Promotion of Mitosis by Activated Protein Kinase B After DNA Damage Involves Polo-Like Kinase 1 and Checkpoint Protein CHFR

Emma Shtivelman

Cancer Research Institute, Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA

Abstract
The role of the protein kinase B (PKB/Akt) in the regulation of cell survival and proliferation is well established. PKB is a key effector in the phosphatidylinositol 3-kinase pathway and plays a role in the initiation of S phase and in the G2-M transition. I report here that activated PKB shortens the G2 arrest induced by DNA damage and promotes early entry into mitosis. Activated PKB supports high levels of expression and activity of the polo-like kinase 1 (Plk1) after DNA damage as cells accumulate in G2. The checkpoint protein CHFR implicated in degradation of Plk1 is involved in the regulation of Plk1 by PKB. PKB phosphorylates CHFR in vitro and in vivo. Expression of a mutant form of CHFR that cannot be phosphorylated by PKB results in reduction of levels of Plk1 and inhibition of mitotic entry under normal conditions and after DNA damage. Results of this study support a model in which PKB facilitates mitotic resolution of DNA damage-induced G2 arrest by inhibiting the checkpoint function of CHFR. The deregulated activation of PKB that occurs frequently in tumors might inhibit CHFR activity after DNA damage and therefore promote Plk1 accumulation leading to the disruption of the DNA damage checkpoint.

Introduction
The phosphatidylinositol 3-kinase (PI3K) signal transduction pathway emerged as one of the most frequently activated signal transduction cascades in human tumors. Protein kinase B (PKB)/Akt is the central effector of PI3K pathway and appears to be at the crossroad of multiple cellular pathways regulating cell proliferation, survival, transcription, and metabolic responses (1). Activation of PKB occurs after stimulation with growth and survival factors by extracellular stimuli such as cell-matrix and cell-cell contacts and in response to hypoxia (reviewed in Ref. 2). Activation of PKB is initiated by the binding of 3'-phosphorylated phosphoinositide products of PI3K PI (3,4,5)-P and PI (3,4)-P to the pleckstrin homology (PH) domain. This binding results in the subsequent targeting of PKB to the plasma membrane and activating phosphorylation events at residues Thr 308 and Ser 473 by other kinases such as 3'-phosphoinositide-dependent protein kinase-1 (reviewed in Ref. 3). Activated PKB is responsible for survival signals transduced by PI3K by phosphorylating and inhibiting activity of several proapoptotic proteins (reviewed in Ref. 4). PKB also affects the G1-S transition by regulating function of cyclin D through phosphorylation and inhibition of the cyclin-dependent kinase (cdk) inhibitors p27KIP and p21WAF (5).

The dual-specificity phosphatase PTEN dephosphorylates PI (3,4,5)-P and therefore acts as negative regulator of PKB (reviewed in Ref. 6). PTEN is frequently lost or inactivated in human tumors (7), which underscores the importance of PKB activation in tumorigenesis. In addition to the survival and G1-S-promoting activities of PKB, recent evidence demonstrates that PKB participates in regulation of the G2-M transition. Activity of PKB is increased in the G2-M phase of cell cycle in epithelial cells and is necessary for the timely progression through mitosis (8). Inhibition of the PI3K/PKB pathway prevents or delays the transition from G2 to mitosis and results in apoptosis of cells arrested in G2. PKB plays a key role in these events because expression of a constitutively active PKB (caPKB), which is unresponsive to phosphatidylinositol signaling, alleviates all the effects of PI3K inhibition in G2-M (8).

To monitor genomic integrity, cells are equipped with a variety of checkpoint mechanisms that are frequently compromised in tumors. The G2 DNA damage checkpoint prevents mitotic entry when DNA is damaged, therefore guarding against genomic instability. The G1-S checkpoint is often inactivated in tumors due to the functional deficiency of p53, and abrogation of G2 checkpoint has emerged as an attractive target for tumor therapy. Because PKB is frequently activated in a variety of human tumors and because of its role in the regulation of G2-M transition, I examined the effects of activated PKB on the G2 DNA damage checkpoint and identified a potential mechanism for these effects.

Results
capKB Disrupts the G2 Arrest Induced by DNA Damage
Expression of capKB was introduced into two cell lines of epithelial origin: breast carcinoma MCF7 and canine renal epithelial line MDCK (8). capKB lacks the PH domain, and to ensure the constitutive activity of this enzyme in the absence of membrane targeting, two residues that are phosphorylated in activated PKB (Ser 473 and Thr 308) were substituted by

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Requests for reprints: Emma Shtivelman, Cancer Research Institute, Comprehensive Cancer Center, University of California San Francisco, 2340 Sutter Street, San Francisco, CA 94115. Phone: (415) 502-1985; Fax: (415) 502-3179.
E-mail: eshtivel@cc.ucsf.edu

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aspartic acid residues (9). Clones of MDCK, control (neo) and expressing caPKB, were subjected to ionizing irradiation and analyzed at different times. Determination of mitotic indices showed that in MDCKneo cells mitotic activity was inhibited for at least 4 h after irradiation but resumed before that time in MDCKcaPKB cells (Fig. 1A), indicating disruption of the G2 arrest/delay. Moreover, MDCKneo cells showed only a transient peak of mitotic activity and ceased dividing at 12–14 h after irradiation while MDCKcaPKB cells remain mitotically active (Fig. 1, A and B).

**FIGURE 1.** caPKB compromises the G2 DNA damage checkpoint. A. MDCK clones were irradiated at 6 Gy, harvested at different times, and processed for determination of mitotic indices. The experiment was performed twice with highly reproducible results. An independent pair of MDCKneo and MDCKcaPKB clones showed a highly similar response to irradiation. B. DNA content and mitotic indices of MDCK clones treated as in A. Nocodazole (250 nm), where indicated, was added for the last 12 h of incubation after irradiation. Numbers in the X-axis of the DNA histograms, percentages of cells with 2n and 4n amount of DNA. Bottom panels, percentages of apoptotic or subdiploid cells. Numbers to the right of the 4n peaks, mitotic indices. Experiments were performed with several independent clones with similar results.

C. MCF7 clones were untreated (UT), subjected to 6-Gy irradiation (IR), or treated with 1-A Adriamycin (Adri) for 1 h. Mitotic indices were calculated 18 h later. D. MCF7 clones were treated with 6-Gy irradiation or mock-treated and 300-nM nocodazole was added 1 h later. Percentages of mitotic cells were calculated 18 h later. Columns, percentages of mitotic activity in irradiated cultures relative to mock-irradiated nocodazole-treated cultures; bars, SD. Experiments were performed with several independent clones with similar results.
Analysis of DNA content showed that majority of MDCK cells initially accumulated with a 4n amount of DNA after ionizing irradiation (Fig. 1B, 8 h). This G2 accumulation was not affected by caPKB, and both types of clones eventually resolved it through mitotic division (Fig. 1, A and B). The generation time in MDCK cells is quite short (about 9–10 h) and by 12 h after irradiation majority of cells that arrested/delayed in G2 returned to interphase (Fig. 1B). At this time, only 0.5% of MCFneo cells remained mitotically active; however, in MDCFcaPKB cells, mitotic activity approached normal levels (Fig. 1B). Therefore, after resolution of the initial delay/arrest in G2, irradiated MDCFcaPKB but not MDCFneo cells continued to enter mitosis. Treatment with nocodazole for 12 h after irradiation revealed profound differences between responses of MDCFneo and MDCFcaPKB clones. MDCFneo cells underwent a partial G1 arrest (31%) but only 13% of MDCFcaPKB cells remained in G1, indicating impairment of G1 arrest by PKB (Fig. 1B). Of cells arrested by irradiation and nocodazole with 4n amount of DNA, only a third of MDCFneo transited to mitosis (20% mitoses of 60% cells with 4n DNA content). In MDCFcaPKB cells, 61% of G2-M cells were in mitosis (52% mitoses of 83% G2-M cells). Notably, when MDCK cells are treated with nocodazole alone for 12 h, 50–60% of all cells arrest in mitosis (data not shown). Therefore, irradiation of MDCFcaPKB cells did not affect total number of cells capable of entering mitosis in a span of 12 h, indicating a complete abrogation of the G2 arrest by caPKB.

Cell cycle progression of MDCK clones was analyzed also at 24 h after irradiation. In MDCFneo cells, the rate of mitosis was very low and fewer cells than normally had a 4n DNA content, indicating suppressed proliferation. In MDCFcaPKB cells, both parameters were significantly higher (Fig. 1B). Treatment with nocodazole for the last 12 h allowed to calculate total number of cells that entered mitosis after first 12 h of irradiation as well as to estimate percent of cells remaining arrested in G1. Within the second 12 h after irradiation, 4% of MDCFneo cells entered mitosis compared with 42% of MDCFcaPKB cells. This dramatic difference in mitotic rates could be ascribed in part to the fact that significantly more MDCFneo cells remained arrested in G1 (Fig. 1B, bottom panel). However, of cells arrested with 4n amount of DNA, only a small minority overcame G2 arrest (4% of 32%). In contrast to control cells, more than half (42% of 75%) of MDCFcaPKB cells with 4n amount of DNA were in mitosis. Clearly, introduction of caPKB into MDCK cells leads to the abrogation of both G1 and G2 DNA damage checkpoints.

Breast carcinoma MCF7 clones, control (neo) and expressing caPKB, were γ-irradiated or treated with the DNA damaging drug Adriamycin. Mitotic activity in MCF7 cells after these treatments ceased temporarily and resumed at later time points; however, in MCFcaPKB cells, resumption of mitosis occurred earlier and with higher frequency (data not shown). At 18 h, mitotic indices were significantly higher in treated MCFcaPKB than MCFneo cells (Fig. 1C). Treatment of cells with nocodazole after irradiation was used to calculate the total number of cells that overcame the G2 delay and entered mitosis within 18 h. As shown in Fig. 1D, the number of cells entering mitosis was reduced in irradiated MCFneo clones >2-fold compared with unirradiated nocodazole-treated cultures. However, in MCFcaPKB cells, the number of cells entering mitosis after irradiation was reduced only by about 20% (Fig. 1D), indicating that the G2 DNA damage checkpoint is severely compromised by caPKB. MCF7 cells undergo both transient G1 and G2 arrest after DNA damage, and these data show that the G2 arrest is compromised by caPKB. However, the partial G1 arrest observed in MCF7 cells was not affected by caPKB (data not shown).

PKB has been extensively characterized as a kinase with strong antiapoptotic activity (reviewed in Ref. 4). Having established that caPKB compromises the G2 DNA damage checkpoint, I have examined the consequences of its expression for the long-term survival and proliferation of cells after DNA damage using a colony-forming assay. Interestingly, the colony-forming capacity of MCFcaPKB cells in absolute numbers was higher than that of MCFneo cells (average of 10.2% vs. 4.8% for MCFneo, respectively), indicating an advantage conferred by caPKB expression for survival and/or proliferation of plated isolated cells. However, colony survival rate of MCFcaPKB cells after treatment with γ-irradiation or Adriamycin was actually somewhat lower than that of MCFneo cells (Fig. 2). No significant differences were observed in the long-term survival of MDCFneo and MDCFcaPKB cells subjected to DNA damage (data not shown), although, as with MCF7 cells, caPKB enhanced the colony-forming potential of untreated MDCK cells. In addition, MCFcaPKB and MDCFcaPKB clones transiently treated with staurosporine, a potent inducer of apoptosis, had a 10-fold higher long-term survival rate than control (neo) clones (data not shown). Therefore, the antiapoptotic activity of caPKB is well expressed but is not protective in the context of DNA damage.

Presence of Activated Exogenous PKB Alleviates Inhibition of Plk1 After DNA Damage

The serine/threonine kinase polo-like kinase 1 (Plk1) promotes G2-M progression by regulating activity of several key mitotic regulators (reviewed in Ref. 10). Phosphorylation by Plk1 contributes to the activation of the positive mitotic regulator phosphatase Cdc25C (11). Plk1 also phosphorylates cyclin B, promoting its association with activated cdk1 (12, 13). Plk1 protein levels begin to accumulate in late S-G2 phase of normal cell cycle and its activity peaks at mitosis (14, 15). Activity of Plk1 is targeted by DNA damage checkpoint, and inhibition of Plk1 is instrumental in ensuring G2 arrest (16, 17). I examined if expression of caPKB affects the activity of Plk1 after DNA damage. In MCFneo cells at 2 h after irradiation, Plk1 activity was inhibited 2-fold but no inhibition was observed in irradiated MCFcaPKB cells (data not shown). This indicated that caPKB might rescue Plk1 from inhibition after DNA damage. To better examine the effect of PKB on Plk1 after DNA damage, I used HeLa cells in which irradiation initially leads to a very strong inhibition of Plk1 (see below). Attempts to introduce constitutive expression of caPKB into HeLa cells have not produced stably transfectcd caPKB-expressing clones. Therefore, an inducible form of PKB, PKB-ER, was stably introduced into HeLa cells. PKB-ER is a
fusion of PKB sequences with the hormone-binding domain of estrogen receptor (18). Its expression is not detectable in transfected cells unless they are treated with 4-hydroxytamoxifen (4OHT; 19), which induces expression of a PKB fusion protein that possesses a high kinase activity.

First, I examined the effect of PKB-ER on irradiation-induced G2 arrest. HeLa cells undergo a transient G2 arrest resolved eventually through mitosis. Induction of PKB-ER in stably transfected clonal populations of HeLa-PKB-ER did not affect mitotic indices of unirradiated cultures (Fig. 3A). However, the G2 delay observed after irradiation of uninduced HeLa cells was compromised in 4OHT-treated cells expressing activated PKB-ER (Fig. 3A). DNA content analysis showed that HeLa-PKB-ER cells resolved the irradiation-induced accumulation of cells with 4n amount of DNA faster when expression of PKB-ER was induced (data not shown). As a control for possible undesirable effects of tamoxifen, identical experiments were performed using HeLa cells and a vector-transfected control clone of HeLa with and without tamoxifen treatment. Mitotic responses to irradiation of these control cell populations were not affected by treatment with tamoxifen and were identical to those of HeLa-PKB-ER cells in the absence of tamoxifen (data not shown).

Next, I examined activity of Plk1 after irradiation of HeLa-PKB-ER with and without 4OHT treatment. In uninduced HeLa-PKB-ER clones, activity of Plk1 was strongly inhibited after irradiation (Fig. 3B). However, induced HeLa-PKB-ER cells failed to significantly down-regulate Plk1 activity (Fig. 3B). These data indicate that exogenously expressed activated PKB abrogates inhibition of Plk1 by DNA damage.

Activation of Exogenous PKB After DNA Damage Correlates With Accumulation and Activation of Plk1

Having observed that exogenously expressed PKB prevents inhibition of Plk1 by DNA damage, I examined possible correlation between activities of endogenous Plk1 and PKB. Cells lines chosen for this analysis were osteosarcoma U2OS that respond to irradiation by a strong and lasting G2 arrest and a glioblastoma line U373 that resolves the G2 arrest through

FIGURE 2. caPKB impairs survival of MCF7 cells after DNA damage. MCFneo and MCFcaPKB clones were plated at 1500 cells/well of six-well plates and treated on the next day with the indicated doses of γ-irradiation (left panel) or adriamycin (mM; right panel) for 1 h. Colonies of >50 cells were counted 2 weeks later. Columns, averages of three experiments conducted in triplicates per point; bars, SD. Two additional MCFcaPKB clones also had a lower colony-forming ability than control clones after DNA damage.

FIGURE 3. Activated PKB promotes mitosis after DNA damage and prevents inhibition of Plk1. A, HeLa-PK-BER cells (incubated in the presence or absence of tamoxifen; 4OHT) were subjected to 10-Gy γ-irradiation and analyzed for mitotic activity at indicated times. B, HeLa-PKB-ER cells were treated as in A and analyzed for Plk1 activity. The autoradiograph shows incorporation of 32P into γ-casein used as a substrate in kinase assays. The graph shows results of Plk1 kinase activity assays in arbitrary units, where 1 is activity in untreated cells.

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mitotic progression (Fig. 4). PKB activity was found to be very low in untreated U2OS cells by Western blot analysis with a phosphospecific antibody to phospho-Ser 473 (Fig. 4). After irradiation, PKB activity dropped to levels undetectable by Western blot analysis with this antibody and became marginally higher 2 days later as confirmed by direct measurement of kinase activity in vitro (Fig. 4). Plk1 activity remained low in U2OS cells throughout the course of experiment (Fig. 4) in agreement with published results (16). During normal cell cycle progression, cells reaching G2 phase accumulate high levels of Plk1. However, in irradiated U2OS, although majority of cells arrested in G2, the levels of Plk1 protein were not elevated (Fig. 4). I suggest that lack of Plk1 activation in G2-arrested U2OS cells is a direct consequence of the low Plk1 protein levels, and this could be related to the strong G2 arrest.

Glioblastoma U373 cells have very high levels of active deregulated PKB due to the inactivation of the tumor suppressor PTEN (20). Irradiation of U373 cells lead to a moderate increase in the phosphorylation of Ser 473 as detected by Western blot analysis (Fig. 4). Direct measurements of PKB kinase activity in vitro actually showed activation of PKB kinase in U373 after irradiation (Fig. 4). Both activity and

![Graphs showing PKB and Plk1 activity in U2OS and U373 cells at different time points after irradiation.](https://example.com/graph.png)

**FIGURE 4.** Plk1 levels and activity are differentially regulated after DNA damage in cell lines with different levels and regulation of PKB activity. Cells were irradiated at 10 Gy, harvested at indicated times, and processed for Western blot analysis of the activation status of PKB with phosphospecific antibody to Ser 473 (pPKB), PKB protein levels (PKB), Plk1 levels (Plk1), and DNA content and mitotic indices. Percentages of cells with 4n amount of DNA (G2/M) and mitotic cells (M) are below the Western blots. Bottom, representative assays of activities of PKB (PKB kinase), Western blot analysis of PKB immunoprecipitated for kinase activity assays (PKB IP), and results of the quantitative analysis of the kinase activity. Analogous results are shown for Plk1 (Plk1 kinase and Plk1 IP). Antibodies raised in different species were used for immunoprecipitation and Western analysis to prevent detection of the IgG heavy chains. Columns, kinase activity normalized to activity in untreated cells and a median result of two experiments that produced very similar results.
levels of Plk1 were strongly increased as progressively more irradiated cells accumulated in G2-M (Fig. 4). Apparently, neither Plk1 levels nor its activity in U373 cells reaching G2-M after irradiation were adversely affected by DNA damage.

To further illustrate the differences between G2 responses to DNA damage in U2OS and U373 cells, nocodazole treatment after irradiation was used to examine cumulative Plk1 activity and levels (Fig. 5). There was only a minimal reduction in both Plk1 activity and levels in irradiated U373 cells compared with control unirradiated cultures, further demonstrating that Plk1 activity is not subject to inhibition after DNA damage in U373. In a stark contrast to U373 cells, in U2OS cells, both levels and activity of Plk1 were dramatically inhibited by irradiation.

These results show that activity of Plk1 after DNA damage in cell lines analyzed and in HeLa cells (data not shown) correlates strongly with the levels of Plk1 protein. The failure of U2OS cells to accumulate higher levels of Plk1 protein could be directly responsible for the low Plk1 activity observed. Correspondingly, high Plk1 activity in irradiated U373 cells directly corresponds to the accumulation of Plk1 protein. These results also show a good correlation between activation of PKB and accumulation of Plk1 protein after irradiation in two different tumor lines, although this correlation does not imply a direct relationship.

**CHFR, a Mitotic Checkpoint Protein and an E3 Ubiquitin Ligase for Plk1, Is a PKB Substrate in Vitro and in Vivo**

Data in Figs. 4 and 5 suggest that inhibition of Plk1 activity by DNA damage might be directly related to the failure of the G2-arrested irradiated cells such as U2OS to accumulate high levels of Plk1 protein normally found in G2 (21, 22). A negative regulatory mechanism for controlling Plk1 levels was identified recently (23) that involves the mitotic checkpoint protein CHFR (24). CHFR delays mitotic entry of cells subjected to mitotic stress such as interference with microtubule polymerization or stability (24). CHFR was shown to possess an E3 ubiquitin ligase activity that is specific for Plk1 protein (23). Overexpressed CHFR can ubiquitinate Plk1 and lead to its degradation in *Xenopus* extracts (23). CHFR protein contains a forkhead-associated domain (FHA) at its NH2 terminus (24), and several FHA proteins were shown to be direct phosphorylation targets of PKB (reviewed in Ref. 25). Indeed, the MotifScan search of human CHFR sequence predicted two high probability consensus phosphorylation sites for PKB at both ends of the FHA domain of CHFR (Thr 39 and Ser 205), a localization similar to that shown for the PKB-phosphorylated sites in forkhead transcription factors (25). Using *in vitro* phosphorylation assays, I found that a baculovirus-expressed partially purified PKB protein phosphorylated the glutathione S-transferase (GST)-CHFR fusion protein. When T39A and S205A substitutions were introduced into CHFR protein, phosphorylation was strongly reduced (Fig. 6A).

To examine if CHFR is phosphorylated by PKB *in vivo*, phosphospecific antibodies were raised to phospho-Thr 39. Extremely low levels of endogenous CHFR prevent identification of this protein in total cell lysates; therefore, I have examined phosphorylation *in vivo* of exogenously expressed CHFR. Mammalian expression vectors for the *myc*-tagged wild-type CHFR and the double mutant T39A and S205A (CHFR-TASA) were transiently transfected into HEK293 cells. Both proteins were efficiently detected with an antibody against *myc* epitope. The phosphospecific antibody recognized only the wild-type protein, indicating that it is phosphorylated on Thr 39 in *Xenopus*. Exogenously expressed CHFR protein containing Ala 39 was not recognized (Fig. 6B).

To further demonstrate that CHFR is phosphorylated by PKB *in vivo*, I have transfected CHFR into HeLa-PKB-ER cells in the absence or presence of tamoxifen and analyzed phosphorylation of T39. As shown in Fig. 6C, phosphorylation on T39 was increased when expression of activated PKB was induced, suggesting that PKB is indeed responsible for the phosphorylation of Thr 39 in CHFR.

Because CHFR is an E3 ubiquitin ligase and auto-ubiquitiniates *in vivo* (20), I have examined if this activity of CHFR is affected by the phosphorylation status on T39 and S205. Proteins expressed from wild-type CHFR, TASA mutant, and I306A mutant (the RING finger domain mutant deficient in ubiquitin ligase activity; 23) were immunoprecipitated from transiently transfected HEK293 cells and analyzed for the presence of ubiquitin conjugates. These were detected in both wild-type and TASA mutant but not in I306 mutant (Fig. 6D). Moreover, the amount of ubiquitinated species detected in CHFR-TASA was not reduced compared with the wild-type CHFR protein, although expression levels of TASA are consistently and significantly lower than levels of the wild-type protein (Fig. 6, B and D). Inhibition of CHFR phosphorylation by PKB might therefore lead to an increase in CHFR ubiquitin ligase activity and could account for the lower expression of transfected CHFR-TASA due to faster degradation.

**FIGURE 5.** Irradiation inhibits accumulation and activation of Plk1 in U2OS but not U373 cells. Plk1 activity was quantified in untreated cells or in cells treated with 300-nM nocodazole alone for 18 h or irradiation at 10 Gy followed by nocodazole for 18 h. Top, levels of Plk1 protein in total cell lysates (Plk1 WB), amounts of Plk1 precipitated for the kinase activity assays (Plk1 IP), and autoradiographs of kinase activity assays (Plk1 kinase). Bottom, quantitative analysis of kinase activity assay.
To examine in a more direct manner how phosphorylation by PKB might affect auto-ubiquitination of CHFR, CHFR and TASA were transfected into HeLa-PKB-ER cells in the absence and presence of tamoxifen. Presence of ubiquitinated conjugates was examined in immunoprecipitated CHFR proteins. Ubiquitin conjugation of CHFR was somewhat inhibited when activity of PKB-ER was induced (Fig. 6E), suggesting that phosphorylation by PKB has a negative effect on the E3 ligase activity of CHFR.

**CHFR Protein That Cannot Be Phosphorylated by PKB Inhibits Entry Into Mitosis**

Wild-type CHFR and CHFR-TASA were transiently transfected into HeLa cells to examine their effect on Plk1 levels, kinase activity, and mitotic activity of cells. Expression of exogenous CHFR-TASA but not wild-type CHFR lead to a noticeable decrease in Plk1 levels and its activity (Fig. 7A). Activity of Plk1 precipitated from cells transfected with CHFR-TASA was 40% of Plk1 activity from mock-transfected or wild-type CHFR-transfected cells (average of three experiments). Considering that only about 30–40% of HeLa cells were transfected in these experiments (data not shown), these results are significant.

I have examined how mutations T39A and S215A affect the checkpoint function of CHFR that was shown to inhibit/delay entry into mitosis. Expression vectors for the myc-tagged wild-type CHFR and the double mutant T39A and S205A (CHFR-TASA) were transiently transfected into HeLa cells to examine their effect on mitotic transition under normal conditions as well as genotoxic and mitotic stress.

Mitotic indices were calculated specifically in transfected cells that were identified by expression of the cotransfected
enhanced green fluorescent protein (EGFP). The wild-type CHFR had no effect on the frequency of mitoses (Fig. 7B). However, a significant reduction in mitotic indices was observed in cells transfected with the mutant CHFR-TASA (Fig. 7B). Transfected HeLa cells were γ-irradiated and harvested 18 h after irradiation. This time was chosen because in HeLa cells mitotic activity is highest at 16–20 h after irradiation and eventually results in resolution of the G2 arrest (data not shown). Cells expressing CHFR-TASA but not wild-type CHFR had a significantly lower rate of mitosis, indicating that the G2 arrest is enforced by CHFR-TASA (Fig. 7B). These results show that CHFR not phosphorylated by PKB contributes to inhibition of the G2-M transition under both normal conditions and when DNA is damaged.

The effect of CHFR-TASA on mitotic entry under conditions of mitotic stress was also examined. After taxol treatment (Fig. 7B) and nocodazole treatment (data not shown), both wild-type and mutant CHFR were able to inhibit mitotic entry. I have also used the RING finger mutant of CHFR (23) and found that transient expression of CHFR-I306A had no effect on mitosis under any of conditions (data not shown).

These results suggest that phosphorylation of CHFR by PKB might inhibit its ability to enforce DNA damage-induced G2 arrest but could be irrelevant to its function as a mitotic checkpoint. The E3 ligase activity of CHFR is necessary for inhibition of transition to mitosis under all conditions as the RING finger domain mutant has no effect on chromosomal condensation under any conditions tested.

Discussion

This study was initiated to examine the effect of PKB on the G2-M transition of cell cycle after DNA damage. The rationale for the experiments described here was based on recent findings (8) showing that (i) activity of PKB is increased in the G2-M phase of cell cycle and is necessary for the timely progression through mitosis, (ii) inhibition of the PI3K/PKB pathway prevents or delays the transition from G2 to mitosis and results in apoptosis of epithelial cells arrested in G2, and (iii) inhibition of PKB is largely responsible for these events because introduction of a caPKB alleviates all the consequences of PI3K inhibition on the G2-M transition. My results, as well as recently published results of Roberts et al. (26), showed that inhibition of PI3K/PKB pathway hails mitotic progression most likely by inhibiting activation of cyclin B/cdk1 kinase.

While this manuscript was in preparation, a report was published that demonstrated a role for activated PKB in abrogation of the G2 DNA damage checkpoint (27). Experiments reported here lead to similar conclusions about the negative effect of activated PKB on the DNA damage-induced G2 arrest. Deregulated activity of PKB promotes early mitotic entry of cells with damaged DNA (i.e., leads to the abrogation of the G2 DNA damage checkpoint).

**FIGURE 7.** Exogenously expressed CHFR that cannot be phosphorylated by PKB induces down-regulation of Plk1 levels and activity and inhibits mitosis. **A.** HeLa cells were transfected with either wild-type mycCHFR or the mutant form (TASA). Levels of mycCHFR and Plk1 were determined by Western blotting with antibodies to the myc epitope and Plk1, respectively. **Lower panel,** Activity of Plk1 was measured in the kinase assay with α-casein as a substrate. **B.** HeLa cells were cotransfected with the indicated plasmids and pEGFP at 3:1 ratio. Mitotic indices were calculated from cyto spun preparations in which only EGFP-positive transfected cells were counted. In untreated transfected cultures (UT), mitotic frequency was determined 32–36 h after transfection. Irradiated cells (IR) were subjected to 10-Gy γ-radiation at 24 h after transfection and harvested 18 h later. For taxol treatment (Tax), cells were treated with 100-nM taxol for 8 h starting at 24 h after transfection. **C.** A model proposed to explain the observed effect of PKB on G2-M transition. Question marks, unknown mechanisms that result in up-regulation of PKB activity in normal G2-M and its modulation after DNA damage and unknown factors that modulate activity of Plk1 in an ATM/ATR-dependent manner.
of G₂ arrest). Kandel et al. (27) reported protection from DNA damage-induced apoptosis by myristoylated Akt in Rat1a cells. I found that the caPKB lacking the PH domain does not protect cells from death induced by DNA damage, although it confers a survival advantage after non-DNA damaging cytotoxic treatments. On the contrary, MCF7 clones with caPKB had a somewhat lower survival rate after DNA damage inflicted by irradiation of Adriamycin in long-term assays. These differences could be due to the different forms of PKB used. Indeed, the caPKB used in this study is apparently not subject to any known form of regulation of PKB activity and is truly constitutively active being independent of PIP3 binding, membrane recruitment, and phosphorylation by 3’-phosphoinositide-dependent protein kinase-1 (9). Apparently, by promoting mitosis soon after DNA damage, caPKB contributes to the frequency of mitotic catastrophe that leads to increased cell death through a nonapoptotic pathway.

I suggest that activated PKB in tumor cells actually affects the resolution of the DNA damage-induced G₂ arrest rather than establishment of the initial G₂ arrest. It was shown that the cessation of mitotic activity within first 1 or 2 h after irradiation is an axatia telangiectasia mutated (ATM)-dependent process that represents failure of cells that are in G₂ during irradiation to enter mitosis (28). I have not observed significant effects of activated PKB on the mitotic activity at 2 h after irradiation in MDCK, MCF7, or HeLa-PKB-ER cells (data not shown). However, activated PKB promotes mitosis after the initial checkpoint response, indicating that it might affect an ATM/ATR and Rad3 related (ATR)-independent step in the resolution of the G₂ arrest.

This study identifies a likely G₂-M target of PKB and provides one possible mechanism of how PKB positively regulates normal G₂-M transition and inhibits DNA damage-induced G₂ arrest. Data reported here suggest that the abrogating effect of activated PKB on the G₂ arrest is mediated at least in part through alleviation of the Plk1 inhibition that follows DNA damage (16). Suppression of Plk1 activation appears to be a conserved mechanism for preventing mitotic division when DNA is damaged. Inhibition of Cdc5, the yeast homologue of Plk1, was shown to be involved in the prevention of mitotic exit after DNA damage (29). Regulation of Plk1 activity occurs at multiple levels: Plk1 protein can apparently be degraded before the onset of mitosis (data not shown). Plk1 protein accumulates in the G₂-M phase of cell cycle (14, 15) and is degraded at the exit from mitosis by the anaphase-promoting complex (21). In addition, activation of the Plk1 kinase in G₂-M involves phosphorylation (30, 31).

Plk1 protein can apparently be degraded before the onset of mitosis in the presence of high levels of the ubiquitin ligase CHFR at least in Xenopus extracts (23). CHFR was originally described as a mitotic checkpoint protein (24) capable of delaying or preventing entry into mitosis. Expression of CHFR is frequently absent in tumors due to hypermethylation (32–35). My results show that the checkpoint function of CHFR in DNA damage but not in mitotic stress responses is regulated by PKB that directly phosphorylates CHFR on Thr 39. Phosphorylation of CHFR by PKB appears to negatively affect its activity as an ubiquitin ligase. Unphosphorylated CHFR (TASA mutant) shows a constitutive checkpoint function and prevents mitotic transition even under normal conditions.

These data suggest a simple mechanism that can explain at least partially the PKB effects on G₂-M transition both under normal growth conditions and after DNA damage (Fig. 7C). Under normal conditions, an unknown mechanism induces high PKB activity in G₂-M (9) that results in the inhibition of CHFR checkpoint activity possibly through negative effect on its E3 ligase activity. This is supported by the data that CHFR-TASA auto-ubiquitinites at a higher rate than the wild-type CHFR (Fig. 6D) and that in the presence of activated PKB, CHFR appears to contain less ubiquitin conjugates (Fig. 6E). When the ubiquitin ligase activity of CHFR is suppressed through phosphorylation by PKB, Plk1 protein can accumulate, undergo activating phosphorylation, and promote the G₂-M transition. DNA damage might act to prevent the increase in PKB activity in G₂ by unknown mechanisms (Fig. 7C, question mark on the left), therefore allowing expression of the checkpoint function of CHFR that results in ubiquitination and degradation of Plk1 and a consequent arrest in G₂. DNA damage also could lead to inhibition of Plk1 kinase activity in ATM/ATR-dependent manner (17) via yet unidentified factors (Fig. 7C, question mark on the right). In tumor cells such as U373 where tumor suppressor PTEN is inactive, DNA damage apparently does not inhibit activation of PKB. PKB activity remains low as cells accumulate in G₂ after DNA damage, although PKB activity is high in normal G₂-M (data not shown). U2OS cells express CHFR. Although CHFR from U2OS cells was initially reported to be a mutant form (24), the single nucleotide change in U2OS CHFR was found to represent a common polymorphism (32). Therefore, CHFR may possibly be involved in regulation of Plk1 levels in U2OS cells after DNA damage.

Data show that while CHFR phosphorylated by PKB on T39 can prevent chromosome condensation in response to interference with microtubule dynamics, it does not inhibit mitotic transition in response to DNA damage. Only the version that cannot be phosphorylated by PKB is able to prevent mitotic transition after DNA damage and even under normal conditions acting as a constitutive checkpoint. The role of the FHA domain would appear to be different in responses of CHFR protein to different types of stress.

CHFR is likely to be not the only G₂-M target of PKB. A role was described for transcription factor FOXO3a, a PKB target, in the G₂-M delay induced by stress and DNA damage repair (36). Published results (8) and work in progress strongly suggest a direct modulation (inhibition) of the activity of the DNA damage effector kinase Chk1 by activated PKB. Other G₂-M substrates of PKB remain to be identified.

Regulation of the G₂ DNA damage responses is very complex (37) and findings described here and by others (27) add an extra layer of complexity to it by implicating a major signal transduction pathway in the abrogation of the DNA damage-induced G₂ arrest. Deregelation of the PI3K/PKB pathway is a frequent event in tumorigenesis (38), and effects of activated PKB on G₂ checkpoint could have profound consequences for the tumor cell responses to DNA damage.
Materials and Methods

Cell Culture and Transfections

Cell lines were cultured either in RPMI (HeLa and U373) or in DMEM (MCFC7, MDCK, and U2OS) supplemented with 10% fetal bovine serum. Transfections were performed using Fugene (Roche, Switzerland) according to the manufacturer’s instructions. Transient transfections of HeLa cells were performed 1.5–2 h after plating, resulting in transfection efficiencies of at least 30%. Stable transected clones were propagated from isolated single cell colonies following selection with an appropriate concentration of geneticin G418.

Plasmids and Antibodies

Plasmids used for transfections were pCS-mycCHFR (a gift of G. Fang), pWZLneoAkt-ER (a gift of M. McMahon), and pEGFP-C1 (Clontech Laboratories, Inc., Palo Alto, CA). pWZLneoAkt-ER contains sequences of human PKB/Akt1 with the PHI domain substituted by myristoylation signal of human ε-src (18). Mutations T39A and S215A were introduced into pCS-mycCHFR and pGEX-CHFR according to standard procedures. Antibodies used were rabbit anti-PKB (Upstate Biotechnology, Inc., Charlottesville, VA) for Western blotting, antibodies were preincubated with an excess of a peptide identical to the immunizing peptide, except that Thr 39 was not phosphorylated.

Phosphospecific Antibodies

Antibodies were raised in rabbits against phosphopeptide LLRKREWpTIGRRRC corresponding to positions 32–46 in the sequence of human CHFR. Immune serum was affinity purified on a column of phosphopeptide immobilized on Sulfolink (Pierce Chemical Co., Rockford, IL). Prior to use in Western blotting, antibodies were preincubated with an excess of a peptide identical to the immunizing peptide, except that Thr 39 was not phosphorylated.

PhosphorImager.

Mitotic Spread Preparation and Quantitation

For mitotic spreads, cells were harvested 1.5-2 h after plating, resulting in transfection efficiencies of at least 30%. Stable transected clones were propagated from isolated single cell colonies following selection with an appropriate concentration of geneticin G418.

Kinase Activity Assays

Cell lysates were prepared in kinase lysis buffer: 125-nM NaCl, 50-nM Tris (pH 7.5), 1-nM EDTA, 1-nM EGTA, 10-nM sodium β-glycerophosphate, 1-nM NaVO4, 10-nM NaF, 1-nM DTT, 10% glycerol, and protease inhibitor cocktail (Roche). Active kinases (Plk1 or PKB) were precipitated with appropriate antibodies, and immunoprecipitates were collected on protein G-Sepharose (Life Technologies, Inc., Carlsbad, CA) and washed thrice in kinase lysis buffer and twice in kinase buffer (50-mM NaCl, 50-mM Tris [pH 7.5], 1-mM EDTA, and 1-mM DTT). Dephosphorylated α-casein (Sigma Chemical Co., St. Louis, MO) was used as a substrate for Plk1 and GST-AHNK-C213 for PKB (39) in kinase reaction containing 10-μM ATP and 5-μCi [32P]γ-ATP. Reactions were resolved by PAGE. Incorporation of 32P into substrates was quantified using PhosphorImager.

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References


Promotion of Mitosis by Activated Protein Kinase B After DNA Damage Involves Polo-Like Kinase 1 and Checkpoint Protein CHFR

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Emma Shtivelman


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