A Role for PI 3-Kinase and PKB Activity in the G2/M Phase of the Cell Cycle

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Summary

The role of the PI 3-kinase cascade in regulation of cell growth is well established [1]. PKB (protein kinase B) is a key downstream effector of the PI 3-kinase pathway and is best known for its antiapoptotic effects [2, 3] and the role it plays in initiation of S phase [4]. Here, we show that PKB activity is high in the G2/M phase of the cell cycle in epithelial cells. Inhibition of the PI 3-kinase pathway in MDCK cells induces apoptosis at the G2/M transition, prevents activation of cyclin B-associated kinase, and prohibits entry of the surviving cells into mitosis. All of these consequences of the inhibition of PI 3-kinase are relieved by expression of a constitutively active form of PKB (caPKB), indicating that PKB plays a role in regulation of the G2/M phase. Inhibition of PI 3-kinase results in activation of Chk1, whereas caPKB inhibits the ability of Chk1 to become activated in response to treatment with hydroxyurea. Preliminary data show that PKB phosphorylates the Chk1 polypeptide in vitro on serine 280. These results not only implicate PKB activity in transition through the G2/M stage of the cell cycle, but they also suggest the existence of crosstalk between the PI 3-kinase pathway and the key regulators of the DNA damage checkpoint machinery.

Results and Discussion

PKB Activity Is Highest in the G2/M Phase of the Cell Cycle in Epithelial Cells

We have examined the kinase activity of PKB throughout the cell cycle in cells synchronized at the G1/S boundary by a double thymidine block (HeLa and MDCK) or in G1 phase by serum starvation (NIH 3T3). The activity of PKB in HeLa cells increases as cells enter G2 and progress to mitosis and drops precipitously as cells complete mitosis and return to the G1 phase (Figure 1A). Results of the immune complex kinase assays correlate with the levels of active PKB determined by Western blot analysis with phosphospecific antibodies to phosphoserine 473 (Figure 1B).

Similar to HeLa cells, PKB activity in MDCK cells is highest in the late G2/early M phase of the cell cycle (Figure 1); but, in NIH 3T3 cells, activity of PKB is not increased in G2/M (see the Supplementary Material available with this article online). These results suggest a possible role for PKB in mitotic progression in epithelial cells.

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Inhibition of PI 3-Kinase Induces Apoptosis in HeLa and MDCK Cells

We analyzed the effect of inhibiting PI 3-kinase on survival and cell cycle progression. Cells were collected at different time points after the addition of LY294002, a PI 3-kinase inhibitor, and were analyzed for DNA content. In MDCK cells, the transition from G1 to S phase was fully inhibited by 6–8 hr; however, a significant proportion of cells arrested with a 4n amount of DNA, and some cells succumbed to apoptotic death (Figure 2). Treatment of HeLa cells with LY294002 also resulted in a cell subpopulation with a 4n amount of DNA as well as accumulation of apoptotic cells (Figure 2 and Table S1). NIH 3T3 cells showed a slight delay in progression through G2/M but eventually arrested predominantly in the G1 phase (Figure 2). About 20% of cells of glioblastoma U373 treated with LY294002 remained trapped with a 4n amount of DNA for at least 24 hr (see the Supplementary Material).

Inhibition of the PI 3-Kinase Pathway during S Phase Induces a G2 Arrest and Apoptosis of MDCK and HeLa Cells

Because the majority of cells in asynchronous populations are in G1 phase, the potential effects of the inhibition of PI 3-kinase in the later stages of the cell cycle might be obscured by the well-known inhibition of the G1 to S transition. Therefore, we have synchronized MDCK cells by a double thymidine block and added LY294002 in the late S phase (at 2 hr after release). Between 4 and 5 hr after release from the thymidine block, when untreated cells accumulate in G2 and start entering mitosis, we observed a massive apoptosis of MDCK cells and an accumulation of surviving cells with a 4n amount of DNA (Figures 3A and 3B).

To distinguish between G2 versus mitotic arrest, we calculated mitotic indices of MDCK cells treated with LY294002. The data clearly showed that treatment of MDCK cells with LY294002 results in a G2 arrest (Figure 3B) and that apoptosis occurs as cells attempt to transit from G2 to M phase.

The caspase inhibitor zVAD, but not unrelated protease inhibitor zFA, almost completely inhibited the appearance of cells with fragmented DNA when added simultaneously with LY294002, strongly indicating involvement of caspases and the apoptotic nature of cell death. zVAD had no effect on the G2 arrest of LY294002-treated MDCK cells (see the Supplementary Material).

Inhibition of PI 3-kinase in NIH 3T3 cells in late S phase (Figure 3C) resulted in a delay in both the S phase and mitotic progression, but not apoptosis (Figures 3C and 3D). Delay in mitotic progression paralleled the observed delay and decrease in activation of the mitotic cyclin B/cdk1 complex (see the Supplementary Material).

Treatment of synchronized HeLa cells with LY294002 in the late S phase resulted in a significant reduction in the number of cells entering mitosis, delay of the mitotic timing, and accumulation of apoptotic cells (Figure 3D). About 20% of cells died while attempting to transit

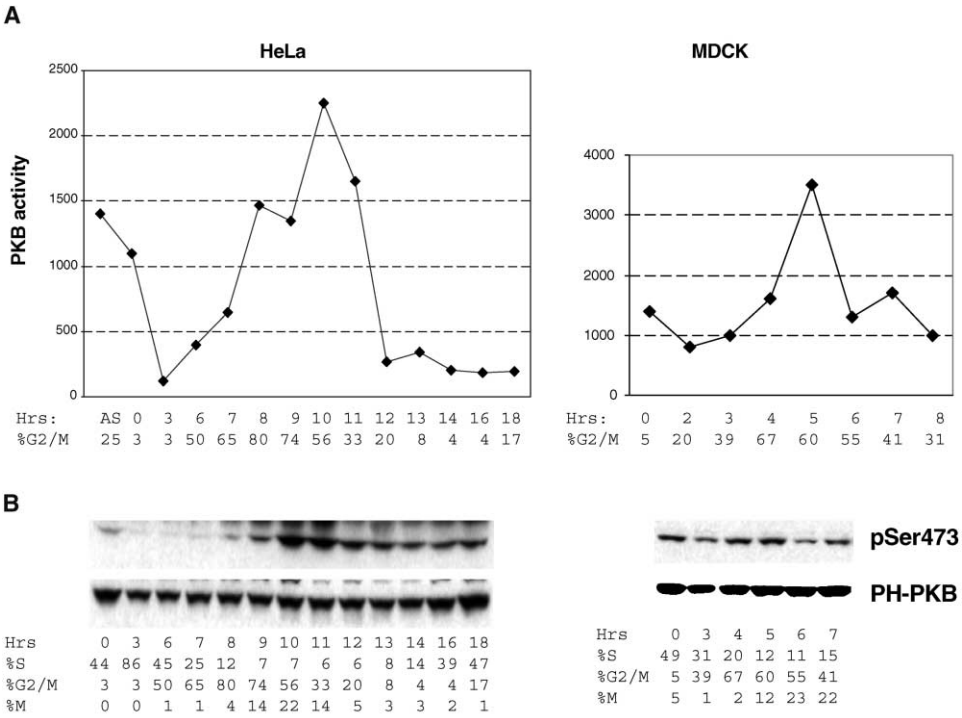

Figure 1. PKB Activity Is Highest in the G2/M Phase of the Cell Cycle in Epithelial Cell Lines

(A) Quantitation of PKB activity in the in vitro kinase assay. Protein extracts were prepared with HeLa and MDCK cells synchronized by a double thymidine block. Activity of endogenous PKB was determined in the immune complex in vitro kinase assay with Crosstide substrate as described [5]. Incorporation of ^{32}P into substrate was quantified using Phosphorimager. The numbers directly below the *x* axis indicate hours after release from the double thymidine block. Percentages of cells with a 4n amount of DNA (G2/M) are shown below the hours after release. Cell cycle progression was monitored by flow cytometric analysis of cellular DNA content in fixed propidium iodide-stained cells. The data shown represent one out of two experiments that produced practically identical profiles of PKB activity.

(B) Western blot analysis of PKB phosphorylation on serine 473 and total PKB protein levels in synchronized cell lines. In each set, the upper panel shows Western blots probed with the antibody specifically recognizing phosphorylated PKB (pSer473), and the lower panel shows the same blot reprobed with the anti-pleckstrin homology domain antibody (PH-PKB). The numbers directly below the lower panel indicate time in hours elapsed after release of cells from the double thymidine block (HeLa and MDCK). The percentages of cells in different phases of the cell cycle are also shown. The mitotic indices were determined by examining the nuclear morphology of DAPI-stained cells.

through the G2/M phase, and about 20% of cells did not progress from G2 to M (Figure 3E).

Constitutively Active PKB Prevents Apoptosis and Promotes Mitotic Progression when PI 3-Kinase Signaling Is Inhibited

Next, we examined if the G2 arrest and apoptosis resulting from the inhibition of the PI 3-kinase pathway in MDCK cells is at least partially a consequence of the inhibition of PKB activity within this pathway. To address this possibility, a form of PKB that is unresponsive to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) signaling and therefore resistant to treatment with LY294002 was introduced into MDCK cells. This constitutively active PKB (caPKB) lacks the pleckstrin homology domain, and, to ensure the constitutive activity of this enzyme in the absence of membrane targeting, two residues that are phosphorylated in activated PKB, serine 473 and threonine 308, were changed to aspartic acid [5]. Exogenous caPKB introduced into MDCK cells showed a very high activity toward a peptide substrate in vitro (Figure 4A). Importantly, while endogenous PKB, as expected, was inhibited by treatment with LY294002, the caPKB remained active (Figure 4A).

MDCKneo and MDCK-caPKB clones were synchronized and subjected to treatment with LY294002 in S phase as described above. caPKB fully protected MDCK from apoptosis during the G2/M phase of the cell cycle (Figures 4B and 4C). Moreover, the MDCK-caPKB cells underwent only a delay in G2 and progressed through mitosis, as shown by the calculation of mitotic indices and specific staining of mitotic cells with the MPM2 antibody [6] (Figures 4B and 4C).

Cyclin B-associated kinase activity is inhibited in MDCKneo cells treated with LY294002 compared to untreated cultures progressing through G2/M (Figure 4D). In MDCK-caPKB cells treated with LY294002, mitotic kinase is active at 8 and 10 hr after release, corresponding to the increase in mitotic indices and MPM2 positivity (Figures 4C and 4D). These data indicate that PKB activity might be necessary for activation of mitotic kinase and progression from G2 to mitosis in MDCK cells.

Inhibition of the PI 3-Kinase/PKB Pathway in Epithelial Cells Is Associated with an Increase in Kinase Activity of Chk1

In search of potential substrates of PKB that might be relevant to the G2 arrest resulting from PI 3-kinase inhibi-

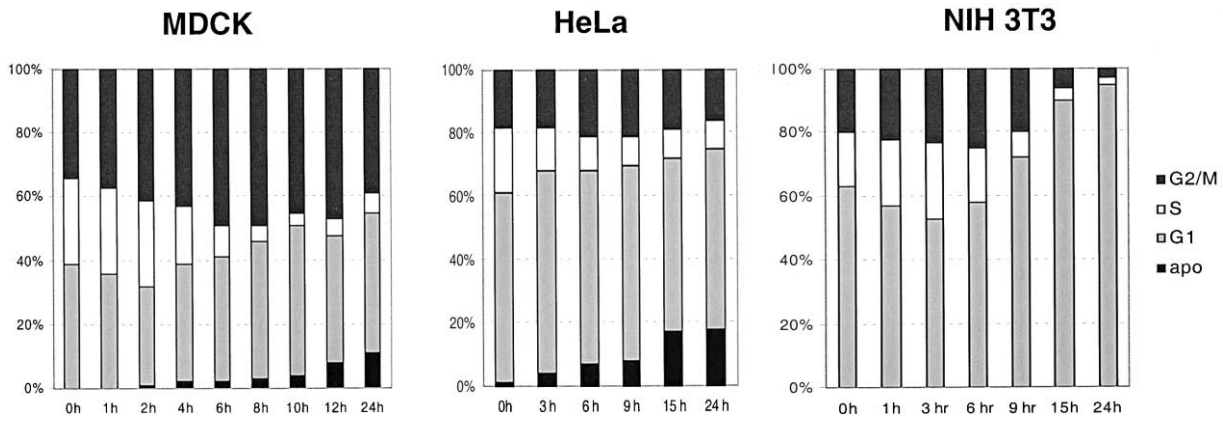

Figure 2. Inhibition of the PI 3-Kinase Pathway Results in Delay/Arrest with a 4n Amount of DNA

Asynchronously growing cell lines were harvested at different times after the addition of 30 μM LY294002. The percentage of cells in different stages of the cell cycle and of apoptotic cells was quantified by flow cytometric analysis of DNA content in fixed cells stained with propidium iodide.

tion, we have identified a conserved potential PKB phosphorylation site at serine 280 of the human Chk1 kinase sequence [7]. This residue is phosphorylated by PKB in vitro (see the Supplementary Material).

We examined if inhibition of PI 3-kinase and the presence of caPKB affect the basal activity or hydroxyurea-induced activity of Chk1. In addition to MDCK clones, we have derived stable clones of breast carcinoma line MCF7 expressing caPKB. Although the activity of exogenous caPKB in MCF7 clones was much lower than in MDCK, it was also resistant to treatment with LY294002 (data not shown). Treatment of MCF7 and MDCK control clones with LY294002 resulted in a significant increase in the kinase activity of Chk1 (Figure 4E). These results indicate that inhibition of the PI 3-kinase results in activation of Chk1 in the absence of hydroxyurea. However, treatment of caPKB-expressing clones of MCF and MDCK cells with LY294002 or hydroxyurea failed to elicit an increase in Chk1 activity (Figure 4E). This indicates that inhibition of PKB is a culprit in the activation of Chk1 by inhibition of the PI 3-kinase pathway or in response to stalled DNA replication by hydroxyurea.

In conclusion, we have found an unanticipated role for the PI 3-kinase signal transduction pathway and PKB in regulation of the G2/M transition. PKB activity protects MDCK cells from apoptosis during the G2/M transition and is necessary for activation of the mitotic kinase. High activity of PKB in G2/M appears to be a specific feature of cell cycle progression in epithelial cells, although the limited number of cell lines analyzed in this study does not allow us to make generalizations about our findings. It is possible that epithelial cells entering mitosis need additional antiapoptotic mechanisms in view of their susceptibility to anoikis [8], which might be triggered by the partial loss of cell-cell and cell-substrate adherence during mitosis. PKB might provide protection against this. Indeed, MDCK cells entering mitosis in suspension are temporarily protected by caPKB from anoikis (data not shown).

We have addressed the potential mechanism of PKB activation in G2/M by analyzing levels of PtdIns(3,4,5)P3 throughout the cell cycle in HeLa and MDCK cells. In both lines, the levels of PtdIns(3,4,5)P3 did not change significantly as cells progressed through S and G2/M phases (data not shown). Therefore, changes in total PtdIns(3,4,5)P3 levels are not likely to explain changes in PKB activity, and other mechanisms such as regulation of PKB activity by phosphatases [9] might be involved.

Our results implicate Chk1 as a potential G2/M target of the PI 3-kinase/PKB pathway. In vitro phosphorylation of Chk1 by PKB suggests that PKB directly phosphorylates Chk1 in vivo and regulates its activity. This possibility is supported by the data demonstrating the inhibition of Chk1 activation by caPKB in vivo. Inappropriate activation of Chk1 as a consequence of the PI 3-kinase/PKB inhibition might contribute to the observed G2 arrest, because Chk1 is involved in the regulation of the G2/M transition [10]. However, we have no evidence that caPKB inhibits the basal activity of Chk1 kinase in cells not subjected to DNA damage. We suggest that PKB inhibits Chk1 activation in response to DNA damage. The inhibitory effect of inappropriately active PKB on Chk1 kinase might have important implications for understanding how tumor cells with deregulated PI 3-kinase pathways respond to DNA damage. At least one report indicates a role for the PI 3-kinase/PKB signaling in overriding DNA damage-induced G2 arrest [11]. Our preliminary results support these observations. It remains to be determined whether other regulators of the G2/M transition are targets of PI 3-kinase/PKB pathway. In this respect, a recent report showing a role for PKB in reinitiating meiosis in starfish oocytes through inhibitory phosphorylation of Myt1 kinase [12] is of particular interest.

Supplementary Material

Supplementary Material including tables with numerical values for cell cycle data in Figures 2 and 3, data on inhibition of apoptosis in LY294002-treated cells by zVAD, as well as figures showing PKB activity in NIH 3T3 cells, the effect of LY294002 on cyclin B/cdk1 activity in NIH 3T3 cells, and in vitro phosphorylation of Chk1 by PKB is available at http://images.cellpress.com/supmat/supmatin.htm.

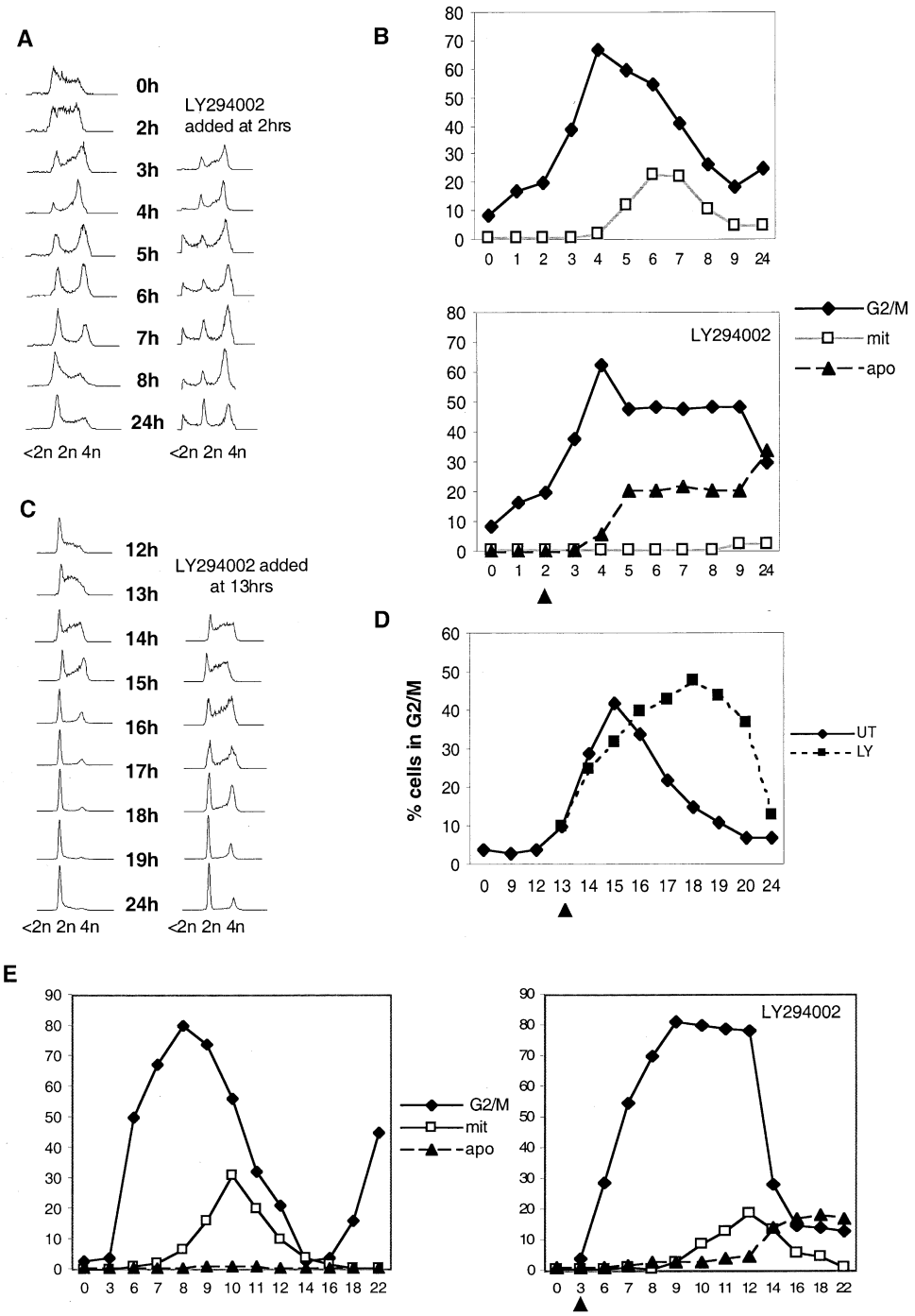

Figure 3. Inhibition of PI 3-Kinase in Late S Phase Induces a G2 Arrest/Delay and Apoptosis

(A) Flow cytometric analysis of DNA content in MDCK cells synchronized by a double thymidine block at G1/S phase and released into full medium. Parallel cultures were treated with 30 μM LY294002 added 2 hr after release. Cells were processed for analysis of DNA content by flow cytometry of PI-stained cells and for determination of mitotic indices in DAPI-stained cytospin preparations (at least 400 cells were counted for each time point).

(B) Cell cycle progression and apoptosis in MDCK cultures treated as in (A). The percentages of cells with a 4n amount of DNA (G2/M), mitotic cells (mit), and cells with less than a 2n amount of DNA (apo) are shown. Control cultures are shown in the top panel, and cultures treated with 30 μM LY294002 added 2 hr after release are shown in the bottom panel. Apoptotic cells in the untreated cultures did not exceed 1%–2% of total at any time.

(C) NIH 3T3 cells were synchronized by serum starvation for 24 hr and were released into full medium. Treatment of parallel cultures with 50 μM LY294002 was initiated 13 hr after release.

(D) Progression through G2/M phase in NIH 3T3 cultures treated as described in (C). UT, untreated cultures; LY, cultures treated with LY29402.

(E) Cell cycle progression in double thymidine block-synchronized HeLa cells with or without treatment with LY294002 (30 μM, added 3 hr after release, right panel). The percentages of cells with a 4n amount of DNA (G2/M) and a <2n amount of DNA (apo) were calculated from flow cytometric analysis of DNA content. The arrowheads below the *x* axis indicate times of the addition of LY294002.

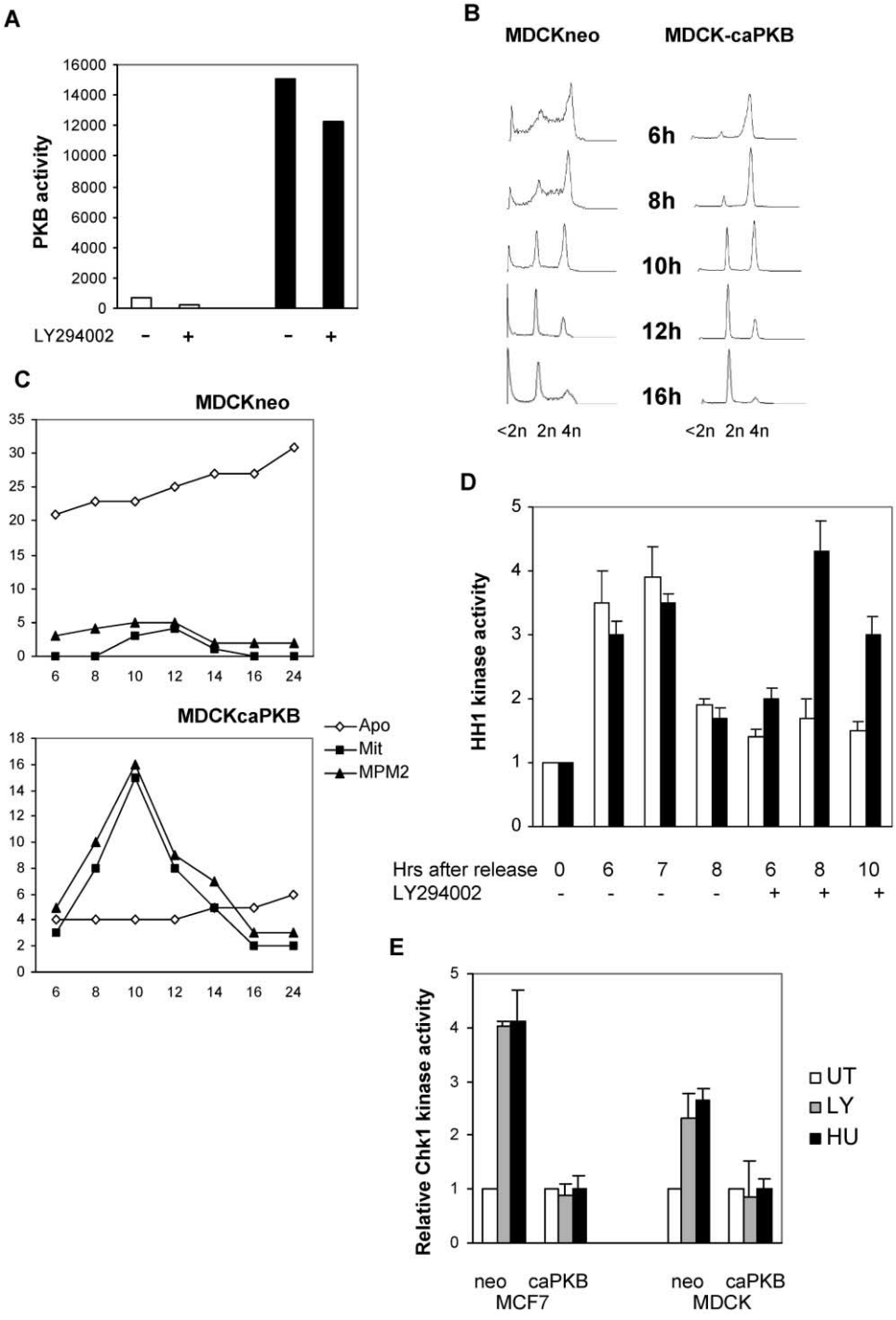

Figure 4. caPKB Is Active in the Presence of LY294002 and Protects MDCK Cells from Apoptosis and G2 Arrest

(A) Activity of PKB in MDCKneo (control) and MDCK-caPKB clones treated with LY294002. Cells were synchronized, treated with LY294002 as described in Figure 3, and harvested at 5 hr after release. PKB (endogenous and exogenous) was precipitated with anti-PKB antibodies and anti-epitope tag antibodies against the Glu-Glu epitope tag on caPKB, respectively. Kinase activity was measured as described in the legend of Figure 1. White bars represent MDCKneo cells, and black bars represent MDCK-caPKB cells.

(B) CaPKB protects MDCK from apoptosis and relieves G2 arrest. MDCKneo and caPKB clones were synchronized by a double thymidine block, released into fresh medium, and treated with LY294002 1.5 hr after release. DNA content was analyzed by flow cytometry.

(C) Cell cycle progression and apoptosis in MDCKneo and MDCK-caPKB clones treated with LY294002 as in (B). Percentages of apoptotic (Apo), mitotic (Mit), and MPM2-positive (MPM2) cells are shown.

(D) Cyclin B1-associated kinase activity is inhibited in synchronized MDCK cells treated with LY294002 but is restored in cells expressing caPKB. Lysates prepared from synchronized MDCKneo (white bars) and caPKB cells (black bars) were analyzed for kinase activity precipitated using anti-cyclin B1 antibodies. Histone H1 was used as a substrate in the immune complex-linked in vitro kinase assay. The numbers directly below the *x* axis indicate hours after release from thymidine block with or without the addition of LY294002 at 1.5 hr. Data are presented as the fold increase of HH1 kinase activity over that precipitated from double thymidine-arrested cells (time point 0) and are the average of three experiments performed.

(E) Activity of endogenous Chk1 was assayed in the immune complex kinase assay with GSTcdc25(200-256) as a substrate as described [13]. Cells were either untreated (UT), treated with 30 μM LY294002 for 3 hr (LY), or treated with 2 mM hydroxyurea (HU) for 16 hr (MCF7) or 5 hr (MDCK). Results are expressed relative to the values observed in extracts from untreated cells and are an average of three experiments performed.

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