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Research Article

Inhibitors of phosphatidylinositol 3-kinase activity prevent cell cycle progression and induce apoptosis at the M/G1 transition in CHO cells

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Abstract. Phosphatidylinositol 3-kinase (PI3-kinase) activity has been implicated in regulating cell cycle progression at distinct points in the cell cycle by preventing cell cycle arrest or apoptosis. In this study, the role of PI3-kinase activity during the entire G1 phase of the ongoing cell cycle was studied in Chinese hamster ovary (CHO) cells synchronized by mitotic shake-off. We show that inhibition of PI3-kinase activity during and 2 h after mitosis inhibited cell cycle progression into S phase. In the presence of the PI3-kinase inhibitor wortmannin or LY294002, cells were arrested during early G1 phase, leading to the expression of the cleaved caspase-3, a central mediator of apoptosis. These results demonstrate that PI3-kinase activity is required for progression through the M/G1 phase. In the absence of PI3-kinase activity, cells are induced for apoptosis in this particular phase of the cell cycle.

Key words. Phosphatidylinositol 3-kinase; cell cycle progression; apoptosis; G1 phase; actin.

The G1 phase of the cell cycle is the period in which growth and attachment factors determine the ultimate fate of the cell [1]. These external factors activate regulatory mechanisms that control the progression through the different stages of the G1 phase or, alternatively, induce cell cycle arrest, cell differentiation or apoptosis. As long as growth factors are present, continuously cycling cells progress through the G1 phase. When growth factors are removed, cell cycle progression stops and cells exit the cell cycle to enter a state that leads to quiescence or apoptosis [2–4]. Whether growth factor-deprived cells arrest progression through the cell cycle and become apoptotic or start to differentiate depends on the position of the cell in the G1 phase. Cells deprived of growth factors in the early G1 phase become susceptible to apoptosis (designated as $G0^-$ cells), whereas cells that are deprived of growth factors during mid or late G1 phase stop cell cycle progression and may start to differentiate (designated as G_R cells) [5].

A well-known signal transduction cascade, capable of protecting cells from apoptosis, is the phosphatidylinositol 3-kinase (PI3-kinase) pathway. The classical form of the heterodimeric lipid kinase PI3-kinase consists of a p85 regulatory subunit and a p110 catalytic subunit, and can be activated by cytokines, G protein-coupled receptors, growth factor receptors, and several kinds of cellular stress including heat shock and hypoxia [6]. Upon receptor tyrosine kinase (RTK) autophosphorylation, the p85 protein binds to RTK by its two SH2 domains, thereby recruiting the p110 subunit to the plasma membrane [7]. Once activated, PI3-kinase phosphorylates phosphatidylinositol (PI) on the 3′ position of the inositol ring producing phosphatidyl-inositol-3-monophosphate [PI(3)P], phosphatidyl-inositol-3,4,-biphosphate [PI(3,4)P2], and phosphatidyl-inositol-3,4,5-triphosphate [PI(3,4,5)P3]. Proteins containing a conserved protein motif, called a pleckstrin homology (PH) domain, bind to PI(3,4,5)P3, and in turn activate signalling proteins involved in cell proliferation and anti-apoptosis [8–10].

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Evidence is accumulating that the PI3-kinase signalling pathway controls cell proliferation at distinct points in the cell cycle. Chemical inhibition of PI3-kinase arrests cell cycle progression at the G2/M transition by preventing activation of maturation-promoting factor (MPF) and centrosome duplication [11–13]. In MDCK cells, cell cycle arrest at the G2/M transition leads to apoptosis [11]. Moreover, inhibition of the PI3-kinase pathway during S phase increases the kinase activity of Chk1, which plays a central role in the cellular response to several types of DNA damage that may occur in the S and G2 phases of the cell cycle [11]. These findings not only demonstrate the importance of PI3-kinase activity in the G2/M transition of the cell cycle, but they also suggest the existence of cross-talk between the PI3-kinase signal transduction pathway and the key regulator of the DNA damage checkpoint machinery. Phosphorylation and activation of the PI3-kinase pathway occur in late S phase of the cell cycle and persist until the end of mitosis. However, it has been suggested that PI3-kinase activity must be inhibited for proper progression through mitosis [12]. Studies using overexpression show that transient activation of PI3-kinase accelerates progression through the different phases of the cell cycle, whereas cells expressing a constitutively active mutant failed to exit mitosis and arrested in telophase [14]. The latter supports the notion that down-regulation of PI3-kinase is required for proper mitotic exit. Other data have been presented showing that upon growth factor stimulation, PI3-kinase activity promotes entry into S phase [15]. Inhibition of PI3-kinase has been suggested to induce cell cycle arrest at mid to late G1 phase by decreasing the level and nuclear localization of the G1-associated cyclin D [16]. Moreover, inhibition of PI3-kinase activity has been shown to decrease both the level of cyclin-dependent kinase (Cdk) 4, the activating partner of cyclin D, as well as the phosphorylation state of the retinoblastoma (pRB) protein, whose phosphorylation is regulated by the activity of G1 Cdks [16, 17]. Another target of PI3-kinase is the Cdk inhibitor $p27^{KIP1}$ [17–19]. Elimination of Cdk inhibitors proceeds through a PI3-kinase-dependent pathway by controlling the expression and subcellular localization of $p27^{KIP1}$ prior to transition through G1 and into S phase.

In this study, the requirement for PI3-kinase activity was studied during the G1 phase of the ongoing cell cycle of Chinese hamster ovary (CHO) cells. The mitotic shake-off method, a selective method for producing undisturbed synchronized cultures [5, 20, 21], was used for cell synchronization. Here, we show that PI3-kinase activity prevents the induction of apoptosis during early G1 phase in continuous cycling CHO cells. Inhibition of PI3-kinase activity by the PI3-kinase inhibitors LY294002 or wortmannin during and 2 h after mitosis inhibited cell cycle progression into S phase as measured by thymidine incorporation and expression of cyclin A. Both inhibitors also caused an inhibition of cyclin D expression and a decrease in MAP kinase phosphorylation, but had no effect on the phosphorylation of FAK, suggesting that the cells were arrested in the early G1 phase. Furthermore, the inhibition of PI3-kinase during mitosis leads to the expression of the cleaved caspase-3, a central mediator of apoptosis. These results demonstrate that PI3-kinase activity is required for progression through the M/G1 phase. In the absence of PI3-kinase activity, cells are induced for apoptosis in this particular phase of the cell cycle.

Materials and methods

Cell culture, synchronization and treatment. CHO cells were grown on Dulbecco's modified Eagle's Medium (DMEM; Gibco) containing 7.5% fetal calf serum (FCS; Gibco) and 5 mM L-glutamine at 37 °C in a humidified 5% CO_2 atmosphere. Three days prior to synchronization, cells were transferred to HEPES (25 mM) buffered DMEM containing 7.5% FCS. One day before mitotic shake-off, cells were plated at a density of 3×10^4 cells/cm^2. Mitotic cells were obtained by shaking an asynchronously growing cell population firmly for 1 min and pipetting off the medium as described previously [20]. The mitotic cells obtained after mitotic shake-off were plated at a density of 15,000 cells/cm^2 on cell culture dishes, either in the absence or presence of the PI3-kinase inhibitors LY294002 (20 μM) or wortmannin (100 nM) (Kordia).

[3H]-thymidine incorporation assay. To measure DNA synthesis by thymidine incorporation, cells obtained after mitotic shake-off were cultured in the presence of [^{3}H]-thymidine (^{3}H-TdR) (specific activity 2 Ci/mmol, Amersham) at a concentration of 1.0 μCi/ml. Cells were grown in 24-well plates either in the presence or absence of the PI3K inhibitors LY294002 or wortmannin. After the completion of one cell cycle, cells were washed twice with PBS and disrupted in 0.1 M NaOH. Incorporated radioactivity of the samples was measured using a liquid scintillation counter (Beckman LS 6000SE).

Statistical analysis. Unpaired Student *t*-tests were performed using GraphPad Prism version 3.00 for Windows and GraphPad Software. Results are expressed as the mean ± SD (n = 6 for each determination). Asterisks indicate values significantly different from the control cells (**$p < 0.05$, ***$p < 0.001$).

Cell extraction and Western blotting. At the indicated times after replating, cells were washed in PBS and lysed in RIPA buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium desoxycholate, 0.1% SDS,

1 mM EDTA, 100 mM NaF, 1 mM benzamidine, 1 mM PMSF and 1 mM ortho-sodiumvanadate (Na_3VO_4)]. Cells that were not allowed to attach to the substratum (mitotic cell samples) were spun down by centrifugation at 1400 rpm for 7 min prior to lysis. For Western blotting, cell lysates of about 1.38×10^5 cells per slot were loaded on 10% polyacrylamide gels. Proteins were electrophoretically transferred to PVDF membrane (Roche Diagnostics). Membranes were blocked with PBS containing 4% dried milk powder and 0.1% Tween 20 and then incubated with one of the following antibodies: mouse anti-cyclin A (2.5 µg/ml; Oncogen Research products), rabbit anti-cyclin D1/D2 (1 µg/ml), mouse anti-MAP kinase R2 (ERK2) (0.5 µg/ml), and rabbit anti-PI3-kinase p85 (1:10,000; Upstate Biotechnology), mouse anti-$p125^{FAK}$ (0.25 µg/ml), and mouse anti-PI3-kinase p110α (0.025 µg/ml; Transduction Laboratories), rabbit anti-phosphorylated $p125^{FAK}$ (pY397) (0.375 µg/ml; Bioscource International), rabbit anti-phosphorylated-MAP-kinase, and cleaved caspase-3 (1:1000; New England Biolabs). Incubations were followed by three washes with blocking buffer and incubated either with 0.8 µg/ml diluted horseradish peroxidase-conjugated donkey anti-mouse IgG (DAM-PO) or donkey anti-rabbit IgG (DAR-PO) (Jackson ImmunoResearch Laboratories) diluted in blocking buffer. Membranes were then washed three times in blocking buffer and twice in PBS, followed by detection of bound antibody using enhanced chemiluminescence (Dupont).

Fluorescence microscopy. Cells were washed twice with PBS (37 °C) and then fixed and permeabilized simultaneously in 4% formaldehyde in PBS containing 0.1% Triton X-100 for 15 min, followed by two washes in PBS, and incubated for 10 min with 50 mM glycine in PBS to block non-specific binding. After washing twice with PBS containing 0.2% gelatin, cells were incubated for 60 min at room temperature with tetramethylrhodamine-5-(and-6) isothiocyanate (TRITC)-conjugated phalloidin and washed again as described above. Finally, cells were mounted in moviol under coverslips (diameter 18 mm; Menzel) [22, 23], and fluorescence was visualized with a Leitz immune fluorescence microscope (Orthoplan Flu 043944) equipped with Leitz objective lenses (×40 1.3 NA and ×63 1.4 NA). Images were acquired, and pictures were processed with Adobe Photoshop 7.0.

DNA staining. Cells were fixed in 4% formaldehyde in phosphate-buffered saline (PBS). Subsequently, cells were incubated with 2 µg/ml DAPI in PBS for 5 min at 37 °C. Cells were washed twice with PBS and fluorescent DNA-DAPI complexes were visualized with a Leica CCD camera (model DC350F) using Leica Image Manager 50 software. Pictures were processed with Adobe Photoshop 7.0.

Results

PI3-kinase activity in early G1 phase is required for cell cycle progression. During the G1 phase, cells decide whether they will continue to progress through the cell cycle, or will undergo apoptosis [4, 24]. The PI3-kinase signal transduction pathway plays an important role in regulating the proliferative response of cells during the different stages of the cell cycle [12, 14, 15, 18, 25]. To examine a possible relationship between the PI3-kinase signal transduction pathway and the fate of the cell during the ongoing cell cycle, CHO cells synchronized by mitotic shake-off were incubated with a PI3-kinase inhibitor at different time points after mitosis, and assayed for ^{3}H-TdR-incorporation, as a measure of DNA synthesis (fig. 1A). Addition of the irreversible wortmannin or reversible LY294002, structurally unrelated inhibitors of PI3-kinase activity [26–28] at 4 and 6 h after mitosis did not cause a significant effect on ^{3}H-TdR incorporation at

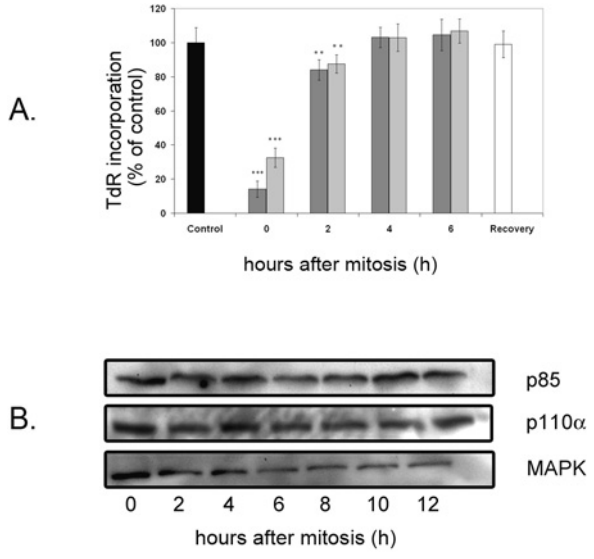

Figure 1. Effect of the PI3-kinase inhibitors wortmannin and LY294002 on cell cycle progression in continuously cycling CHO cells. Mitotic CHO cells were collected by the shake-off procedure described in Materials and methods. The effect of PI3-kinase inhibition at different points during the G1 phase on the proliferative capacity of the cells was examined by determination of thymidine incorporation in the absence or presence of LY294002 [30]. (*A*) ^{3}H-TdR incorporation was measured after 14 h of cell growth in the absence (Control) or presence (0, 2, 4 and 6 h) of the PI3-kinase inhibitors wortmannin (100 nM, left bar) and LY294002 (20 µM, right bar). The PI3-kinase inhibitors were added at different time points after mitosis (0, 2, 4 and 6 h). Reversibility of drug effects was examined by releasing LY294002-treated cells in fresh medium (white bar). Data are the mean ± SD (n = 6). Unpaired t tests were performed using GraphPad Prism version 3.00 for Windows and GraphPad Software. Asterisks indicate values significantly different from the control cells (**$p < 0.05$, ***$p < 0.001$). (*B*) Western blot showing the expression of the class Ia PI3-kinase proteins p85, and p110α. $p42^{MAPK}$ was used as loading control for all blots. Experiments were repeated at least three times and similar results were obtained.

14 h after mitosis, when compared to control cells. However, addition of the PI3-kinase inhibitors during and 2 h after mitosis caused a significant decline in ^{3}H-TdR incorporation at 14 h after mitosis (fig. 1A). When, after 14 h of exposure to LY294002, these cells were incubated in fresh medium without inhibitor, ^{3}H-TdR incorporation returned to the control level. These results demonstrate that cells recover from treatment with LY294002. Altogether, these data indicate that continuously cycling cells require PI3-kinase activity in early G1 phase for transition into S phase.

The differences in effects of the PI3-kinase inhibitors on cell cycle progression of mitotic versus G1 phase cells (fig. 1) may be caused by either a change in protein expression of the p110α and p85 proteins or a change in PI3-kinase activity. Therefore, cells were harvested at different time points after mitosis and expression of the p110α and p85 proteins of an identical number of cells was determined by means of Western blotting. As shown in figure 1B, the expression level of the class Ia PI3-kinase proteins did not change in the time period from mitosis to 12 h after mitosis, like p42^{MAPK}, as described previously [21]. Since no change in p110α and p85 protein expression was observed, the drop in PI3-kinase activity caused by the treatment of cells with LY294002 most probably causes the difference in effect on cell cycle progression of mitotic versus G1 phase cells.

PI3-kinase activity is required for progression through the G1 phase. The progression from one phase of the cell cycle to the next is controlled by the cyclin/Cdk complexes. To establish the phase of G1 in which PI3-kinase activity is required for cell cycle progression, we determined the protein expression level of the G1 phase-specific cyclins in the absence or presence of the inhibitors of PI3-kinase. Synchronized CHO cells were harvested at different time points after mitosis and analyzed for cyclin A expression by Western blot analysis, since cyclin A has been shown to be expressed in late G1/early S phase [29]. In figure 2, the expression pattern of cyclin A is shown 0, 2, 4, 6, 8, 10, and 12 h after mitosis, in the absence and presence of the PI3-kinase inhibitor LY294002, added to the cells during mitosis. At 8 h after mitosis, corresponding to late G1/early S phase, a significant increase in cyclin A expression was observed in control cells, as has been described earlier [30]. The increase in cyclin A expression was not caused by changes in protein as deduced from p42^{MAPK} expression used as a loading control (fig. 2). In contrast to what was observed in control cells, cyclin A expression was severely decreased in cells incubated in the presence of the PI3-kinase inhibitor LY294002 (fig. 2). Similar results were obtained with wortmannin (data not shown). These results suggest that inhibition of PI3-kinase activity arrests cell cycle progression before or at mid G1 phase.

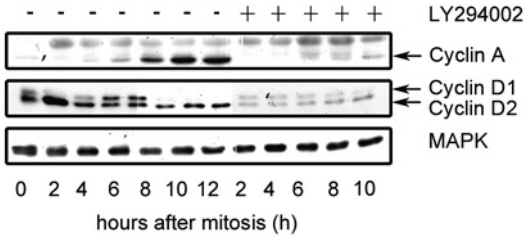

Figure 2. Effect of PI3-kinase inhibition on the expression of cell cycle-related proteins. Western blot showing the expression of cyclin A, cyclin D1/D2 (p34^{cyclinD1} and p36^{cyclinD2}), and p42^{MAPK} in synchronized CHO cells in the absence (–) or presence (+) of PI3-kinase inhibitor LY294002. LY294002 was added to the cells during mitosis. Cells were synchronized by mitotic shake-off, incubated in the absence (–) or presence (+) of the PI3-kinase inhibitor LY294002 (20 µM) and lysed in RIPA buffer. P42^{MAPK} was used as loading control for all blots. Experiments were repeated at least three times and similar results were obtained.

In contrast to cyclin A, cyclin D2 has been shown to be expressed throughout the M and G1 phases of the ongoing cell cycle [31]. During the G1 phase, the level of cyclin D1 fluctuated in control cells. Addition of LY294002 or wortmannin during mitosis resulted in a rapid decrease of both cyclin D1 and D2 expression within 2 h after addition of the inhibitor. These observations indicate that inhibition of PI3-kinase activity inhibits either M phase or early G1 phase.

PI3-kinase activity is required for progression through the early G1 phase. One of the earliest events during the G1 phase of the ongoing cell cycle concerns the phosphorylation of MAP kinase. This phosphorylation has been demonstrated to occur as early as 10 min after mitosis and is required during G1 phase for progression to S phase [21]. To further determine the moment in which cells arrest progression through the G1 phase caused by inhibition of PI3-kinase activity, we determined the phosphorylation status of MAP kinase in the presence and absence of the PI3-kinase inhibitor LY294002 or wortmannin (fig. 3A). In control cells, phosphorylated MAP kinase was not detected during mitosis, whereas 30 min after mitosis, an increase in MAP kinase phosphorylation was observed as has been described before [21]. In contrast, cells treated with the PI3-kinase inhibitor did not exhibit phosphorylated MAP kinase during G1 phase. These observations indicate that cells arrest either at the exit of the M or entry into the G1 phase.

During the entry into the G1 phase, cells attach to the substratum and assemble specialized sites of cell matrix adhesion, so-called focal adhesions. One of the earliest events in focal adhesion assembly concerns the phosphorylation of focal adhesion kinase (p125^{FAK}). To investigate whether PI3-kinase activity is essential either at the exit of M or entry into the early G1 phase, we used p125^{FAK} tyrosine phosphorylation as a parameter for

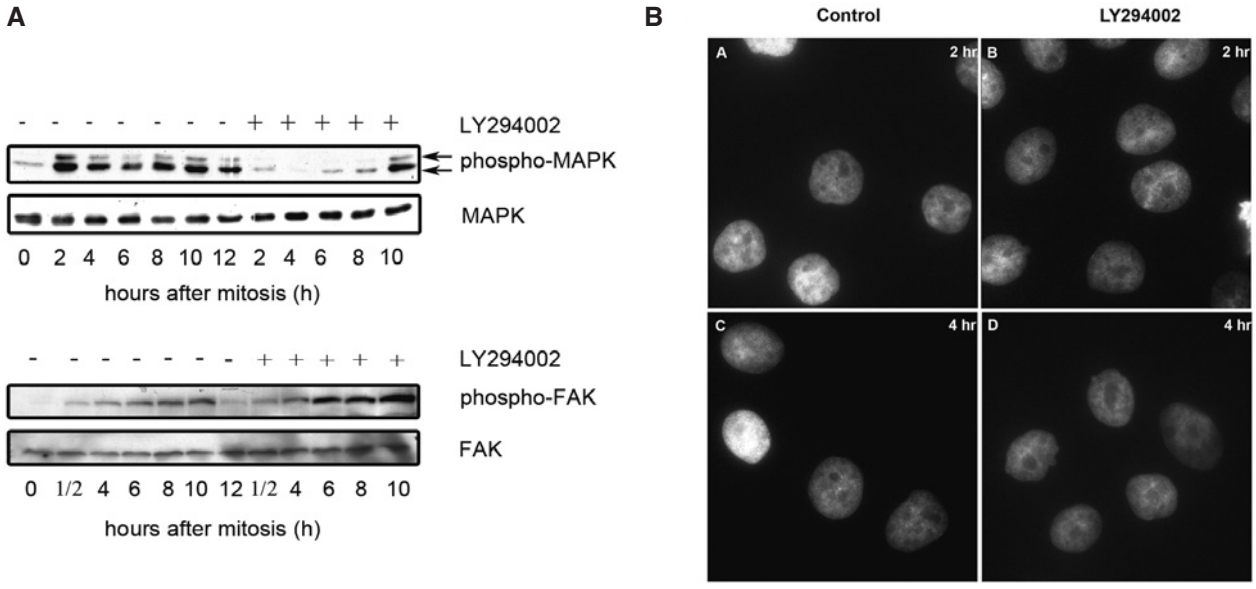

Figure 3. Effect of PI3-kinase inhibition on the phosphorylation status of p42^{MAPK} and p125^{FAK}. (*A*) Western blot showing the phosphorylation of p44/p42^{MAPK}, pY397^{FAK}, and the expression of p125^{FAK} in synchronized CHO cells in the absence (–) or presence (+) of the PI3-kinase inhibitor LY294002. Cells were synchronized by mitotic shake-off, incubated in the absence (–) or presence (+) of the PI3-kinase inhibitor LY294002 (20 µM) and lysed in RIPA buffer. LY294002 was added to the cells during mitosis. p42^{MAPK} was used as loading control for phosphorylated p44/42^{MAPK}, whereas p125^{FAK} was used as loading control for pY397^{FAK}. Experiments were repeated at least three times and similar results were obtained. (*B*) Synchronized CHO cells were incubated in the absence (panel A, C) or presence (panel B, D) of LY294002. Cells were fixed 2 h (panel A, B), and 4 h (panel C, D) after mitosis, labelled with the nuclear stain DAPI, and analysed by fluorescence microscopy.

early G1 phase entry. Mitotic CHO cells incubated with or without the PI3-kinase inhibitor were analysed for the phosphorylation of p125^{FAK} at Tyr397 by Western blotting (fig. 3A). Phosphorylation of p125^{FAK} at Tyr397 was detected 1 h after mitosis, followed by a gradual increase in phosphorylation during the remainder of the G1 phase, compared to p125^{FAK} (fig. 3A). A similar expression pattern of pY397^{FAK} was obtained for the cells treated with the PI3-kinase inhibitor. Similar results were obtained using wortmannin (data not shown).

Furthermore, to investigate whether cells treated with the PI3-kinase inhibitors display mitotic or interphase nuclei, synchronized CHO cells were stained with the nuclear counterstain DAPI at different time points after mitosis in the absence or presence of the PI3-kinase inhibitors, and analysed using immunofluorescence microscopy. Figure 3B shows the DAPI staining of cells 2 h (panel A, B) and 4 h (panel C, D) after mitosis, in the absence (panel A, C) and presence (B, D) of the PI3-kinase inhibitor LY294002. The inhibitor was added to the cells during mitosis. DAPI staining revealed that LY294002-treated cells at 2 and 4 h after mitosis exhibited nuclear staining similar to control cells, displaying interphase nuclei. This indicates that mitotic cells enter G1 phase despite inhibition of PI3-kinase activity, suggesting that PI3-kinase activity is required for progression through the early G1 phase.

PI3-kinase induces actin filament remodelling in the early G1 phase. PI3-kinase induces actin filament remodelling to form lamellipodia and filopodia, resulting in cell spreading [32]. In a variety of cell types, the organization of the actin cytoskeleton is implicated in G1 phase progression since disruption of actin filaments with pharmacological agents leads to G1 arrest [33–36]. Therefore, we investigated whether inhibition of PI3-kinase blocks any actin remodelling event during early stages of the G1 phase. CHO cells incubated in the absence or presence of PI3-kinase inhibitor were fixed at different time points after mitosis and F-actin organization was visualized by fluorescence microscopy (fig. 4). Phalloidin staining of F-actin in mitotic cells showed a distribution of actin filaments in a contractile ring and additional arrangements of short cortical and cytoplasmic filaments (fig. 4A). Post-mitotic substratum adherence was accompanied by the formation of actin-mediated surface protrusions such as lamellipodia and ruffles (fig. 4B). Reorganization of F-actin into stress fibres was visible within 1 h after mitosis and corresponded with cell spreading over the substratum (fig. 4B). Figure 4C shows that at 3 h after mitosis, cells continue with the formation of surface protrusions and the reorganization of F-actin into stress fibres to maximize cell surface attachment, as deduced from cells stained with phalloidin 3 h after mitosis. Phalloidin staining of F-actin in cells

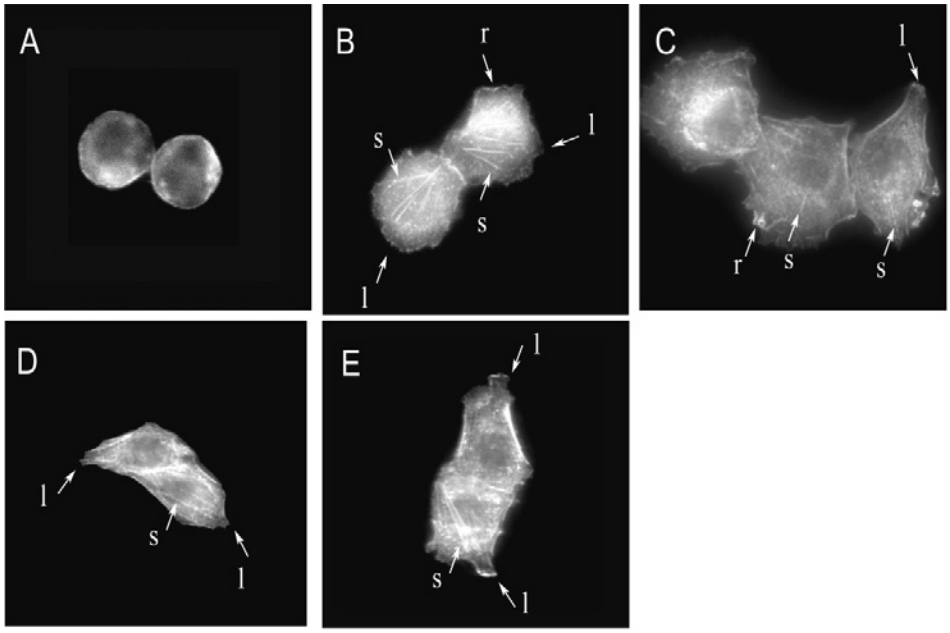

Figure 4. Effect of inhibition of PI3-kinase activity on actin filament remodelling. CHO cells were synchronized by mitotic shake-off and plated in six-well plates in the absence or presence of the PI3-kinase inhibitor LY294002. LY294002 was added to the cells during mitosis. At different times after synchronization, cells were fixed and F-actin was labelled with TRITC-conjugated phalloidin and visualized by fluorescence microscopy. Cells were fixed in mitosis (*A*), 1 h (*B*), and 3 h (*C*) thereafter. Cells in the presence of the PI3-kinase inhibitor were only fixed at 1 h (*D*) and 3 h (*E*) after mitosis. Stress fibers (s), lamellipodia (l), and ruffles (r) are indicated. Experiments were repeated at least three times and similar results were obtained.

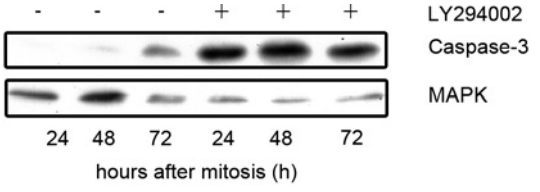

Figure 5. Effect of inhibition of PI3-kinase activity on the cleavage of caspase-3. Western blotting showing the appearance of cleaved caspase-3 and $p42^{MAPK}$ in synchronized CHO cells in the absence (–) or presence (+) of the PI3-kinase inhibitor LY294002. The inhibitor was added to the cells during mitosis. Cells were synchronized by mitotic shake-off and incubated for 24, 48, and 72 h in the absence (–) or presence (+) of LY294002. Thereafter, cells were lysed in RIPA buffer and an equal number of cells was loaded on the gel as described in Materials and methods. $p42^{MAPK}$ was used as loading control. Experiments were repeated at least three times and similar results were obtained. Representative blots are shown.

treated with PI3-kinase inhibitor exhibited distributed actin filament bundles at the leading edge of the cells within 1 (fig. 4D) and 3 h after mitosis (fig. 4E). At 1 and 3 h after mitosis, however, the formation of lamellipodia was decreased in the presence of the PI3-kinase inhibitor (fig. 4D, E). These data indicate that PI3-kinase activity induces the formation of actin structures at the cell periphery, but not the distribution of actin filaments during the early stages of the G1 phase. This suggests that PI3-kinase does not exert its effect on cell cycle progression through the actin cytoskeleton.

PI3-kinase activity prevents induction of apoptosis in early G1 phase. Growth factors have recently been shown to prevent apoptosis by activating PI3-kinase in several cell types, including PC12 pheochromocytoma cells, Rat-1 fibroblasts, T lymphocytes, and haemopoietic progenitors [37–40]. We have previously demonstrated that cells deprived of growth factors in the early G1 phase become susceptible to apoptosis and this decision point in the early G1 phase was designated as $G0^{-}$ [5]. This, with the fact that cells treated with PI3-kinase inhibitor enter cell cycle arrest in early G1, suggests that the $G0^{-}$ decision point is regulated by the PI3-kinase signal. PI3-kinase has been shown to protect cells from apoptosis by suppressing the activation of caspase-3 [41]. Therefore, we investigated the role of PI3-kinase in induction of caspase-3 activity during the early G1 phase of the continuous cell cycle in CHO cells. After mitotic shake-off, CHO cells were incubated with or without the PI3-kinase inhibitor for 24, 48 and 72 , and analysed for cleaved caspase-3 by Western blotting using an antibody recognizing the 17/19-kDa fragment of activated caspase-3. As shown in figure 5, cells not treated with the PI3-kinase inhibitor LY294002 or wortmannin did not exhibit cleaved caspase-3, whereas cells treated with the PI3-kinase inhibitor exhibited cleaved caspase-3 as early as 24 h after the start of mitosis. These observations suggest that PI3-kinase protects the cells from apoptosis by suppressing the activation of caspase-3, and that it consequently plays

an important role in the decision of the cells to progress through the cell cycle or to undergo apoptosis.

Discussion

In this study we investigated the role of the PI3-kinase signal transduction pathway during the G1 phase of the cell cycle in CHO cells. Inhibition of the PI3-kinase activity by the PI3-kinase inhibitors LY294002 and wortmannin resulted in a reduced cell cycle progression into S phase, as measured by thymidine incorporation and expression of cyclin A. This reduction in DNA synthesis and cyclin A was not found when PI3-kinase activity was inhibited in mid or late G1 phase, but only when PI3-kinase was inhibited during mitosis or in the early G1 phase. PI3-kinase exerts its effects on cell cycle progression by inhibition of cyclin D expression and phosphorylation of MAP kinase. Inhibition of PI3-kinase activity did not have an effect on the phosphorylation of FAK or on actin filament formation, suggesting that the cells were arrested in the early G1 phase. The point in the G1 phase in which cells arrest in the presence of the PI3-kinase inhibitor seems to coincide with the $G0^-$ decision point, as identified recently in the continuous cell cycle of CHO cells by serum withdrawal [5]. In that particular study, the $G0^-$ decision point was correlated with apoptosis. As induction of apoptosis is associated with reduced PI3-kinase activity, it was tempting to suggest that PI3-kinase activity in the early G1 phase regulates the anti-apoptotic signal [40–46]. Indeed, cells exposed to LY294002 during mitosis exhibit cleaved caspase-3, a central mediator of apoptosis, suggesting that the cell cycle arrest induced by LY294002 leads to the induction of the apoptotic pathway in continuously cycling CHO cells. The findings support the view that the decision point in which the cells decide to progress through the cell cycle or to undergo apoptosis at the $G0^-$ point occurs in early G1 phase [5]. Moreover, these data are consistent with studies suggesting that PI3-kinase is required for G1 cell cycle progression [15]. However, the observation that inhibition of PI3-kinase causes cell cycle arrest at early G1, but not in the mid and late G1 phase, seems to contradict studies suggesting a role for PI3-kinase in this particular phase [47–49]. However, most of these studies have focused on cells entering G1 phase from the quiescent state [18, 47]. In many G0 cells, high levels of the p21 and p27 Cdk inhibitors ensure that the cyclinD/Cdk4,6 complexes that may be present in the cell remain inactive [50]. Essential for progression through G1 and entry into S phase is the elimination of increased levels of Cdk inhibitors and the up-regulation of cyclin D expression in growth factor-arrested G0 cells [51]. In G0 cells, this proceeds through a PI3-kinase-dependent pathway [18, 19, 52]. In continuously cycling cells, such regulation is not required, since no change in the expression of the p27 Cdk inhibitor in the absence of growth factor is observed [1]. Cell cycle progression through G1 is initially regulated by the activation of the cyclinD/Cdk4 complex, which induces the phosphorylation of pRB protein and the release of E2F [16, 52]. An additional function of cyclin D in the regulation of G1 cell cycle progression is thought to be the formation of cyclin D-Cdk complexes. These may titrate and sequester Cdk inhibitors away from Cdk2 complexes, thereby promoting cell cycle progression [51]. Our study indicates that inhibition of the PI3-kinase by LY294002 decreases the expression of the G1-associated protein cyclin D1/D2 when continuously cycling cells progress from M to G1. These data indicate a role for the PI3-kinase pathway in regulating cyclin D1/D2 expression. Our observation is in line with studies [16, 17] suggesting that PI3-kinase activity is required for cyclin D1 expression either through regulating the stabilization or the transcriptional activation of cyclin D1. We have previously reported a positive correlation between $p42^{MAPK}$ phosphorylation and expression of cyclin D1/D2 at the M/G1 transition. In continuously cycling CHO cells, $p42^{MAPK}$ is phosphorylated early in the G1 phase and translocates to the nucleus at the mid or end of the G1 phase [21]. Once $p42^{MAPK}$ phosphorylates, cyclin D1/D2 expression remains and cells appears to continue early G1 progression [21]. There is evidence that PI3-kinase acts as an earlier intermediate of the $p42^{MAPK}$ signal transduction pathway in response to either tyrosine kinase receptors or G-coupled receptor activation [53, 54]. In this study, we have shown that treatment with LY294002 markedly inhibits $p42^{MAPK}$ phosphorylation in CHO cells. This finding, along with the above results, may suggest a role for PI3-kinase in phosphorylating $p42^{MAPK}$ to stabilize cyclin D1/D2 expression and progression through early G1 phase. However, the possibility that LY294002 exerts the effect on cyclin D1/D2 expression and the phosphorylation of $p42^{MAPK}$ by arresting cells just before $p42^{MAPK}$ phosphorylation occurs cannot be excluded.

Overall, we conclude that PI3-kinase is required for progression through the M/G1 transition. In the absence of PI3-kinase activity, the cells are induced for apoptosis in this cell cycle phase. Molecular targets of PI3-kinase involved in protecting cells from apoptosis during the early G1 phase remains to be determined. Evidence is accumulating that the downstream target of PI3-kinase, AKT, is involved in G1 cell cycle arrest as well as in delivering anti-apoptotic signalling. Many studies support a model whereby PI3-kinase activates intracellular serine/threonine AKT/PKB, which probably acts through inhibition of the activation of the caspases [43].

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