

Disruption of G1-phase phospholipid turnover by inhibition of Ca²⁺-independent phospholipase A₂ induces a p53-dependent cell-cycle arrest in G1 phase

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Summary

The G1 phase of the cell cycle is characterized by a high rate of membrane phospholipid turnover. Cells regulate this turnover by coordinating the opposing actions of CTP:phosphocholine cytidyltransferase and the group VI Ca²⁺-independent phospholipase A₂ (iPLA₂). However, little is known about how such turnover affects cell-cycle progression. Here, we show that G1-phase phospholipid turnover is essential for cell proliferation. Specific inhibition of iPLA₂ arrested cells in the G1 phase of the cell cycle. This G1-phase arrest was associated with marked upregulation of the tumour suppressor p53 and the expression of cyclin-dependent kinase inhibitor p21^{cip1}. Inactivation of iPLA₂ failed to arrest p53-deficient HCT cells in the G1 phase and caused massive apoptosis of p21-deficient HCT cells, suggesting that this G1-phase arrest

requires activation of p53 and expression of p21^{cip1}. Furthermore, downregulation of p53 by siRNA in p21-deficient HCT cells reduced the cell death, indicating that inhibition of iPLA₂ induced p53-dependent apoptosis in the absence of p21^{cip1}. Thus, our study reveals hitherto unrecognized cooperation between p53 and iPLA₂ to monitor membrane-phospholipid turnover in G1 phase. Disrupting the G1-phase phospholipid turnover by inhibition of iPLA₂ activates the p53-p21^{cip1} checkpoint mechanism, thereby blocking the entry of G1-phase cells into S phase.

Key words: Ca²⁺-independent Phospholipase A₂, phospholipid turnover, p53-dependent G1 arrest

Introduction

Phospholipids are the major building blocks of cell membranes, which are crucial to the life of the cell. To successfully form daughter cells, cells must double their phospholipid mass during cell-cycle progression. Phosphatidylcholine (PtdCho) is a major component of phospholipids in mammalian cells, and regulation of its biosynthesis and turnover is crucial in maintaining membrane structure and function (Lykidis and Jackowski, 2001).

PtdCho metabolism varies throughout the cell cycle (Jackowski, 1996; Lykidis and Jackowski, 2001). Although cells in G1 phase rapidly synthesize and degrade PtdCho, they maintain a constant total membrane phospholipid mass (Jackowski, 1994). By contrast, PtdCho turnover ceases in S phase to allow the cells to double their membrane phospholipid content in preparation for cell division, and the synthesis and degradation of membrane phospholipids components are at their lowest point in G2 and M phases (Jackowski, 1994; Jackowski, 1996). It is obvious that a cell must have stringent control mechanisms to keep the phospholipid content in tune with the cell cycle.

Many signals influence cell division and the deployment of the developmental program of a cell during G1 phase. Diverse metabolic, stress and environmental cues are integrated and

interpreted during this period to determine whether the cell enters S phase or pauses in its cell cycle. The G1-phase cells maintain a constant membrane phospholipid content by coordinating the opposing actions of CTP:phosphocholine cytidyltransferase (CCT) and the group VIA Ca²⁺-independent-phospholipase A₂ (iPLA₂) (Baburina and Jackowski, 1999; Barbour et al., 1999); several lines of evidence indicate that this coordination is crucial to normal cell proliferation (Jackowski, 1996; Lykidis and Jackowski, 2001). First, enforced CCT expression stimulates both the incorporation of choline and glycerol into PtdCho as well as the degradation of PtdCho to glycerophosphocholine (GPC) by upregulating iPLA₂ expression (Baburina and Jackowski, 1999; Barbour et al., 1999). Second, cellular proliferation is inhibited when PtdCho is modified to prevent its degradation to GPC (Baburina and Jackowski, 1999). Third, overexpression of iPLA₂ in cells of the insulinoma (INS-1) cell line increased the rate of cell proliferation (Ma et al., 2001).

iPLA₂ hydrolyzes the sn-2 fatty acyl bond of phospholipids to liberate free fatty acids and lysophospholipids (Ma and Turk, 2001). It has also been reported to be involved in cell proliferation (Ma et al., 2001; Roshak et al., 2000; Sanchez and Moreno, 2001; Sanchez and Moreno, 2002). Since the regulated deacylation of PtdCho to GPC is a key process in

membrane phospholipid homeostasis, and the inability to degrade excess PtdCho inhibits cellular proliferation (Baburina and Jackowski, 1999), it is possible that degradation of excess PtdCho controls cell proliferation by tethering phospholipid metabolism to endogenous pathways of cell-cycle control. To investigate this possibility, we studied whether disrupting phospholipid turnover by specifically inhibiting iPLA₂ can induce cell-cycle arrest without affecting cell viability. Here, we demonstrate that inhibition of iPLA₂ directly regulates cell proliferation, arresting cells in the G1 phase of the cell cycle. This G1-phase arrest requires activation of the tumour suppressor p53 and expression of the cyclin-dependent kinase inhibitor p21^{cip1}. These findings indicate that iPLA₂ cooperates with p53 to monitor a membrane phospholipid turnover in G1 phase.

Results

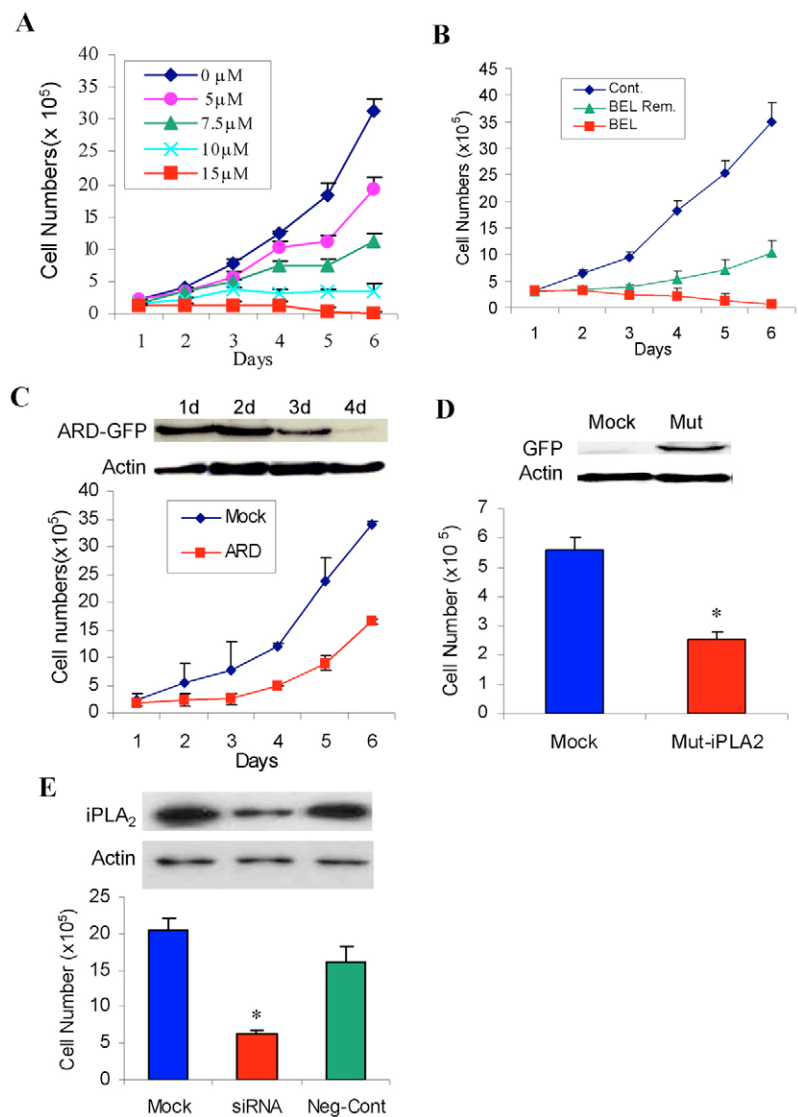
G1-phase phospholipid turnover is essential for cell proliferation

iPLA₂ plays a key role in the regulation of G1-phase phospholipid turnover by degrading PtdCho (Baburina and Jackowski, 1999; Barbour et al., 1999). We and others

previously reported that iPLA₂ is involved in cell proliferation (Ma et al., 2001; Roshak et al., 2000; Sanchez and Moreno, 2001; Sanchez and Moreno, 2002). To investigate whether iPLA₂-mediated PtdCho degradation is coupled to cell-cycle progression, we examined the effect of disrupting G1-phase phospholipid turnover by inhibiting iPLA₂.

We first used bromoenol lactone (BEL), an irreversible and mechanism-based specific inhibitor of iPLA₂, to inhibit the iPLA₂ activity in INS-1 cells (Ackermann et al., 1995; Hazen et al., 1991). We found that BEL inhibited INS-1 proliferation in a concentration-dependent manner (Fig. 1A). Cell proliferation was completely inhibited by 10 μM of BEL without causing cell death, consistent with a previous report (Fuentes et al., 2003). Cells treated with up to two days of 15 μM BEL can proliferate after removal of the inhibitor, but persistent treatment caused reduced viability (Fig. 1B). BEL has recently been reported to inhibit Mg²⁺-dependent cytosolic phosphatidate phosphohydrolase-1 (PAP-1) (Balsinde and Dennis, 1996). To determine whether PAP-1 is involved in BEL-induced inhibition of cell proliferation, we treated the cells with propranolol, a well-known PAP-1-specific inhibitor (Fuentes et al., 2003; Pappu and Hauser, 1983). In accordance

Fig. 1. Inactivation of iPLA₂ inhibits INS-1 cell proliferation. (A) BEL inhibits INS-1-cell proliferation in a concentration-dependent manner. Cells (10⁵) were treated with different BEL concentrations for up to 6 days and counted daily. Error bars represent the mean ± standard deviation (s.d.) of three experiments. (B) Proliferation of BEL-treated cells recovers upon BEL withdrawal. INS-1 cells were cultured in the presence of BEL (15 μM) for 2 days and then continuously cultured with (red) or without (green) BEL. Untreated cells were used as controls (blue). Error bars represent standard deviation from the mean of three experiments. (C) Transient expression of ARD-iPLA₂-GFP reduces INS-1 cell proliferation. INS-1 cells (2 × 10⁵) were transfected with ARD-iPLA₂-GFP (5 μg/100 mm plate), then counted daily, starting 24 hours after transfection. Mock-transfected cells (no DNA) were used as controls. The cell lysates were prepared and analyzed for expression of ARD-iPLA₂-GFP over 6 days, by western blotting with anti-GFP and anti-actin antibodies (top). Graph shows analysed data (bottom). Error bars represent the mean ± s.d. of three experiments. (D) Expression of Mut-iPLA₂-GFP inhibits the proliferation of INS-1 cells. iPLA₂-expressing INS-1 cells were transfected with Mut-iPLA₂-GFP (labelled Mut or Mut-iPLA₂ at 10 μg/100 mm plate) or mock transfected (no DNA) with FuGENE. Counting of cells began on the second day after transfection (bottom). After counting, cells lysates were prepared and analyzed by western blotting for GFP and actin (top). Error bars represent the mean ± s.d. of three experiments. **P* < 0.05. (E) Expressing siRNA against iPLA₂ decreases cell proliferation. Mock transfected INS-1 cells, and those transfected with psiRNA-iPLA₂ were counted on the fifth day after transfection (bottom), and the cell lysates were prepared for Western blot analysis for iPLA₂ (top). **P* < 0.05. A scrambled siRNA construct was transfected to serve as a negative control. Error bars represent the mean ± s.d. of three experiments.



with a previous study (Fuentes et al., 2003), inhibition of PAP-1 by 200 μ M propranolol caused a massive cell death rather than cell-proliferation arrest. This result indicates that the suppression of cell proliferation by BEL is not attributable to inhibition of PAP-1.

We next overexpressed the ARD-iPLA₂-GFP [ankyrin-repeat domain of iPLA₂ fused to green fluorescent protein (GFP)], an alternatively spliced form of the iPLA₂ gene known to function as a negative regulator of iPLA₂ (Larsson et al., 1998; Manguikian and Barbour, 2004), in INS-1 cells. Transient expression suppressed cell proliferation in these cells, which was reversed when ARD-iPLA₂-GFP expression decreased (Fig. 1C). We then mutated the serine in the GxSxG motif that confers iPLA₂ enzymatic activity (Tang et al., 1997) to alanine, and transfected this Mut-iPLA₂-GFP construct into INS-1 cells. Expression of this iPLA₂ mutant markedly suppressed cell proliferation (Fig. 1D). Lastly, we utilized small interfering RNA (siRNA) against iPLA₂ to downregulate iPLA₂ expression in INS-1 cells (Song et al., 2005), which also markedly inhibited cell proliferation (Fig. 1E). Taken together, our data unambiguously demonstrate that specific inhibition of iPLA₂ blocks proliferation of INS-1 cells.

Disruption of phospholipid turnover arrests cells in G1 phase

Since phospholipid turnover is a characteristic of G1-phase cells (Jackowski, 1996; Lykidis and Jackowski, 2001), we asked whether inhibition of iPLA₂-mediated PtdCho degradation affects S-phase entry. We measured 5-bromodeoxyuridine (BrdU) incorporation by fluorescence-activated cell sorting (FACS) after inhibition of iPLA₂. BrdU incorporation markedly decreased with increasing BEL concentrations; 15 μ M BEL almost completely blocked the incorporation of BrdU (Fig. 2A), suggesting that phospholipid turnover in G1 phase is required for cells to enter S phase. Furthermore, staining for the mitosis biomarker phosphohistone H3 (Nowak and Corces, 2004) showed that, in contrast to the G2-M-transition arrest caused by nocodazole treatment, few cells progressed to G2-M transition after 30 hours of BEL treatment (Fig. 2B).

To better characterize this cell-cycle arrest, we treated cells with both BEL and nocodazole and analyzed them by FACS (Agami and Bernards, 2000). Although nocodazole treatment alone showed an accumulation of G2-M-transition cells with time (Fig. 2C), treatment of cells with both BEL and nocodazole showed a BEL-concentration-dependent increase in the number of cells in G1 phase and a concomitant decrease in S phase and G2-M transition, confirming the G1-phase arrest.

To verify that BEL affects only iPLA₂, we overexpressed wild-type iPLA₂-GFP or ARD-iPLA₂-GFP in INS-1 cells and used FACS to sort cells with varying levels of GFP (Fig. 3A). Consistent with previous data (Ma et al., 2001), overexpression of iPLA₂ increased the proliferation rate of INS-1 cells. After FACS sorting, a mixed population (R1 population) of GFP-containing cells showed a similar cell-cycle distribution for both fusion proteins. When we selected a cell population with higher levels of ARD-iPLA₂-GFP (R2 or R3), we observed more cells in G1 phase and fewer in S phase and G2-M transition. By contrast, selection for higher iPLA₂-GFP expression gave a similar cell-cycle distribution, regardless of

the gate used (Fig. 3A). These results indicate that cells are arrested in G1 phase by ARD-iPLA₂ in a concentration-dependent manner.

To further confirm that expression of ARD-iPLA₂ prevents cell progression to G2-M transition, we used fluorescence microscopy to analyze transfected cells treated with nocodazole (Fig. 3B). In the ARD-iPLA₂-GFP-transfected plate, the cells expressing ARD-iPLA₂-GFP exhibited interphase characteristics: noncondensed chromosomes and an intact nuclear envelope, visualized by the ring structure of the nuclear pore complex protein mAB414 staining (Fig. 3Bb,c). However, two cells on the same plate that did not express ARD-iPLA₂-GFP had clearly progressed to G2-M transition, exhibiting condensed chromosomes and disrupted nuclear envelopes (Fig. 3Bb and c, respectively).

By contrast, the iPLA₂-GFP transfected cells did not stay in interphase under the same conditions. Several cells progressed to G2-M transition, with obviously condensed chromosomes and broken-down nuclear envelopes. FACS analysis of the cells in the presence of nocodazole showed that 61.5% cells with ARD-iPLA₂-GFP, but only 37% of the cells with iPLA₂-GFP, were in G1 phase (Fig. 3C). These data strongly suggest that G1-phase phospholipid turnover is a programmed cellular event required for cell-cycle progression.

Inhibition of iPLA₂ induces upregulation of p53

The above results prompted us to explore the mechanisms by which cells might modulate progression through the cell cycle when G1-phase phospholipid turnover is disrupted. LPA (lysophosphatidic acid), a mitogen that stimulates cell proliferation (Mills and Moolenaar, 2003), is converted from iPLA₂-generated lysophospholipids by lysophospholipase D. We examined the effect of LPA or Lyso-PC (lysophosphatidylcholine) on cell proliferation when iPLA₂ was inhibited. As shown in Fig. 4, LPA or Lyso-PC did not reverse the inhibitory effect of BEL on cell proliferation under our culture conditions, indicating that iPLA₂ must act directly on phospholipid turnover to control cell proliferation.

Since G1-phase phospholipid turnover is required for cells to enter S phase, we asked whether preventing this turnover would halt cell-cycle progression by activating a protection mechanism similar to the DNA damage checkpoint that induces p53 stabilization and G1-phase arrest. To answer this question, we transfected INS-1 cells with ARD-iPLA₂-GFP to inhibit cell proliferation and then analyzed p53 expression by western blot. Remarkably, we found that increasing expression of ARD-iPLA₂-GFP caused a dramatic accumulation of p53 (Fig. 5A). To verify this finding, we treated INS-1 cells with BEL for 30 hours followed by immunoblotting and fluorescence microscopy analyses. Similar to ARD-overexpression, BEL induced considerable increases in p53 levels (Fig. 5B).

In situ immunofluorescent staining showed that p53 colocalized with DAPI, indicating that the protein was in the nucleus (Fig. 5C). p53 acts primarily as a transcription factor and an essential transcriptional target of p53 in the induction of G1-phase arrest is p21^{cip1}, a cyclin-dependent kinase inhibitor (CKI). Accumulation of p21^{cip1} inhibits cyclin-E/CDK2 activity and the G1-S transition (Kastan and Bartek, 2004; Vogelstein et al., 2000). We found that, although p21^{cip1} was barely detected in control cells, it was dramatically

elevated in ARD-iPLA₂-GFP-transfected (Fig. 5A) and BEL-treated INS-1 cells corresponding to the accumulation of p53 (Fig. 5B). Our finding reveals first time that the inhibition of iPLA₂ leads to G1-phase arrest by activating the p53/p21^{cip1} checkpoint pathway.

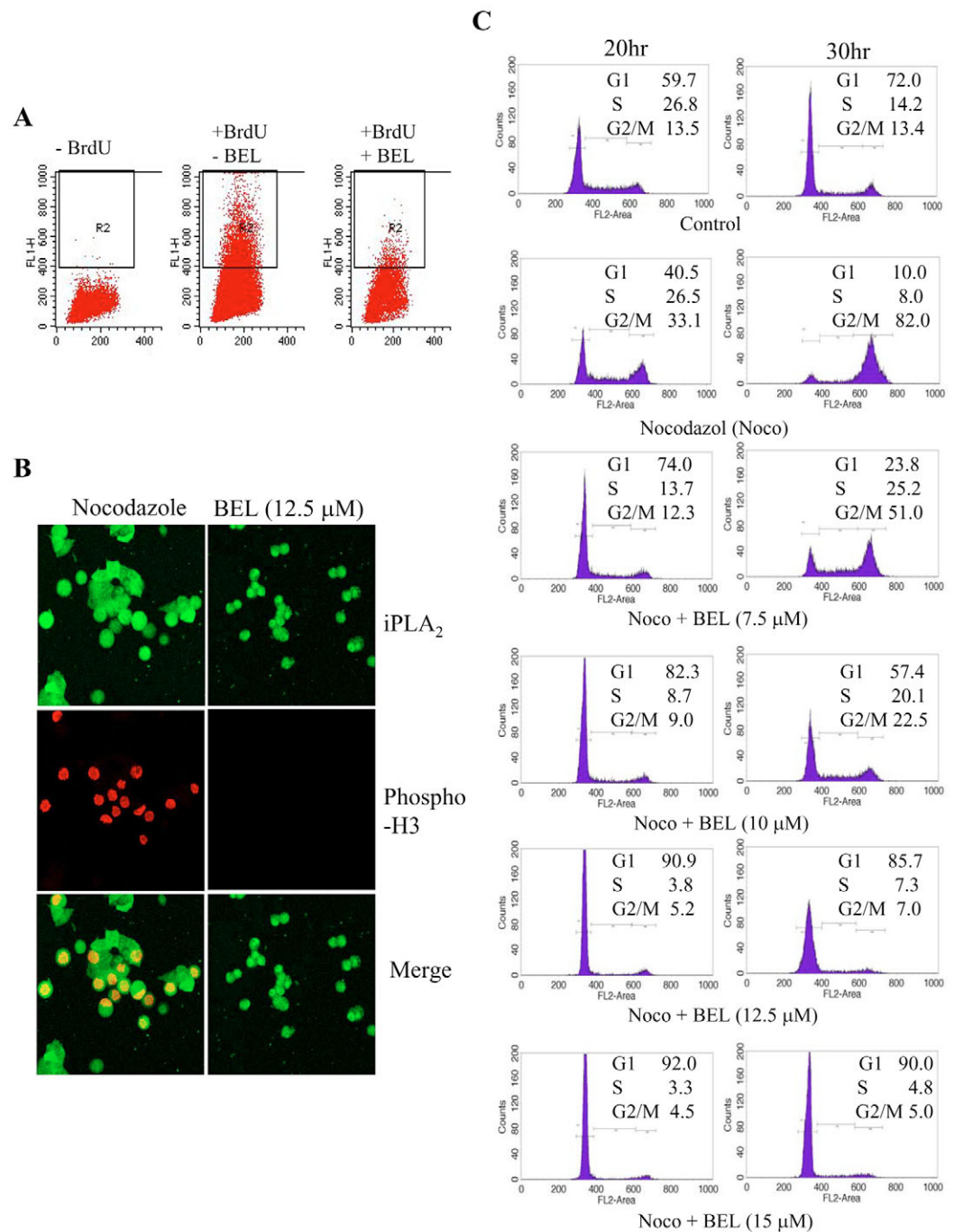
Activated cyclin-E/CDK2 phosphorylates p27^{kip1}, a CKI that inhibits cyclin-E/cdk2 activity, targeting it for polyubiquitination and degradation (Massague, 2004). This suggests that inhibition of cyclin-E/CDK2 activity by p21^{cip1} might in turn lead to stabilization of p27^{kip1}. Indeed, immunoblotting showed that p27^{kip1} levels were increased in BEL-treated INS-1 cells (Fig. 5B). Moreover, inhibition of the cyclin E/cdk2 kinase results in persistence of the RB-E2F

interaction and blocks the transcription of S-phase genes such as cyclin A, which is required for formation of the functional cyclin A/cdk2 complex needed for DNA synthesis (DeGregori et al., 1995). Not surprisingly, levels of cyclin A in BEL-treated INS-1 cells were, therefore, barely detectable (Fig. 5B). Taken together, these results demonstrated that inhibition of iPLA₂ results in a G1-phase arrest through activation of p53, and this, in turn, leads to accumulation of p21^{cip1} and the subsequent inhibition of cyclin-E/CDK2 activity.

G1-phase arrest induced by inactivation of iPLA₂ requires p53

To substantiate the role of p53 in G1-phase arrest induced by

Fig. 2. Inactivation of iPLA₂ by BEL arrests INS-1 cells in G1 phase. (A) Inactivation of iPLA₂ inhibits DNA synthesis. Cells were cultured for 24 hours in with or without BEL (12.5 μM) and labelled with BrdU or not. Cells were stained with fluorescein-conjugated anti-BrdU for BrdU and PI for DNA. R2 indicates cells in S phase. (B) BEL prevents cell progression to G2-M transition. INS-1 cells were treated with BEL (12.5 μM) followed by immunofluorescent staining of the mitosis marker phosphohistone H3. Treatment of cells with nocodazole served as a control for G2-M-transition arrest. Cells were fixed and stained for phosphohistone H3 and for iPLA₂. Magnification, 40×. (C) Inhibition of iPLA₂ in the presence of nocodazole increases the number of cells in G1 phase. INS-1 cells were treated either with nocodazole (0.5 μg/ml) only or with both nocodazole and different concentrations of BEL for 20 hours and 30 hours. Nontreated cells were used as controls. The DNA content of the cells was then analyzed by FACS after PI staining. The percentages of each indicated phase represents the mean of three FACS analyses.



inhibition of iPLA₂, we compared the effect of BEL on the cell-cycle distribution in isogenic human HCT116 colon cancer (HCT) cells that either expressed wild-type p53 (HCTp53^{+/+}) or not (HCTp53^{-/-}). Both HCTp53^{+/+} and HCTp53^{-/-} cells were treated for 10 hours with increasing concentrations of BEL before FACS analyses. The number of HCTp53^{+/+} cells in G1 phase was decreased 50% in the presence of BEL, whereas the number of cells in S phase was decreased threefold compared with cells that had not been treated with BEL (Fig. 6A). Consistent with a role for p53 in this cell-cycle arrest, BEL treatment for 10 hours induced the accumulation of p53 and p21^{cip1} in a concentration-dependent manner (Fig. 6B). Interestingly, p27^{kip1} levels showed little variation after 10 hours of treatment with increasing concentrations of BEL, confirming that p27^{kip1} does not play a major role in this event.

By contrast, HCTp53^{-/-} cells showed no significant change in the number of cells in G1 phase and only a moderate decrease in the number of cells in S phase and G2-M transition (Fig. 6A). This suggests that the effect of BEL on HCTp53^{-/-} cells is not phase-specific and that p53 is required for G1-phase arrest induced by a disruption of the phospholipid turnover.

To further demonstrate the role of p53 in this G1-phase arrest, we analyzed the proliferation of both HCTp53^{+/+} and HCTp53^{-/-} cells after 28 hours of BEL treatment. Similar to INS-1 cells, this extended BEL treatment inhibited the proliferation of HCTp53^{+/+} cells without resulting in cell death (Fig. 6C), and caused the accumulation of p53, p21^{cip1}, and p27^{kip1} in a concentration-dependent manner (Fig. 6D). By contrast, HCTp53^{-/-} proliferation was only partially inhibited by treatment with 15 μM BEL (Fig. 6C).

We observed that p21^{cip1} was slightly upregulated in HCTp53^{-/-} cells (Fig. 6D). Since p21^{cip1} has also been implicated in G2-phase arrest (Bunz et al., 1998), this upregulation might have contributed to the accumulation of G2-M-transition cells (Fig. 6A) and the partial inhibition of cell

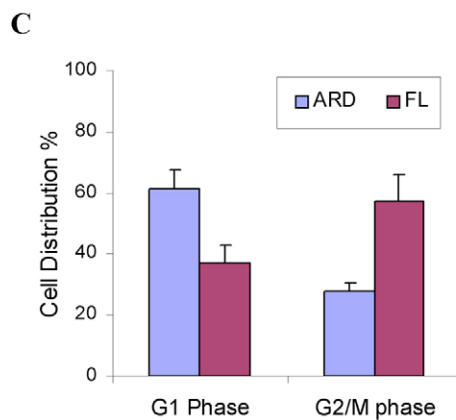
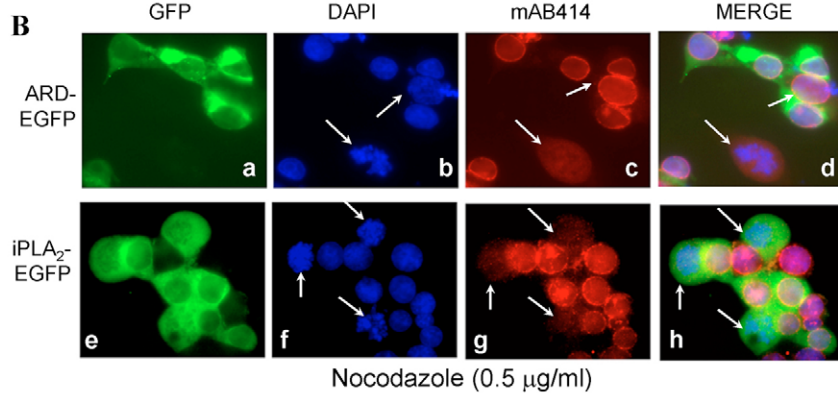
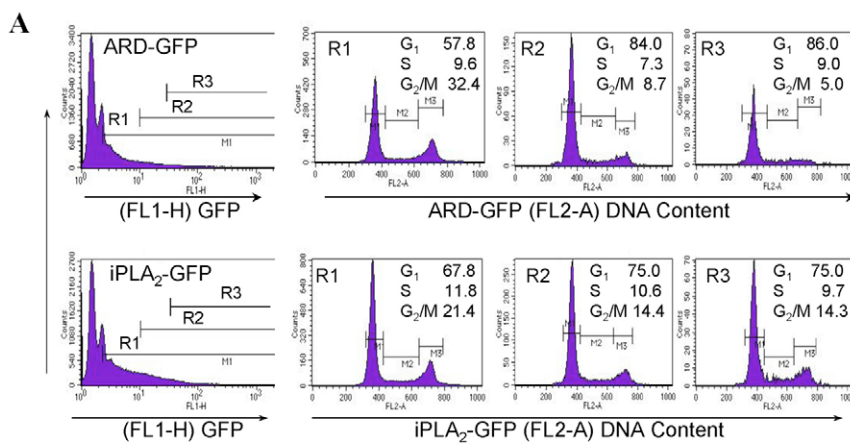


Fig. 3. Enforced expression of the iPLA₂ ankyrin-repeat domain induces G1-phase arrest in INS-1 cells. (A) Cells with higher expression levels of ARD-iPLA₂-GFP have a higher number of cells in G1 phase than cells with lower expression levels. INS-1 cells were transfected with ARD-iPLA₂-GFP or iPLA₂-GFP constructs; 42 hours after transfection, cells were stained with PI and subjected to FACS analysis for GFP-fluorescence (FL1-H) and DNA content (FL2-A). R1, R2 and R3 represent the gated cells with increasing GFP-fluorescence expression (left). Phasic distribution of the gated GFP cells is displayed (right). Figures represent one of four experiments and data represent the mean of four experiments. (B) Cells expressing ARD-iPLA₂-GFP remain in G1 phase even in the presence of nocodazole. INS-1 cells were transfected with ARD-iPLA₂-GFP or iPLA₂-GFP constructs (green), stained for the nuclear pore protein mAB414 (red), counterstained for DNA with DAPI (blue) and analyzed by fluorescence microscopy (Zeiss Axioskop). Arrows in b and f indicate the condensed chromosomes; arrows in c and g indicate the nuclear-envelope-breakdown events; arrows in d and h indicate merged cells of a, b and c, and of e, f and g, respectively. Magnification, 100×. (C) The number of ARD-iPLA₂-GFP-expressing cells in G1 phase far exceeds that of iPLA₂-GFP-expressing cells, even in the presence of nocodazole. Cells of the same experiments as in B were collected and their DNA content was subjected to FACS analysis. When GFP-intensity levels were equalized (FL1-H) in the presence of nocodazole, 61.5% of ARD-iPLA₂-GFP-expressing cells versus 37% of iPLA₂-GFP-expressing cells were in G1 phase. By contrast, 27.7% of ARD-iPLA₂-GFP-expressing cells versus 57.4% of iPLA₂-GFP-expressing cells were in G2-M transition. A total of 200,000 total events were analysed. Error bars represent the mean ± s.d. of three experiments.

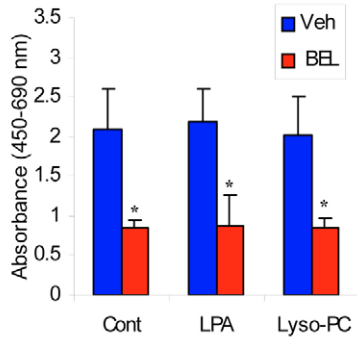


Fig. 4. LPA or Lyso-PC treatment cannot overcome the inhibition of cellular proliferation by BEL. INS-1 cells were treated with or without BEL, and 20 μ M of LPA and lyso-PC were added to the BEL-treated group. BrdU incorporation was then measured. All data represent the mean of duplicates from three individual experiments. Error bars represent the mean \pm s.d. of six experiments; * P <0.05.

proliferation in a p53-independent mechanism. Together, our data indicate that – although we cannot rule out a function in additional pathways – p53 plays a crucial role in the link between the inhibition of iPLA₂ and G1-phase arrest. Disruption of this link through loss of p53 permits unchecked proliferation despite the inhibition of G1-phase phospholipid turnover.

p21^{cip1} is a key effector of the p53-dependent G1-phase arrest induced by iPLA₂ inhibition

To determine the role of p21^{cip1} in the p53-dependent G1-phase arrest induced by iPLA₂ inhibition, we examined the effect of BEL on HCTp21^{-/-} cells, one of several isogenic colon carcinoma cell lines. Surprisingly, we observed increasing rates of cell death when BEL concentrations increased in these p21-deficient cells (Fig. 7A). Cell death was not observed in HCTp53^{-/-} cells after BEL treatment (Fig. 6C). A hallmark of apoptosis is phosphatidylserine (PS) externalization (Fadok and Henson, 1998). Further analysis of PS externalization by staining with Annexin-V-Fluos showed that BEL treatment of HCTp21^{-/-} cells induced early apoptosis and secondary necrosis (Fig. 7B). By contrast, BEL treatment did not induce apoptosis in HCT wild-type cells (Fig. 7B). These results suggest that inhibition of iPLA₂ in the absence of p21^{cip1} activates the p53-dependent apoptotic pathway (Vousden and Lu, 2002). To further determine whether the apoptosis in the p21-deficient cells was p53-dependent, we treated p21-deficient cells with small interference RNA (siRNA) against p53 (Fig. 7C). Indeed, downregulation of p53 in p21-deficient cells overcame the BEL-induced apoptosis (Fig. 7C). Taken together, these results demonstrate that p21^{cip1} is a key effector of the p53-dependent G1-phase arrest in response to inactivation of iPLA₂, and depletion of p21^{cip1} leads to p53-dependent apoptosis.

Discussion

It has been long recognized that membrane phospholipid metabolism is regulated in tune with the cell cycle. However, little is known about the key regulatory enzymes or the biochemical mechanisms that link phospholipid levels to cell-cycle control. PtdCho is the major membrane phospholipid in

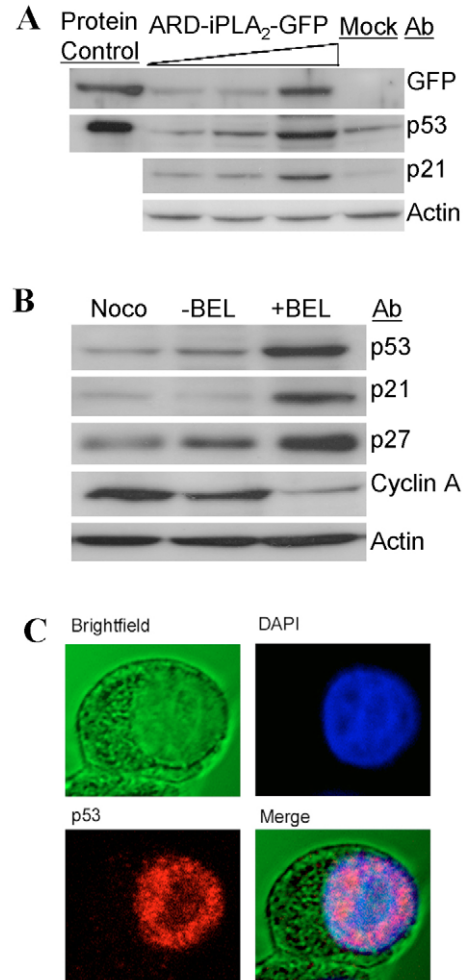


Fig. 5. Inhibition of iPLA₂ induces accumulation of p53 and expression of p21^{cip1}. (A) Expression of ARD-iPLA₂-GFP in INS-1 cells induces the accumulation of p53 and expression of p21. Cells were mock-transfected or transfected with increasing amounts of DNA (2, 5 and 10 μ g/100 mm plate). Cell lysates were prepared and analyzed by western blotting with antibodies against GFP, p53, p21 and actin. Recombinant TNT p53 and ARD-iPLA₂-GFP proteins were loaded as controls. (B) Treatment with BEL for 30 hours increases p53, p21 and p27 levels and decreases cyclin A levels in INS-1 cells. Cells were treated with or without BEL (15 μ M) for 30 hours. Cell lysates were prepared and analyzed by western blotting for p53, p21, p27, cyclin A and actin. (C) p53 accumulates in the nuclei of BEL-treated INS-1 cells. Cells treated with 15 μ M BEL were fixed and stained with anti-p53 (FL-393, Santa Cruz Biotechnology) and counterstained with DAPI followed by analysis with a confocal scanning microscope (Zeiss LSM 510 META). Magnification, 100 \times .

mammalian cells, and regulation of its biosynthesis and turnover is crucial in maintaining membrane structure and function (Kent, 1995).

It has been suggested that mammalian cells have developed a system to maintain a constant membrane phospholipid content during G1 phase by coordinating PtdCho biosynthesis and degradation through the opposing actions of CCT and iPLA₂ (Jackowski, 1996; Baburina and Jackowski, 1999; Barbour et al., 1999). Whereas the bulk of phospholipid production is needed to construct cell membranes for cell

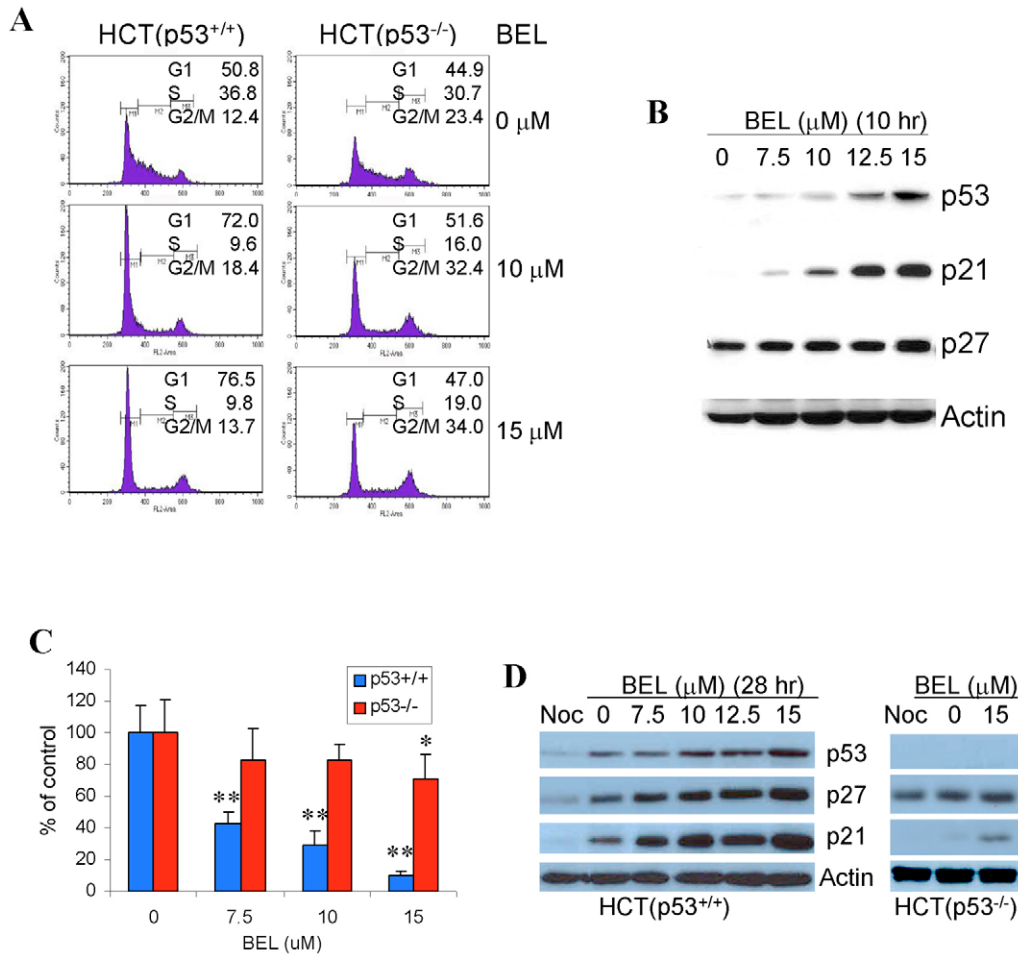


Fig. 6. G1-phase arrest induced by inhibition of iPLA₂ requires p53. (A) BEL treatment for 10 hours increases the number of HCTp53^{+/+} cells but not of HCTp53^{-/-} cells in G1 phase. Both HCTp53^{+/+} and HCTp53^{-/-} cells were treated with increasing concentrations of BEL for 10 hours. The DNA contents of each sample was analyzed by FACS. Data represent the mean of three experiments. (B) Accumulation of p53 and expression of p21 increases with increasing concentrations of BEL. HCTp53^{+/+} and HCTp53^{-/-} cells were treated with increasing concentrations of BEL for 10 hours. Cell lysates were prepared and analyzed by western blot for p53, p21, p27 and actin. (C) Inhibition of iPLA₂ with increasing concentrations of BEL dramatically inhibits the proliferation of HCTp53^{+/+} but only mildly inhibits the proliferation of HCTp53^{-/-} cells. HCTp53^{+/+} cells (blue bars) and HCTp53^{-/-} cells (red bars) were cultured with increasing concentrations of BEL in 24-well microplates for 28 hours, followed by 6 hours of BrdU labelling. Cell proliferation was then determined on a μ Quant microplate reader. Error bars represent the mean \pm s.d. of three experiments; * P <0.05, ** P <0.005. (D) Accumulation of p53 and expression of p21 increases with increasing concentrations of BEL in HCTp53^{+/+} (left). In HCTp53^{-/-} cells, by contrast, p21 expression increased only slightly (right). Both HCTp53^{+/+} and HCTp53^{-/-} cells were treated with increasing concentrations of BEL for 28 hours in 24-well microplates. Cell lysates were prepared and analyzed by western blot for p53, p21, p27 and actin.

division, iPLA₂-mediated deacylation of PdtCho might also act as a feedback control to ensure membrane in optimal condition. Indeed, during mitosis, the cellular membranes undergo dramatic structural and functional changes, such as the complete breakdown and reformation of the nuclear envelope, and the fragmentation and stack reformation of the Golgi membrane (Burke and Ellenberg, 2002; Colanzi et al., 2003). After mitosis, the cell must restructure or remodel its membrane system to ensure proper DNA synthesis and cell-cycle progression. This suggests that the feedback mechanism is capable of restraining cell-cycle progression until particular conditions are satisfied.

Here, we report that inhibition of iPLA₂ arrests cells in the G1 phase of the cell cycle by inducing p53 accumulation and expression of p21^{cip1}. We show that DNA synthesis is blocked

and cells remain in interphase after inhibition of iPLA₂, as evidenced by lack of biomarkers of mitosis, DNA condensation and nuclear envelope breakdown. Moreover, even in the presence of nocodazole, the number of cells in G1 phase increases dramatically with increasing BEL concentrations, suggesting a rapid G1-phase arrest.

Our results demonstrate that, G1-phase phospholipid turnover is essential for cell progression and iPLA₂ is a key component for maintaining G1-phase phospholipid homeostasis by mediating PdtCho degradation. iPLA₂ hydrolyzes the sn-2 fatty acyl bond of phospholipids to liberate free fatty acids and lysophospholipids (Ma and Turk, 2001). It has been proposed to play a role in signal transduction through the actions of the hydrolytic products of iPLA₂, which vary according to the particular cell type and

situation. For example, it plays a role in insulin secretion in pancreatic islet β -cells (Ma and Turk, 2001; Song et al., 2005) and in the generation of lysophospholipids that activate store-operated Ca^{2+} (SOC) channels and capacitative Ca^{2+} influx in smooth-muscle cells (Smani et al., 2004). It also releases LPC to recruit phagocytes to clear apoptotic debris (Lauber et al., 2003).

It has also been proposed that iPLA₂ plays the housekeeping role in phospholipid metabolism, which includes participation in the remodelling of membrane phospholipids (Balsinde et al., 1997; Balsinde et al., 1995) and the regulation of membrane phospholipid turnover (Baburina and Jackowski, 1999; Barbour et al., 1999). This represents a feedback mechanism to fine-tune the phospholipid composition of

membranes and to monitor changes in phospholipid mass during cell-cycle progression. In addition, iPLA₂ – like several proteins involved in cell-cycle control – contains an ankyrin-repeat domain (Ma et al., 1997; Ma and Turk, 2001; Tang et al., 1997). Cell-cycle progression is governed by cyclin-dependent kinases (CDKs) that are activated by cyclin-binding (Morgan, 1995) and inhibited by the CDK inhibitors (Sherr and Roberts, 1999). Inhibitors of the CDK4 (INK4) family include four proteins with four or five tandem ankyrin repeats: p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p16^{INK4d}. These proteins bind to and inhibit CDK4 and CDK6 through their ankyrin repeat domains (Baumgartner et al., 1998; Serrano, 1997). In addition, the p53-binding protein 2 (53BP2) and the oncoprotein gankyrin use ankyrin-repeat domains to interact with their partners (Gorina and Pavletich, 1996; Higashitsuji et al., 2000; Krzywda et al., 2004). Our data suggest that iPLA₂ – with its structural similarities to several cell-cycle control proteins and its housekeeping role in phospholipid metabolism – couples membrane phospholipid turnover to cell-cycle control.

Many previous studies have shown that p53 mediates growth arrest or apoptosis in response to many types of stress: DNA damage, telomere attrition, oncogene activation, hypoxia as well as loss of normal growth and survival signals (Ryan et al., 2001). Recently, it has also been shown that glucose restriction induces activation of a reversible cell-cycle arrest through AMP-activated protein kinase (AMPK)-mediated activation of p53 (Jones et al., 2005). Cell membranes are essential components of all cells; without them the cell can not assert its identity or subdivide its functions into specialized compartments. In addition to this structural role, membrane phospholipids are a reservoir from which cells generate intracellular and intercellular messengers to regulate cell function. The present data indicate that p53, in addition to responding to the types of cellular stress mentioned above, is also essential for cell-cycle arrest induced by phospholipid metabolic stress caused by the inhibition of iPLA₂.

Importantly, inactivation of iPLA₂ fails to arrest p53-deficient cells in G1 phase, indicating that this cell-cycle arrest is p53-dependent. p53 exerts its cell-cycle arrest through its transcriptional target, the cyclin-dependent kinase inhibitor

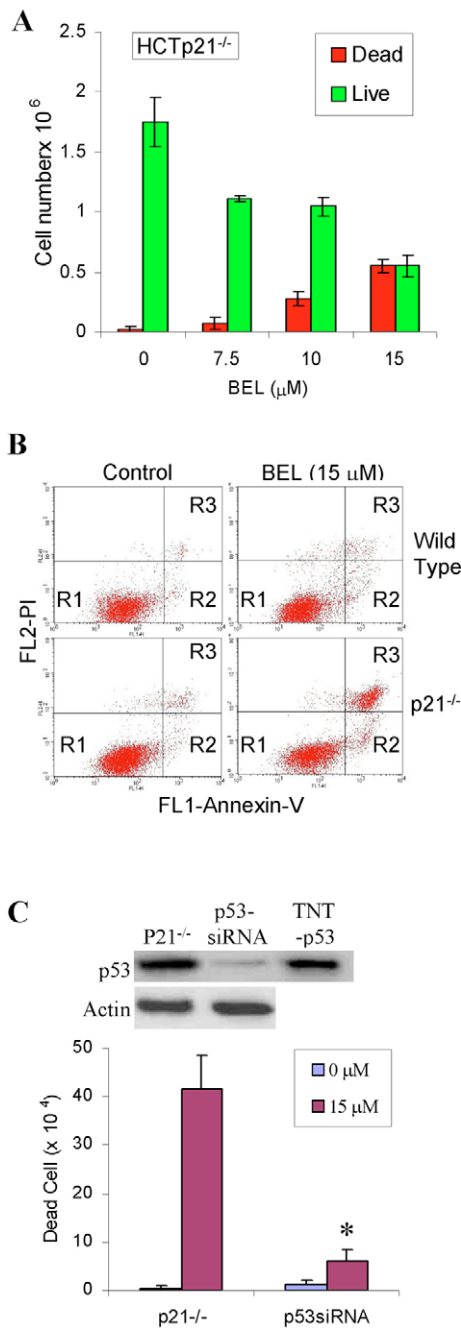


Fig. 7. BEL treatment induces p53-dependent apoptosis in p21-deficient HCT cells. (A) HCTp21^{-/-} cells have a high death rate after inactivation of iPLA₂ by BEL. HCTp21^{-/-} cells (5×10^5) were treated with varying concentrations of BEL for 28 hours. Viable and nonviable cells were determined by Trypan Blue staining and counted. Error bars represent the mean \pm s.d. of six experiments. (B) Inactivation of iPLA₂ induces apoptosis of p21-deficient cells. Both wild-type HCT and HCTp21^{-/-} cells were incubated with or without BEL for 10 hours and then analyzed for apoptosis by Annexin-V-Fluos staining and FACS. R1 represents living cells. R2 represents cells undergoing early apoptosis, and R3 represents secondary necrotic cells. Each panel shows a typical flow-cytometric histogram of 10,000 cells/sample from one of three experiments. (C) BEL-associated death of HCTp21^{-/-} cells can be prevented by p53 depletion. HCTp21^{-/-} cells were cultured overnight in the presence of BEL with 1 nmol of SMARTpool-p53. dsRNA was used as a negative control. The cells were harvested for both western blot analysis for p53 (top) and cell death analysis (bottom). Recombinant TNT p53 was loaded as indicated. * $P < 0.05$.

(CKI) p21^{cip1}. Accumulation of p21^{cip1} inhibits cyclin-E/CDK2 activity and, therefore, the G1-S transition (Kastan and Bartek, 2004; Vogelstein et al., 2000). Interestingly, we also observed that p21^{cip1} is slightly upregulated in HCTp53^{-/-} cells after BEL treatment, which is correlated to an increase in the number of G2-M-transition cells, suggesting the existence of a p53-independent G2-M-transition arrest through p21 expression (Niculescu et al., 1998). This might explain the apparently unchanged phasic distribution in some cell lines after BEL treatment alone (Sanchez and Moreno, 2001).

Furthermore, we showed that HCTp21^{-/-} cells undergo extensive apoptosis when treated with the iPLA₂ inhibitor BEL, indicating that p21^{cip1} is required for p53-dependent G1-phase arrest. The fact that this apoptosis can be overcome by siRNA downregulation of p53 further indicates that p53 is essential for the cell-cycle arrest and might, in some cases, commit cells to apoptosis. Disruption of this checkpoint through loss of p53 permits uncontrolled proliferation in response to the disturbance of phospholipid homeostasis, which may explain the viability of iPLA₂ knockout mice (Bao et al., 2004).

In normal cells, p53 activity is maintained at low levels by the MDM2 protein, which inhibits the transcriptional activity of p53 and targets it for degradation (Haupt et al., 1997; Kubbutat et al., 1997). Cellular insults trigger posttranslational modification of p53, including phosphorylation of Ser15, which stabilizes the protein in part by disrupting its interaction with MDM2 (Shieh et al., 1997). This process is mediated by the ataxia telangiectasia-mutated (ATM) kinase (Banin et al., 1998; Canman et al., 1998) and ataxia telangiectasia and Rad-3-related (ATR) kinase (Tibbetts et al., 1999) or by AMPK (Jones et al., 2005). Whether accumulation of p53 by inhibition of iPLA₂ uses the similar pathways remains to be determined.

iPLA₂ contains an ankyrin-repeat domain (Ma et al., 1997; Ma and Turk, 2001; Tang et al., 1997), and it is interesting that 53BP2 and Ankrd2 proteins bind to p53 through their ankyrin-repeat domains (Gorina and Pavletich, 1996; Kojic et al., 2004). BEL irreversibly inhibits iPLA₂ through a covalent modification (Hazen et al., 1991) that may cause conformational changes, which expose the ankyrin repeat domain of iPLA₂ to p53. Moreover, we showed that forced expression of ARD-iPLA₂ enhances p53 levels and G1-phase arrest in a concentration-dependent manner. Interestingly, the endogenous expression of an ankyrin-repeat domain of iPLA₂ has recently been described by another group of researchers, who postulate that this expression serves as a way to regulate iPLA₂ activity throughout the cell cycle (Manguikian and Barbour, 2004). It remains to be seen whether alternative splicing of iPLA₂ represents a way for the protein to be involved in cell-cycle control by interaction with p53.

In summary, our study reveals hitherto unrecognized cooperation between p53 and iPLA₂ to monitor membrane phospholipid turnover in G1 phase. iPLA₂ has been shown to mediate proliferation of human peripheral blood B and T lymphocytes (Roshak et al., 2000), murine 3T6 fibroblasts (Sanchez and Moreno, 2001) and human colorectal carcinoma (Caco-2) cells (Sanchez and Moreno, 2002), suggesting that this effect is not cell-type specific. Our data suggest that G1-phase phospholipid turnover is systematically monitored in

G1-phase cells and that the cells respond to disruption of this turnover by turning on protective mechanisms.

Materials and Methods

DNA constructs

Wild-type iPLA₂-GFP and ARD-iPLA₂-GFP expression vectors were constructed by ligation of cDNA of either the full-length or the 1375 bp ankyrin-repeat-domain of iPLA₂, in-frame upstream of GFP in the vector pEGFP-N2 (Clontech), respectively. The Mut-iPLA₂-GFP expression vector was constructed by mutating Ser to Ala in the catalytic motif GX SXG in wild-type iPLA₂-GFP using the QuickChange site-direct mutagenesis kit (Stratagene) according to the manufacturer's protocol. The primers used for to generate the mutation by PCR are 5'-GGGTGGCAGGAACCGCCACAGCGGCATCCTGG-3' and 5'-CCAGGATGCCGCTGTGGCGGTTCCCTGCCACCC-3'. The psiRNA-iPLA₂ construct has been described previously (Song et al., 2005).

Cell culture, transfection and treatments

Cells of the insulinoma (INS-1) cell line and human HCT116 colon cancer cells [HCT cells expressing wild-type p53^{+/+} (HCTp53^{+/+}, and its isogenic p53-deficient (HCTp53^{-/-}) and p21^{cip1}-deficient (HCTp21^{-/-}) cells] were cultured as described elsewhere (Bunz et al., 1998; Song et al., 2005). FuGENE 6 Transfection Reagent (Roche) was used for transient transfection of cells according to the manufacturer's instructions. For BEL treatment, the desired numbers of cells were seeded on plates to which variable concentrations of BEL (freshly prepared from stocks kept at -80°C, Cayman) were added simultaneously. Because BEL is stable in aqueous solutions for about 12 hrs, BEL was added every 12 hrs.

Depletion of p53 by siRNA

We used SMARTpool p53 (Upstate/Dharmacon) to deplete p53 in a p21-deficient cell line, HCTp21^{-/-}. HCTp21^{-/-} cells were freshly seeded and cultured overnight. SMARTpool p53 (1 nmol) was transfected into p21-deficient cells (3 × 10⁶) in a 75-cm flask with FuGENE (Roche). Negative control scramble siRNA (Upstate/Dharmacon) was applied at the same time. The transfected cells were split into 12-well plates with fresh BEL on the second day. The rate of cell death was calculated 20 hours after BEL treatment with Trypan Blue staining. Cell lysates were prepared and analyzed for p53 expression by western blot after 10 hours of BEL treatment.

Western blot analyses

Total cell extracts were prepared in RIPA lysis buffer (United States Biological) with a cocktail of protease inhibitors (Roche) as described previously (Song et al., 2005). The protein concentrations were determined using the DC protein assay kit (Bio-RAD) and equal amounts of protein (20-50 μg) per lane were subjected to SDS-PAGE electrophoresis. Western blot analyses were performed as described previously (Song et al., 2005). Antibodies against iPLA₂ (T-14), GFP (FL), p53 (C-19), cyclin A (H-432), p27 (C-19), PCNA (C-20) and actin (C-19) were from Santa Cruz Biotechnology. Anti-p21 antibody (556430) was from BD Biosciences and anti-α-human p53 antibody (DO-1 and pAB1801, ratio 1:1) was a gift from Moshe Oren. TNT p53, a recombinant p53 protein expressed with a TNT T7 Quick Coupled Transcription/Translation System (Sigma), was a gift from Xinjiang Wang.

Measurement of cell proliferation, and apoptosis viability

Cell proliferation was determined by cell counting and BrdU incorporation ELISA (colorimetric) kit (Roche). For cell counting, desired numbers of cells were plated on 6-well plates or 60-mm Petri-dishes and cultured for various periods of time. Then, the cells were harvested, stained by Trypan Blue and counted. The number of dead cells was estimated by counting the Trypan Blue-stained cells. For BrdU incorporation analysis, cells were cultured in 24-well microplates in the presence or absence of BEL for 24 hours and then incubated with BrdU in the presence of BEL for an additional 4-10 hours. BrdU incorporation ELISA was performed according to the manufacturer's protocol and absorbance was determined by a μQuant microplate reader (BIO-TEK Instruments). Cell apoptosis was analyzed with an Annexin V-FLUOS Staining Kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. The labeled cells were immediately analyzed by flow cytometry on a Becton Dickinson FACSCalibur (Heidelberg, Germany). Living cells were negative in both annexin V and PI staining. Early apoptotic cells were annexin V-positive and PI-negative. Secondary necrotic cells were positive in both annexin V and PI staining.

Detection of BrdU incorporation by FACS

Detection of BrdU-labelled DNA in proliferating cells was accomplished with an in-situ cell proliferation kit FLUOS (Roche). The cells were plated in the presence or absence of the desired concentrations of BEL and incubated for 24 hours. They were then labelled with BrdU or not, and subsequently stained according to the manufacturer's flow-cytometry protocol. Cells were counterstained with 1 μg/ml propidium iodide (PI, Sigma) in phosphate-buffered saline (PBS), and analyzed with FACS.

Cell-cycle analysis by FACS

Around 10^5 cells were grown and treated with the respective compounds for various periods of time. After harvesting by centrifugation, the cells were fixed with 70% ethanol and treated with 5 $\mu\text{g/ml}$ RNaseA followed by PI staining (1 $\mu\text{g/ml}$ in PBS). Cells were analyzed using a FACScan (Becton Dickinson). Doublet exclusion was performed by gating for singlet cells on a height vs area plot. The data was processed with the CellQuest™ Pro (BD Biosciences) for determination of cell numbers in G1 and S phases, and G2-M transition. GFP fluorescence was excited at 488 nm and the emission was measured with a 510 nm filter. PI staining was detected with a 580 nm filter.

Microscopy

Control and treated cells were grown on coverslips for desired periods of time. Cells were then washed and fixed with a 2% (v/v) formaldehyde solution in PBS for 10 minutes. After being permeabilized with 0.25% (v/v) Triton X-100 plus 1% NGS for 1 minute, and blocked with 0.5% BSA or 1% NGS (normal goat serum) or 1% normal donkey serum (Chemicon International) for 1 hour, the primary antibodies against iPLA₂, MAB414 (COVANCE, this antibody recognizes Nup358, Nup 214, Nup 153, Nup62), phosphohistone H3 (06-570, Upstate, 1:400) and p53 (FL-393, Santa Cruz Biotechnology, 1:100-400) were added. Secondary antibodies were then applied: Alexa Fluor 488 donkey anti-goat IgG for iPLA₂ (green), Alexa Fluor 647 donkey anti-rabbit IgG (both Molecular Probe, Invitrogen Corporation) for p53 MAB414, and phosphohistone H3 (red) were applied. The stained cells were washed and mounted with the mounting medium, Vectashield (Vector Laboratories). The cells were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (blue) and analyzed with by fluorescence microscopy with Zeiss Axioskop, a Leica TCS-SP (UV) confocal laser scanning microscope (in an inverted configuration) or a confocal scanning microscope (Zeiss LSM 510 META) with a 40 \times objective or 100 \times objective.

Statistical analysis

Data were expressed as mean \pm s.d. The statistical significance of differences was analyzed using Student's *t*-test, where $P < 0.05$ was considered significant.

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