an effect on p53 destabilization during doxorubicin treatment leading to the attenuation of checkpoint mechanisms at the G2/M boundary. Examination of additional clonal cell lines validated the effects of ectopic PBK expression on p53 destabilization (please see Supplementary figure).

In order to examine complex formation between PBK and p53, following immunoprecipitations the fibrosarcoma lysates were probed with either the p53 antibody (Fig. 3A) or with the PBK antibody (Fig. 3B). PBK was also coimmunoprecipitated by p53 antibody utilizing Jurkat T-leukemia cells which make endogenous PBK (Fig. 3C). These experiments suggested a direct interaction between PBK and p53.

Yeast two-hybrid analysis provided a milestone in mapping the interactome which will be widely applicable to functional genomics for many future studies [33,34]. We decided to test the PBK and p53 interactions by yeast two-hybrid assay where positive interaction is demonstrated by reconstitution of the Gal4 transcriptional activator and induction of a series of reporter genes. Following this approach, we were able to select positive colonies in nutritionally deprived selection medium (-His-Leu-Trp). Furthermore, the alpha-galactosidase reporter gene was turned on producing blue colored colonies on plates containing chromogenic substrate X-alpha-gal (Fig. 3D). Thus, PBK and p53 interactions were tested positive with a different genetic approach in agreement with the biochemical experiment.

In order to have further insight into the possible cell cycle effect of PBK expression following doxorubicin treatment, we estimated the fraction of fibrosarcoma cells in the G2 phase that expressed the mitotic markers. Consequently, the mock transfected (HTV-1) or PBK transfected (HTPC-3, 9) fibrosarcoma cell lines were stained with the fluorescent MPM-2 antibody (BD Biosciences Inc.). This anibody recognizes phospho-Ser/Thr moieties present in a number of epitopes that are specifically phosphorylated during mitosis. Antibody probed cells were stained with propidium iodide and were finally analyzed in the FACScan for orange (PI) and green (MPM-2) fluorescence.

As shown in Fig. 4, the antibody peak shifted toward the G2 peak during doxorubicin treatment and it became overlapping to the G2 peak in the PBK expressing fibrosarcoma clones (HTPC-3, 9) similar to nocodazole treated control (Noc), suggesting an accumulation of mitotic markers in the G2 arrested cells expressing constitutively active PBK protein. Clones expressing PBK were not growth arrested and gave a very similar profile as the control cells at zero hour in this experiment (data not shown).

Table 1 represents the frequency of G2 arrested cells (Fig. 4) expressing the mitotic markers as compared to the nocodazole treated controls (100%). HTPC-3 and HTPC-9 (PBK transfected) contained 64.03%; 81.47% and 95.31%; 95.66% (duplicate samples), respectively of G2 cells expressing the mitotic markers. The vector transfected control cells (HTV-1) showed 31.04% accumulation. These results suggested that a greater proportion of fibrosarcoma cells expressing the constitutively active PBK protein may have entered the mitotic phase during doxorubicin induced growth arrest compared to the control cells transfected with an empty vector.

Discussion

Identifying the cellular machinery which attributes to genomic instabilities of various kinds is crucial for understanding the mechanisms accumulating further genetic alterations that lead to malignant diseases. The genes that are pertinent to this function could be worthwhile targets for cancer therapeutics and are also of immense prognostic value. Logically, emphasis are being bestowed upon a number of cell cycle checkpoints which provide surveillance towards maintaining fidelity at multiple cellular processes leading to accurate duplication of cellular entity into two daughters of identical genetic make-up. Thus, the entry into the mitotic cycle is dependent upon faithful replication of the genome during DNA synthetic S phase as well as duplication of the centrosomal structure which subsequently plays a crucial role in organizing the bipolar spindle apparatus leading to the resolution of sister chromatids to opposite poles and eventually error- free cytokinesis [35,36]. We have examined a mitotic protein kinase PBK/TOPK which, we believe, has an important role in a variety of malignant diseases including hematological malignancies, neural tumors, breast cancers and possibly several others. Upon ectopic expression of PBK in the HT1080 fibrosarcoma cell line, we were able to demonstrate an effect which suggested to us that PBK could be involved in gearing towards malignancies of the many progenitor cell types where it is reported to be induced transiently in vivo.

Fig. 3. Interactions between PBK and p53. Fibrosarcoma cells were preincubated with 100 nM doxorubicin for 24 h for induction of endogenous p53. Lysates from fibrosarcoma cells (HT1080-Vector, HT1080-PBK) treated with doxorubicin were incubated with PBK antibody in the presence of suitable affinity capture matrices overnight at 4 °C (Upstate Inc.). Flow through, wash and the bound fractions were run on gel and immunoblotted with either p53 antibody (A) or PBK antibody (B) as designated. Lanes 1, 3, 5, and 7 represent lysates from vector transfected control and hence show endogenous proteins while lanes 2, 4, 6, and 8 represent PBK transfected cell lysates. (C) Coimmunoprecipitation of PBK from Jurkat T-leukemia cell lysates utilizing monoclonal antibody against p53 (Upstate Inc.). Jurkat cells express PBK protein endogenously. Lanes 1, 2, 3, 5, 7 and 4, 6, 8 represent lysates from 18 to 24 h doxorubicin-induced (300 nM) Jurkat cells respectively. (D) Following mating between yeast clones carrying fusion proteins derived from PBK and p53 (1-10-1/PYA10), diploid cells were plated onto -His-Leu-Trp medium for growth and on -Leu-Trp medium for alpha-galactosidase activity in the presence X-alpha-gal as chromogenic substrate. T/PYA10 represents diploid carrying two-hybrid clones of SV40 T-antigen and PBK which is being utilized as a negative control.

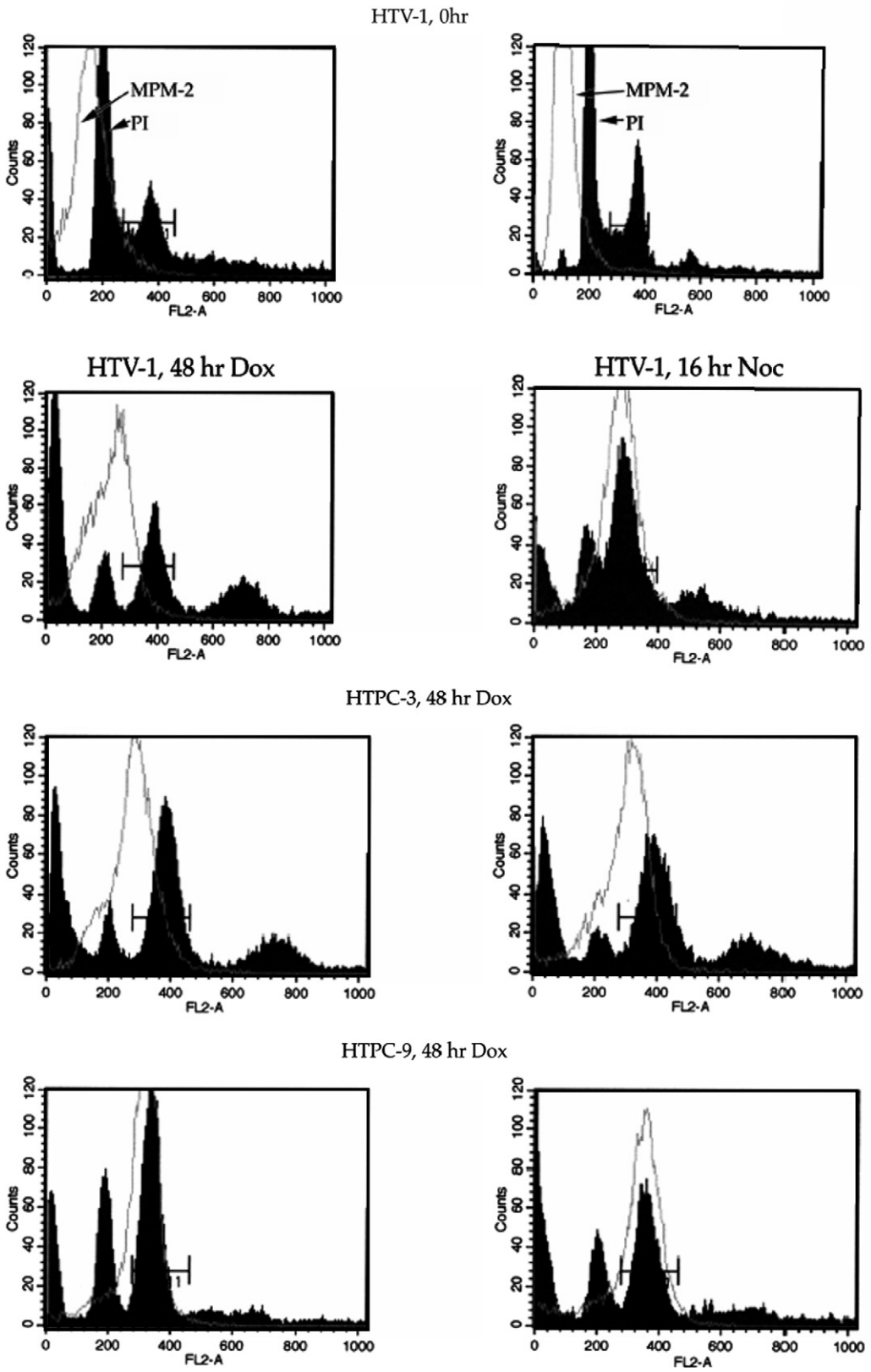


Fig. 4. Aberrant entry into mitoses by G2/M arrested cells expressing activated PBK. Fibrosarcoma cells carrying empty vector (HTV-1) or activated PBK (HTPC-3 and HTPC-9) were treated with doxorubicin for indicated periods of time. Cells were then harvested, fixed and permeabilized and stained with FSE-conjugated MPM-2 antibody for 1 h at 4 °C. Following washing out the excess antibody, cells were stained with propidium iodide (PI) in the presence of RNase and analyzed for orange (PI) and green (MPM-2) fluorescence in BD LSRI flow cytometer (BD Biosciences Inc.). Vector transfected control cells (HTV-1) were also treated with 300 nM nocodazole (for mitotic arrest) for a period of 16 h and analyzed similarly. Individual PI and MPM-2 peaks were indicated in the histogram.

Table 1
Population of G2-arrested cells expressing mitotic markers

Percent G_2 cells stained with MPM2	
HTV-1, 0 h	5.967; 1.09
HTV-1, 48 h Dox; HTV-1, 16 h Noc	31.04; 100
HTPC-3, 48 h Dox	64.03; 81.47
HTPC-9, 48 h Dox	95.31; 95.66

Areas designated by the horizontal bars in Fig. 4 were considered as G2 peak. Proportion of G2 arrested cells accumulating MPM-2 marker (M-phase-specific) were determined by measuring cell count in the area covered in the G2 peak which is overlapping to MPM-2. Nocodazole treated control (16 h Noc) was considered as 100%. Results of duplicated experiments are presented.

Our efforts led to the demonstration of adaptation of G2/M checkpoint in fibrosarcoma cells expressing PBK, causing unabated replication even under the influence of doxorubicin resulting in a significant population of cells acquiring polyploidy. Transient polyploidization leading to aneuploidy in human cells has been reported [37]. Accumulation of polyploid cells or aberrant mitoses as an indicator of G2/M checkpoint attenuation following DNA damage was demonstrated previously to be contingent upon overexpression of oncogenic factors such as E1A, Src, and c-Myc in a variety of cell types including the HT1080 fibrosarcoma cell line [32,38,39]. Our experiments show that PBK overexpression in an oncogenic background exacerbate the tendency to bypass the natural surveillance mechanism associated with G2/M checkpoint and enter into the next cell cycle phase i.e. the mitotic phase. Thus, under the influence of doxorubicin, we have demonstrated the accumulation of a mitotic phase specific marker MPM-2 in the PBK-overexpressing cells above the background level.

We suggest that loss of p53 function as a result of direct physical interaction with PBK, demonstrated by coimmunoprecipitations and yeast two-hybrid analyses, is the basis for such an effect. p53 inactivation was correlated in several recent reports to chromosomal instability and a defect in centrosomal duplication [16,40]. In murine model systems, elimination of p53 function either by targeted loss of p53 gene or by gain-of-function p53 mutations, it led to centrosomal amplifications and aberrant mitoses [41–44]. A ternary complex formation involving another mitotic kinase, Polo- like kinase 1, p53 and Cdc25c has been reported [45]. Further studies in the laboratory to characterize the binding interactions between PBK and p53 and particularly the ability to cause inhibition, may be of significance from the standpoint of therapeutic development in order to treat disparate types of cancer where PBK is upregulated.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.04.125.

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