

Cytokine-driven cell cycling is mediated through Cdc25A

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Lymphocytes are the central mediators of the immune response, requiring cytokines for survival and proliferation. Survival signaling targets the Bcl-2 family of apoptotic mediators, however, the pathway for the cytokine-driven proliferation of lymphocytes is poorly understood. Here we show that cytokine-induced cell cycle progression is not solely dependent on the synthesis of cyclin-dependent kinases (Cdks) or cyclins. Rather, we observe that in lymphocyte cell lines dependent on interleukin-3 or interleukin-7, or primary lymphocytes dependent on interleukin 7, the phosphatase Cdc25A is

the critical mediator of proliferation. Withdrawal of IL-7 or IL-3 from dependent lymphocytes activates the stress kinase, p38 MAPK, which phosphorylates Cdc25A, inducing its degradation. As a result, Cdk/cyclin complexes remain phosphorylated and inactive and cells arrest before the induction of apoptosis. Inhibiting p38 MAPK or expressing a mutant Cdc25A, in which the two p38 MAPK target sites, S75 and S123, are altered, renders cells resistant to cytokine withdrawal, restoring the activity of Cdk/cyclin complexes and driving the cell cycle independent of a growth stimulus.

Introduction

The cytokines interleukin-7 (IL-7) and interleukin-3 (IL-3) are important mediators of T and B lymphocyte survival and growth. IL-7, in particular, is required for the homeostasis of peripheral CD4 and CD8 T cells through mechanisms not fully understood (Schluns et al., 2000; Tan et al., 2001; Khaled and Durum, 2002; Kondrack et al., 2003; Li et al., 2003; Schluns and Lefrancois, 2003; Seddon et al., 2003). Cytokines are known to prevent cell death through the induction of the anti-apoptotic proteins BCL-2 or BCL-X_L and the inhibition of pro-apoptotic proteins like BAX, BAD, or BIM (Maraskovsky et al., 1997; Khaled et al., 1999b, 2001b; Vander et al., 1999; Kim et al., 2003). Overexpression of *bcl-2* protects cells from apoptosis after IL-7 or IL-3 withdrawal, however cells also undergo growth arrest, indicating that these cytokines, in addition to promoting survival, induce replication (Maraskovsky et al., 1997; Khaled et al., 2001b; Li et al., 2004; unpublished data). IL-7 has been shown to be required for *homeostatic* T cell

proliferation in mice (Schluns et al., 2000; Geiselhart et al., 2001). It therefore appears that the replication of lymphocytes in the presence of IL-7 (and similarly IL-3) may not be merely a default pathway reflecting the survival effect of the cytokine, but rather may be a distinct replication signal from the cytokine receptor.

Cell cycle progression is normally mediated by enzymatic complexes containing Cdks, which phosphorylate substrates such as the Retinoblastoma (Rb) protein, releasing E2F and inducing transcription of genes needed for cell division (Nevins, 2001). Cdks are partially activated by binding to specific cyclins and then are fully activated by phosphorylation of a threonine (T160) located in a conserved domain, the T-loop. Progression through G₁ and S phase of the cell cycle requires G₁-Cdk/cyclins, like Cdk4, Cdk6, and cyclin D, as well as the G₁-S Cdk/cyclins, like Cdk2 and cyclin E, and later cyclin A. Though transcriptionally regulated, Cdk activity is primarily controlled through phosphorylation of two conserved residues found in the ATP binding loop, T14 and Y15, mediated by Wee1 kinase and Myt1 (Pines, 1999). In this manner, a pool of phosphorylated Cdks can accumulate during the G₁ and G₂ phases. Activation of Cdks, through dephosphorylation of T14 and Y15, is mediated by members of the phosphatase family,

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Abbreviations used in this paper: DP, dominant-positive; PI, propidium iodide; Rb, Retinoblastoma; RPA, RNase protection assay.

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Cdc25A, B, and C (Nilsson and Hoffmann, 2000). Cdc25A is necessary for promoting the G₁-S phase transition by removing an inhibitory phosphate on Cdk2, promoting Cdk2-cyclin E/A activity (Mailand et al., 2000; Coulonval et al., 2003), but may also have a role during mitotic entry, activating Cdk1 (Mailand et al., 2002). Throughout the cell cycle, Cdc25A is tightly regulated at the protein level through phosphorylation which induces ubiquitination leading to degradation (Donzelli et al., 2004), a process that ultimately contributes to cell cycle arrest through the absence of active Cdks. In addition to inhibitory phosphorylations, Cdks are also inhibited by negative regulation mediated through the p16 (INK4) family (p16, p15, p18, and p19) and the p21 (Cip/Kip) family (p21, p27, and p57), which bind to and inhibit the enzymatic activity of the Cdks (Vidal and Koff, 2000).

Though inhibition of lymphocyte cell death has been accorded the major function of IL-7, an activity in cell cycle progression also is observed. Naive T cells proliferated in vitro when stimulated with IL-4, IL-7 or IL-15, but only IL-7 was essential for the homeostatic proliferation of naive T cells in vivo (Schluns et al., 2000; Tan et al., 2001). In our previous studies, we found that withdrawal of IL-7 induced growth arrest in dependent cells (Kim et al., 2003). The present study examines the mechanisms by which IL-7 and IL-3 induce replication in lymphocyte lines and concludes that a key pathway involves the stress kinase p38MAPK inactivating the phosphatase Cdc25A.

Results

The cytokines IL-3 and IL-7 have been previously studied for their anti-apoptotic effects in lymphocytes. We showed that withdrawal of IL-3 or IL-7 induced the translocation of the death protein BAX (Khaled et al., 1999b) and that overexpression of the anti-apoptotic proteins BCL-2 or BCL-XL (Khaled et al., 2001b; Li et al., 2004) prevented cytokine withdrawal-induced death but did not restore proliferation. Recently, we found that IL-7 also promoted the expression of cell cycle regulators such as p55Cdc and Cdk4 and proliferative factors like c-myc (Kim et al., 2003), furthering supporting a proliferative function, in addition to an anti-apoptotic role, for these cytokines. To identify and characterize components of cell cycle regulation targeted by cytokines like IL-3 or IL-7, we evaluated the effect of these cytokines on the proliferation of two different cytokine-dependent cell lines, as shown in Fig. 1 (A and B) and primary lymphocytes shown in Fig. 1 C. Withdrawal of IL-3 from the pro-B cell line, FL5.12A, induced G₁ arrest beginning at 8 h, progressing through 12 h, and was complete by 17–24 h, with cells accumulating in G₁ as they exited from S phase (Fig. 1 A and Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200409099/DC1>). These results indicate that IL-3 is required for cell division. Withdrawal of IL-7 from the thymocyte line, D1, similarly produced a G₁ arrest after 24 h of cytokine withdrawal (Fig. 1 B) that initiated after 12 h of IL-7 deprivation (not depicted). In both the IL-3- and IL-7-dependent cell lines, growth arrest began many hours before the apoptotic processes of mitochondrial breakdown, effector caspase activation

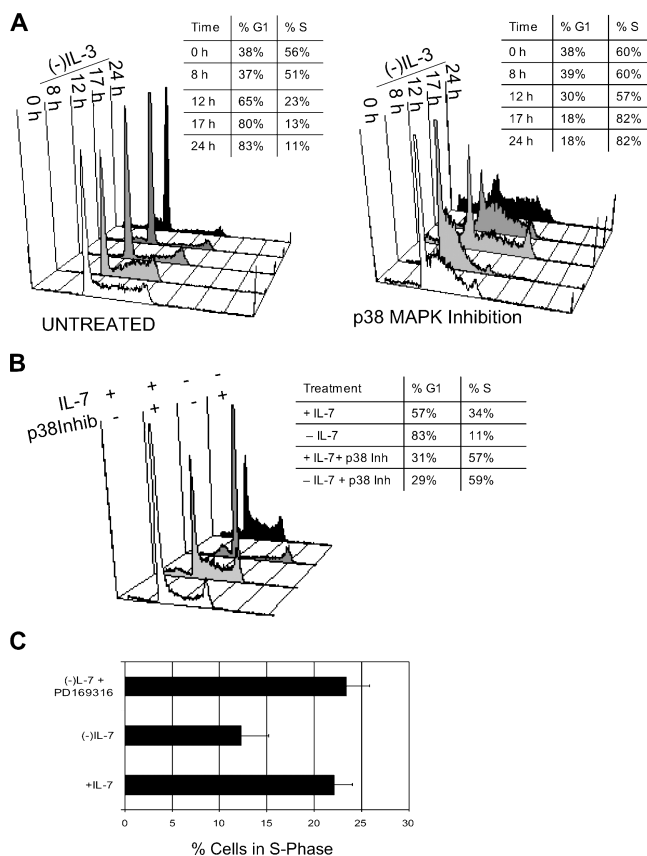


Figure 1. Inhibition of p38 MAPK prevents G₁/S arrest after withdrawal of IL-3 or IL-7 from cytokine-dependent lymphoid cells. Cell cycle was analyzed by incorporation of propidium iodide (PI) as described in Materials and Methods. Percent of cells in G₁ and S phases was calculated using ModFit LT software. (A) IL-3 was withdrawn from FL5.12A, pro-B cells for 8, 12, 17, or 24 h. The p38 MAPK inhibitor, PD169316 (20 μM), was added at the time of IL-3 withdrawal. Shown is a representative example of four experiments. (B) IL-7 was withdrawn from D1, a thymic cell line, for 24 h. The p38 MAPK inhibitor, PD169316 (20 μM) was added at the time of IL-7 withdrawal. Shown is a representative experiment. (C) Mouse lymph node cells were isolated and placed in culture with IL-7 (100 ng/ml) and a submitogenic concentration of Con A (0.25 μg/ml) for 48 h. Cells were then washed and cultured for 24 h without IL-7 and with PD169316 (20 μM). Shown are the results of two combined experiments, with error bars representing ± SEM.

and DNA damage, which take place 24–36 h after cytokine withdrawal (Khaled et al., 2001b). These findings indicate the induction of G₁ arrest is an immediate response to cytokine withdrawal that initiates as early as 8 h after loss of cytokine signal and is complete 17–24 h later.

To determine if G₁ arrest in response to cytokine withdrawal also occurs in primary lymphocytes deprived of IL-7, we isolated lymph node lymphocytes (~70% T cells) and assayed their proliferative capacity. Most primary T cells are in G₀ until antigen activated (Geiselhart et al., 2001) and we found that addition of a low concentration of Con A, nonmitogenic on its own, conferred a proliferative response to IL-7 in vitro. Lymph node cells were cultured for 48 h with IL-7 (100 ng/ml) and Con A (0.25 μg/ml), which induced 23.4% of the cells to enter S phase. After 24 h of IL-7 withdrawal, the number of cells in S phase was reduced to 12.4% (Fig. 1 C) and this de-

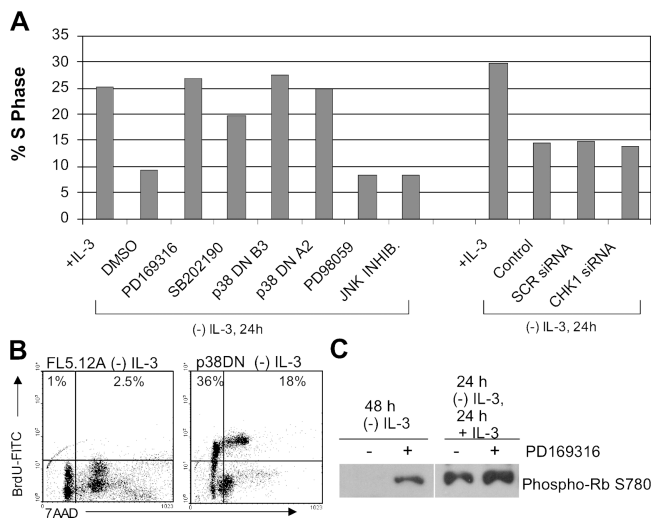


Figure 2. Inhibition of p38 MAPK, but not ERK or JNK, restores S-progression and Rb phosphorylation in the absence of IL-3. Cell cycle was analyzed by incorporation of PI as described in Materials and methods. Percent of cells in S phase was calculated using ModFit LT software. (A) FL5.12A cells were cultured with or without IL-3 for 17 h. The p38 MAPK inhibitors, PD169316 (20 μ M) or SB202190 (20 μ M), the MEK1 inhibitor (activator of ERK), PD98059 (20 μ M), and the Jun kinase inhibitor, JNK inhibitor II (20 μ M), were added at the time IL-3 was withdrawn. DMSO (0.1%) was included as a negative control. Two different FL5.12A clones B3 and A2, stably transfected with a dominant negative (DN) p38 MAPK, were placed in culture without IL-3 for 17 h. Results shown are representative of two experiments. (B) FL5.12A-A2 cells, expressing DN-p38 MAPK, incorporated BrdU during 17 h IL-3 withdrawal, while untransfected cells arrested. (C) Levels of phosphorylated Rb protein were assayed in FL5.12A cells after 48 h of IL-3 withdrawal. Nuclear lysates were resolved by SDS-PAGE and immunoblotting as described in Materials and methods using an antibody specific for Rb protein phosphorylated at serine 780.

clined further to 4% by 48 h (not depicted). Hence, withdrawal of IL-7 from either cytokine-dependent cell lines or primary lymphocytes induces growth arrest before apoptosis.

Previously, we reported that shortly after withdrawal of IL-3 or IL-7, the MAPK stress pathway was activated (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200409099/DC1>) and played an important role in inducing the later events of apoptosis (Khaled et al., 2001a; Rajnavolgyi et al., 2002). Therefore, we examined whether stress kinases could also be involved in cell cycle regulation, specifically the induction of growth arrest observed in the absence of a cytokine signal. PD169316, a potent inhibitor of the stress kinase p38 MAPK, dramatically increased the number of FL5.12A cells in S phase after withdrawal of IL-3, and the weaker p38 MAPK inhibitor SB202190 also showed significant effects (Fig. 1 A and Fig. 2 A). In contrast, inhibition of other MAPKs, ERK or JNK, failed to restore G₁-S phase progression (Fig. 2 A) after IL-3 withdrawal. An effect of pharmacological inhibition of p38 MAPK was accelerated cell death in the absence of cytokines, as can be noted by the increased number of sub-G₁ cells (Fig. 1, A and B). This was not observed upon inhibition of the ERK or JNK pathway. Thus, potent pharmacological inhibitors of p38 MAPK, in addition to relieving the G₁ arrest, also induce some cell death. Perhaps this effect results from mitotic catastrophe or from other drug effects.

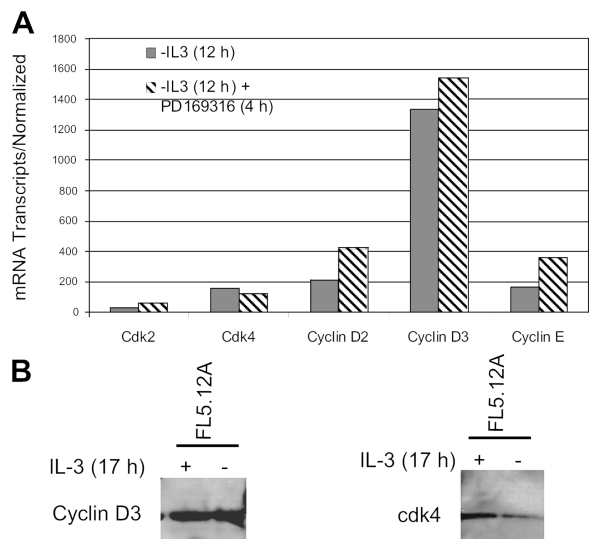
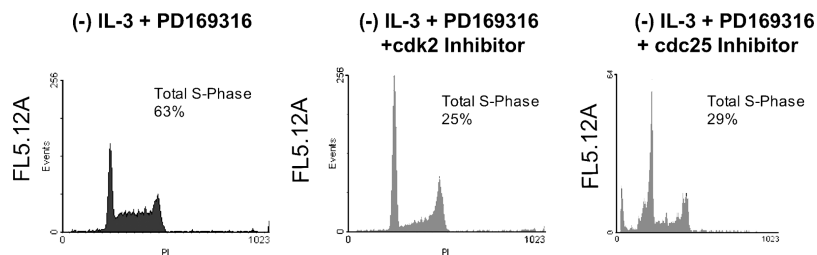


Figure 3. Inhibition of p38 MAPK after IL-3 withdrawal induces minor increases in G₁-S Cdk and cyclins in B lymphocytes. (A) FL5.12A cells were incubated for 12 h in the absence of IL-3 and then further incubated for 5 h with either PD169316 (20 μ M) or IL-3 (not depicted) for a total of 17 h. Cells were then harvested and total RNA isolated. RPAs were performed as described in Materials and methods. Transcription of L32 was measured as a control for total mRNA expressed. Results were quantified using a Bio-Rad Gel Documentation system, and mRNA transcripts for Cdk and cyclins normalized to the levels of L32 expression. (B) Whole cell lysates were prepared from FL5.12A cells deprived of IL-3 for 17 h and immunoblotted as described in Materials and methods for detection of Cdk4 and cyclin D3 with specific antibodies.

To specifically inhibit p38 MAPK without using pharmacological inhibitors, we expressed a dominant negative p38 MAPK, which specifically inhibits p38 MAPK activity, in FL5.12A cells and again observed restoration of cell cycling (~22% in S phase; Fig. 2 A) and DNA synthesis, shown by BrdU incorporation (~18% cells synthesizing DNA; Fig. 2 B), in the absence of IL-3. In the IL-7-dependent thymocyte line, D1, G₁ arrest after IL-7 withdrawal was also dramatically released by inhibiting p38 MAPK with PD169316, resulting in increased progression into S phase (Fig. 1 B). Primary lymphocytes showed a similar pattern upon inhibition of p38 MAPK, relieving the arrest that followed IL-7 withdrawal (Fig. 1 C). These results indicate that activation of p38 MAPK after cytokine withdrawal induces rapid G₁-S phase arrest (well before the onset of apoptosis) and that inhibition of p38 MAPK substantially restores S phase progression, confirmed by measurements of DNA synthesis.

We also assayed for the activity of other checkpoint kinases, specifically CHK1, to determine if these were induced by cytokine withdrawal. Unlike the increased activation of p38 MAPK detected upon cytokine deprivation, we found that protein levels of CHK1 were negligible by 17 h of IL-3 (Fig. S2) or IL-7 (not depicted) withdrawal, though detectable in the presence of cytokines. Therefore, inhibition of CHK1 by RNA interference (Fig. 2 A) did not restore S phase progression in the absence of IL-3, suggesting that CHK1 initiated checkpoint regulation was not the mechanism by which cytokine withdrawal induced growth arrest.

Figure 4. Inhibition of Cdk2 or Cdc25 reverses the effects of p38 MAPK inhibition on the promotion of cell cycling during IL-3 withdrawal. Cell cycling was analyzed by incorporation of PI and percent of cells in S phase was calculated using ModFit LT software as described in Materials and methods. FL5.12A cells were cultured without IL-3 and PD169316 (20 μ M) in the presence of 20 μ M of a pharmacological inhibitor of Cdk2 (Cdk2 inhibitor II) or 20 μ M of a pharmacological inhibitor of Cdc25 (NSC 95397) for 17 h and effects of cell cycling assessed.



Phosphorylation of Rb, which releases E2F and promotes entry into S-phase, was interrupted by IL-3 withdrawal and restored by inhibition of p38 MAPK (Fig. 2 C), suggesting that in these cells p38 MAPK acted upstream of the events leading to Rb phosphorylation. One possibility would be that p38 MAPK negatively regulates the synthesis of the Cdk 4/6-cyclin D complexes (Lavoie et al., 1996), thereby retaining hypophosphorylated Rb in the absence of these complexes. If so, then we would expect levels of mRNA transcripts for G₁-S phase Cdks and cyclins to increase upon p38 MAPK inhibition. However, RNase protection assays (RPAs), to measure mRNA transcripts for Cdks and cyclins, for example Cdk 4, during initiation of G₁ arrest showed decline of transcription after 12 h of IL-3 (Fig. 3 A) and IL-7 (not depicted) withdrawal, and minor, if any, increase in transcription as a result of inhibiting p38 MAPK with PD169316. Re-addition of IL-3 or IL-7 after cytokine withdrawal substantially increased the transcription of Cdks and cyclins as would be expected upon restoration of cytokine signaling pathways (unpublished data). In the absence of IL-3, inhibition of p38 MAPK did result in a slight increase in expression of cyclins D2, D3 and E (Fig. 3 A), which will be further discussed. RPAs were also performed to measure transcription of Cdks 1, 5, 7, and 8 and cyclins A2, B1, C, B2, F, G1, G2, and H, and no effects of p38 MAPK inhibition were observed (not depicted).

To determine if decreased transcription was reflected by loss of protein, we measured the protein levels for one of the Cdks, Cdk4, and one of the cyclins, cyclin D3. We found that these cell cycle mediators could still be detected by immunoblotting cell lysates even 17 h after IL-3 withdrawal when cells are fully arrested (Fig. 3, B and C), demonstrating that, though mRNA levels decreased, the proteins themselves were not limiting in the early stages of cytokine withdrawal. Thus, the mechanism through which p38 MAPK regulates cell cycling in cytokine-dependent lymphocytes is not solely based on controlling the synthesis of Cdks and cyclins.

Whereas other growth factors induce the synthesis of cyclin D, we considered the possibility that posttranslational regulation of cyclin D could be regulated by IL-3 and IL-7, because it has been reported that p38 MAPK could phosphorylate cyclin D1, leading to its ubiquitination and degradation (Casanovas et al., 2000). We therefore overexpressed cyclin D1 in FL5.12A cells to determine whether it would mimic the effect of p38 MAPK inhibition in releasing cells from G₁ arrest after IL-3 loss. This was not the case because overexpression of cyclin D1 in FL5.12A cells failed to relieve cell cycle arrest in the absence of IL-3 whereas the p38 MAPK inhibitor continued to have this effect as it did in untransfected

cells (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200409099/DC1>). Together, withdrawal of IL-3 leads to G₁ arrest by a mechanism unrelated to the levels of cyclin D mRNA (Fig. 3 A) or protein (Fig. 3, B and C).

Inhibition of Cdk2, a kinase involved in the transition into S phase, with a potent and selective pharmacological inhibitor (Cdk2 Inhibitor II – Compound 3), countered the effect of inhibiting p38 MAPK after 17 h of IL-3 withdrawal (Fig. 4). This effect was evident in the absence of IL-3. Because there were minimal effects of p38 MAPK inhibition on the transcription of Cdk2 (Fig. 3 A), we examined a role for regulation of Cdk2 activity, specifically through the Cdc25 family of phosphatases known to dephosphorylate and activate Cdks (Sexl et al., 1999). Treatment of FL5.12A cells with a pharmacological inhibitor (NSC 95397) of Cdc25 phosphatases reversed the S phase progression induced by p38 MAPK inhibition (Fig. 4). These findings suggest that Cdc25 phosphatases, and their activation of Cdks, could be essential components of the pathway through p38 MAPK by which cytokines regulate cell proliferation.

Previous studies had shown that p38 MAPK could directly phosphorylate members of the Cdc25 family, Cdc25B and Cdc25C, inducing their degradation (Bulavin et al., 2001, 2002). However, it is Cdc25A that is involved in the G₁-S phase transition, dephosphorylating Cdk2 on an inhibitory tyrosine, Y15, thereby activating it. We therefore assayed for Cdc25A and observed that the levels of endogenous (Fig. 5 A) and overexpressed HA-tagged Cdc25A rapidly declined 4–8 h after IL-3 withdrawal (Fig. 5 B), suggesting degradation of the protein. Others have shown that the degradation of Cdc25A is proteasome dependent (Mailand et al., 2000) and mediated through ubiquitination (Busino et al., 2004). We found that inhibiting p38 MAPK subsequently restored Cdc25A protein levels, both endogenous levels and overexpressed HA-tagged Cdc25A, promoting stability of the phosphatase (Fig. 5 B). Hence, p38 MAPK appears to act upstream of the decline of Cdc25A after IL-3 withdrawal, and regulating Cdc25A protein stability may be one of the functions of this kinase.

Two critical serines on Cdc25A (S75 and S123) are known to be kinase targets. DNA damage triggers the Chk kinases which phosphorylate Cdc25A on S123 leading to its degradation (Falck et al., 2001; Sorensen et al., 2003), and in *Xenopus* embryos, phosphorylation of S73 (S75 in the human sequence) is mediated by another unknown kinase (Shimuta et al., 2002), perhaps also Chk1 as has been recently shown (Hassepass et al., 2003). However, Chk kinases are known to be activated during cell cycle checkpoints triggered by DNA damage (Bartek and Lukas, 2003), which occurs later in the apoptotic process, and we did not detect Chk1 protein during

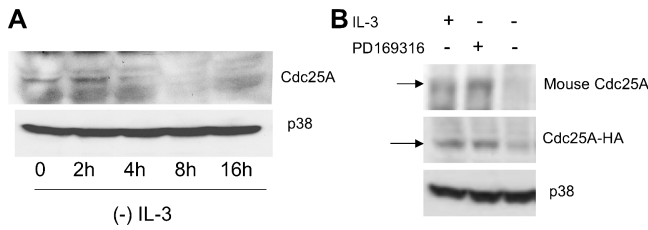


Figure 5. Cdc25A protein is degraded upon cytokine withdrawal and stabilized, in the absence of cytokines, by inhibition of p38 MAPK. (A) Levels of endogenous mouse Cdc25A protein were measured in FL5.12A cells. Whole cell lysates were made from cells deprived of IL-3 for 2, 4, 8, and 16 h, then resolved by SDS-PAGE and immunoblotted as described in Materials and methods using an antibody specific for Cdc25A. Total p38 MAPK was measured as a loading control. (B) Endogenous mouse Cdc25A was measured in FL5.12A cells stably overexpressing wild-type (WT) human Cdc25A. Whole cell lysates were made from cells cultured with or without IL-3 and 20 μ M of PD169316 for 16 h, and then resolved by SDS-PAGE and immunoblotted as described in Materials and methods using antibodies specific for mouse Cdc25A and for human Cdc25A. Levels of p38 were detected as a loading control. Shown are representative experiments of three such performed.

cytokine withdrawal (Fig. S2) nor observed any effects on cell cycle progression upon Chk1 inhibition by RNA interference (Fig. 2 A). This demonstrates that cell cycle arrest due to cytokine withdrawal does not involve Chk1 and suggests that phosphorylation of Cdc25A is mediated by a different kinase activity in cytokine-deprived lymphocytes.

The regulatory sites on Cdc25A, S75 and S123, can be direct targets of p38 MAPK as shown by p38 MAPK in vitro kinase assays using HeLa cells (Goloudina et al., 2003). These results, together with the increased stability of Cdc25A promoted by p38 MAPK inhibition (Fig. 5 B), suggest that p38 MAPK is the kinase principally responsible for phosphorylation of S75 and S123 on Cdc25A, inducing the decline in Cdc25A after cytokine withdrawal.

Having shown that the decrease in Cdc25A proteins correlated with cell cycle arrest after cytokine withdrawal, we tested whether it played an important functional role. Because S75 and 123 were the targets of p38 MAPK, leading to its degradation, we mutated these sites and measured the effect on cell cycling after cytokine withdrawal. Mutation of S123, and S75 to alanines produced a stable form of Cdc25A that significantly restored S phase entry after 24 h of IL-3 withdrawal as shown in Fig. 6 A. The Cdc25A double mutant (S75,123A) functioned like a dominant-positive (DP), promoting cell cycling in the absence of IL-3 compared with expression of the WT protein. Significantly, that activity of Cdc25A-DP in the absence of IL-3 resulted in DNA synthesis, as shown by incorporation of BrdU, and not just accumulation in S phase, with cells progressing into G2/M (Fig. 6 A). This was not observed in cells expressing WT Cdc25A that accumulated in G₁ phase.

The effect of expressing the Cdc25A-DP in IL-7-dependent D1 cells was even more striking, with increased cells cycling in the absence of IL-7 (24 h) compared with WT protein (Fig. 6 B). Using BrdU incorporation to measure DNA synthesis, we also observed restoration of S phase progression by this Cdc25A mutein after IL-7 withdrawal (Fig. 6 B). These effects required mutation of both sites (S75 and S123) on Cdc25A, because

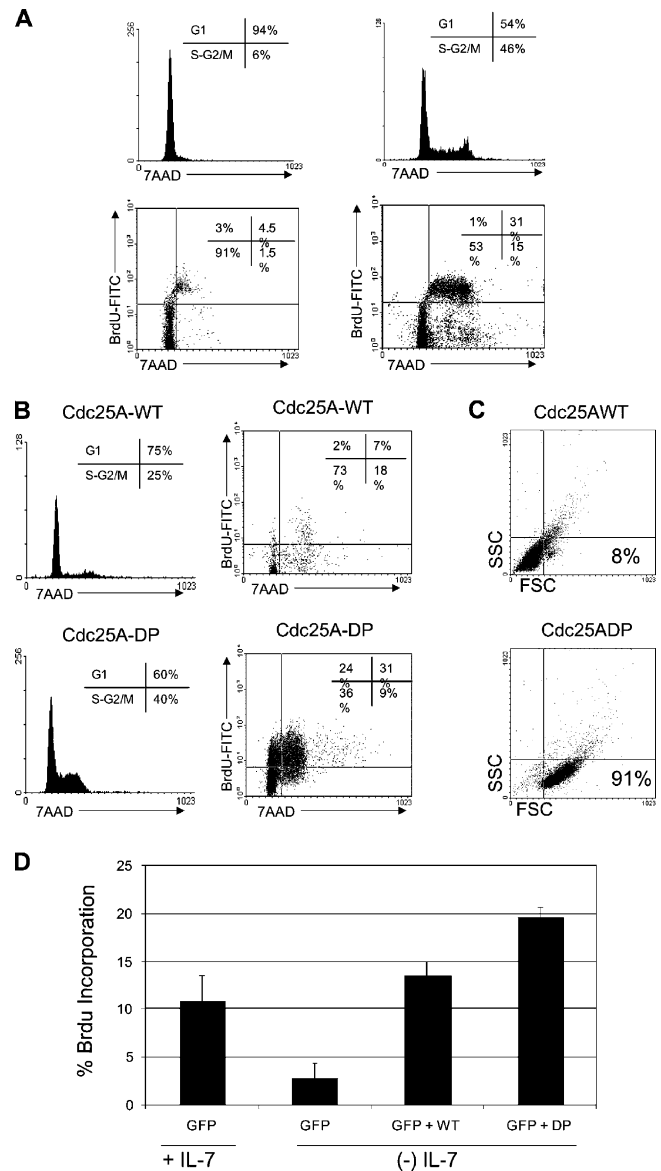


Figure 6. Expression of Cdc25A mutated at S75 and S123 prevents G₁ arrest and sustains S phase progression after withdrawal of IL-3 or IL-7. DNA synthesis was assayed by incorporation of BrdU and cell cycle was analyzed by uptake of 7AAD and assayed by flow cytometry as described in Materials and methods. Percent of cells in G₁ and S phase was calculated using ModFit LT software, excluding apoptotic cells and aggregates. (A) FL5.12A cells stably expressing the dominant-positive (DP) Cdc25A (S75,123A) mutein or Cdc25A-WT were cultured with or without IL-3 for 24 h. (B) D1 cells stably expressing the Cdc25A-DP (S75, 123A) mutein or Cdc25-WT were cultured with or without IL-7 for 24 h. (C) Viability and cell size of D1 cells stably expressing Cdc25A-DP or Cdc25A-WT are shown in dot blots displaying forward (FSC) and side scatter (SSC). Results shown are representative of three or more experiments performed. Apoptotic cells were not excluded (D). Lymph node cells were isolated and grown with IL-7 (100 ng/ml) and Con A (0.25 μ g/ml) as previously described. Primary cells were transfected with Cdc25A-DP or Cdc25A-WT and GFP, as a selection marker. Transfected cells, were selected by gating on GFP expression for analysis of DNA synthesis and cycling by BrdU/7AAD staining after 24 h of IL-7 withdrawal as described in Materials and methods. Shown is a representative experiment of three performed.

either one alone had minimal effects on cell cycling (not depicted). Expression of Cdc25-DP (cotransfected with the selection marker GFP) in primary lymphocytes was able to restore

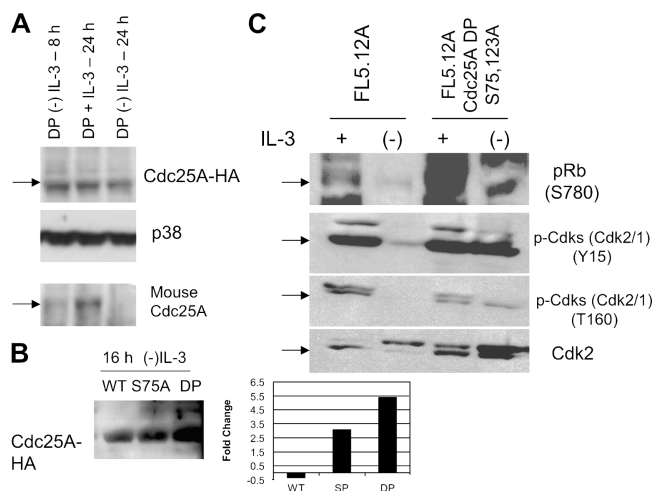


Figure 7. Cdc25A-DP (S75,123A) remains stable and sustains phosphorylation of Rb and Cdk2 after IL-3 withdrawal. (A) Protein levels of Cdc25A-DP (S75,123A) were measured in FL5.12A cells. Cells were deprived of IL-3 for 17 h, and cytosolic extracts were resolved by SDS-PAGE and immunoblotted as described in Materials and methods using an antibody specific for mouse (to detect endogenous protein) or human Cdc25A (to detect the overexpressed HA-tagged protein). Total p38 MAPK was measured as a loading control. Shown is a representative experiment of two. (B) Stability of Cdc25A was determined in FL5.12A cells overexpressing the Cdc25A-DP mutant (DP) and the Cdc25A single mutant (S75A) (SP) after IL-3 withdrawal. The immunoblot was quantified using a Bio-Rad Gel Documentation system and results shown in the adjacent panel. The graph indicates fold differences in protein levels measured with or without IL-3 for 16 h. (C) Levels of phosphorylated Rb protein and Cdk2 were measured in FL5.12A cells and FL5.12A cells stably overexpressing the Cdc25A-DP mutant. Nuclear extracts were made from cells deprived of IL-3 for 17 h, then resolved by SDS-PAGE and immunoblotted, as described in Materials and methods, using antibodies specific for phospho-Rb protein (phosphorylated at serine 780), phospho-Cdk (tyrosine 15) (detects phospho-Cdk2 and phospho-Cdk1) and phospho-Cdk (threonine 160; detects phospho-Cdk2 and phospho-Cdk1). Total Cdk2 levels were also measured with a specific antibody. Note, the same cell lysates (equally loaded) were used in the phospho-Rb, phospho-Cdks, and Cdk-2 blots.

DNA synthesis (measured by BrdU incorporation) in primary cells deprived of IL-7 for up to 48 h (Fig. 6 D; Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200409099/DC1>). Cdc25A-WT also had a positive effect on primary lymphocyte proliferation, though not as striking as that of the DP protein, perhaps due to reduced levels of p38 MAPK in primary cells as compared with cell lines. These findings indicate that Cdc25A is pivotal regulator of IL-7-driven proliferation of lymphocytes, cell lines and primary cells, and a novel target of IL-7 signal transduction through p38 MAPK.

In addition to promoting cell cycling, expression of the Cdc25A-DP was able to extend the life of cells grown in the absence of IL-7. Shown in Fig. 6 C, are forward (FSC)/side scatter (SSC) plots of D1 cells grown in the absence of IL-7 for 48 h. Cells expressing the Cdc25A WT protein, lacking IL-7, had begun to undergo apoptosis and shrink in size, as indicated by decreased FSC, whereas cells expressing Cdc25A-DP remained viable and did not undergo atrophy. Therefore, expression of Cdc25A-DP replaced the IL-7 signal for cycling and cell maintenance for 2–5 d of cytokine withdrawal, after which cells expired likely due to loss of nutrient uptake (Khaled and Durum, 2003). Untransfected cells or cells expressing the WT

protein shrink and die after 36–48 h of cytokine deprivation. As comparison, expression of the anti-apoptotic proteins, BCL-2 (Li et al., 2004) or BCL-XL (Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200409099/DC1>), though protecting cells from death, did not promote cell cycling, but rather inhibited growth and instead maintained cells in a shrunken, vegetative state, likely undergoing autophagy in the absence of cytokines.

Mutation of S75 and S123 on Cdc25A also mimicked the effect of inhibiting p38MAPK in stabilizing the Cdc25A protein after IL-3 withdrawal (Fig. 7, A and B) and IL-7 withdrawal (not depicted). Mutation of S75 alone had a partial effect of stabilizing Cdc25A (Fig. 7 B), although cell cycle progression was not restored, whereas mutation of S123 alone did not restore protein stability (not depicted). Hence, mutation of both S75 and S123 are required to deregulate Cdc25A, promoting protein stability and function in the context of cytokine deprivation in lymphocytes.

The Cdc25A-DP mutant protein was also fully competent for phosphatase activity. The DP mutant sustained phosphorylation of Rb and Cdks (Cdk2 and Cdk1) after withdrawal of IL-3 or IL-7 (Fig. 7 C). Specifically, we detected the accumulation of hyperphosphorylated Cdks at T160 and Y15, correlating with active cell cycling despite cytokine deprivation. It is possible that the increased expression of cyclin E (and perhaps cyclins D2 and D3) transcripts observed during p38 MAPK inhibition (Fig. 3 A) resulted from phosphorylated Rb which in turn would result in the release of E2F and the subsequent induction of various genes, such as cyclins, involved in cell cycle progression. Hence, expression of Cdc25A-DP was sufficient to induce cell cycling and maintain cell integrity in the absence of a cytokine growth stimulus, and this in part resulted from the activation of Cdks/cyclins and the phosphorylation of Rb.

Discussion

Lymphocytes depend on external signals from cytokines for survival and proliferation. Here we have examined the mechanisms by which IL-3 and IL-7 induce proliferation of lymphoid cell lines and primary lymphocytes and find that this pathway differs from that of better-studied factors that induce growth of mesenchymal cells. Rather than inducing synthesis of cyclins, these cytokines appear to protect lymphocytes from a stress response. Withdrawal of IL-3 or IL-7 induced cell cycle arrest through activation of a stress kinase, p38 MAPK, which occurred in the first few hours after cytokine withdrawal. p38 MAPK then directly phosphorylated the phosphatase Cdc25A at S75 and S123, targeting the phosphatase for degradation. Because Cdc25A is required to remove an inhibitory phosphate (Y15) from Cdk2, the latter kinase was inactive, failed to phosphorylate Rb and the cells arrested at the G₁-S boundary. We show that inhibiting either component of this pathway, blocking p38 MAPK activity or expressing a p38 MAPK-resistant form of Cdc25A, prevented growth arrest in the absence of a cytokine receptor signal, restoring cell cycle progression as well maintaining cell viability and size. We have produced time courses (some are shown) for most of these phenomena.

The sequence of events after cytokine withdrawal is: (1–12 h) p38 activation, Cdc25a phosphorylation and degradation; (12–48 h) G₁ arrest; (24–48 h) apoptosis. The time points selected for the figures were intended to illustrate each of these phases.

Another mechanism reported for cell cycle arrest after cytokine withdrawal is through an increase in p27^{kip1}, a negative regulator of proliferation through its interactions with Cdks, especially Cdk2 (for review see Olashaw and Pledger, 2002). Protein levels of p27^{kip1} accumulate in serum-starved and growth factor-deprived cells, and we have confirmed this increase occurs in cells deprived of IL-7 or IL-3. However, we noted that p27^{kip1} levels also increased after cytokine withdrawal in cells overexpressing Cdc25A-DP (unpublished data) that were not arrested in G₁ (Fig. 6); thus these cells replicated normally in the absence of cytokines despite their elevation in p27^{kip1}. This suggests that the positive effect of Cdc25A-DP on cell cycle progression can dominate over the negative effect of p27^{kip1}.

Microinjection of antibodies to Cdc25A have been shown to arrest cells in G₁ (Jinno et al., 1994), indicating the importance of this phosphatase in the cell cycle. Overexpression of wild-type Cdc25A in rat-1 cells accelerated entry into S phase and the activation of Cdk2 (Blomberg and Hoffmann, 1999). However, in the lymphocytes in our studies, overexpressed wild-type Cdc25A was degraded in the absence of IL-3 or IL-7 and did not restore cell cycle progression in cell lines (Figs. 5 and 6), whereas overexpression of the stable Cdc25A-DP sustained cell growth (Figs. 6 and 7). Thus, in our studies, the failure to rescue cell division with overexpressed wild-type Cdc25A in cell lines is presumably because it is rapidly degraded after phosphorylation by p38 MAPK, which is activated by cytokine withdrawal. However, in primary cells, expression of Cdc25A-WT did have growth promoting effects likely because the levels of active p38 MAPK may be reduced in comparison to the cell lines. Even in this case, however, expression of Cdc25A-DP resulted in IL-7-independent DNA synthesis beyond that observed with expression of the WT protein.

In cell lines, Cdc25A-DP supported multiple rounds of cell division in the absence of cytokine. Over the first 2 d a fivefold increase in cell numbers was observed, thereafter, cells continued to divide until the rate of cell death increased to the point that all cells were dead by day 5. The eventual death of cytokine-deprived Cdc25A-DP expressing cells could result both from apoptosis (because they cease expressing Bcl-2) and from metabolic depression because cytokine withdrawal also reduces glucose uptake as we showed for IL-7 (Khaled and Durum, 2003) and others showed for IL-3 (Plas et al., 2001).

If cytokines induce survival via Bcl-2 or Bcl-XL and proliferation via Cdc25A one might predict that transfecting both Bcl-2 and Cdc25A should render cells capable of both survival and proliferation in the absence of cytokine. However we were unable to obtain stable lines of Bcl-2/Cdc25a doubly transfected cells. This could be because, as reported by others (Janumyan et al., 2003; Cheng et al., 2004), overexpression of BCL-2 or BCL-XL, although maintaining the life of the cell, can also inhibit its replication. Or it could be due to lack of glucose uptake, as noted above; thus a cell protected by Bcl-2 or BCL-XL may be able to survive in a quiescent state when de-

prived of cytokine-induced glucose uptake, but when driven to divide by Cdc25A-DP the cell may die from metabolic stress.

Recent studies in HeLa cells showed that osmotic stress or UV irradiation induced cell cycle arrest. This arrest was accompanied by degradation of wild-type Cdc25A, but not Cdc25A-DP, however, in contrast to our studies, cell cycling was not restored by Cdc25A-DP (Goloudina et al., 2003). This suggests that multiple checkpoints, in addition to Cdc25A regulation, must be involved in the growth arrest from osmotic stress or UV irradiation and ensuing DNA damage. One such pathway would be stabilization of p53 leading to p21 induction; this would occur after UV irradiation but does not occur after cytokine withdrawal, for which we show that Cdc25A destabilization is the major mechanism of growth arrest.

CHK1 and CHK2 are also reported to phosphorylate and destabilize CDC25A. We measured levels of CHK1 in cytokine-dependent cells and found that this protein disappeared after cytokine withdrawal (Fig. S2 B). CHK2 is not as critical a regulator of Cdc25A as is CHK1. We also treated cells with CHK1 small interfering RNA and saw no effect on the G₁ arrest induced by cytokine withdrawal (Fig. 2 A). In contrast to the disappearance of CHK1, p38 activity dramatically increased after cytokine withdrawal (Fig. S2 A). These observations favor a role for p38, rather than CHK1 in the down-regulation of Cdc25A after cytokine withdrawal.

We found that p38 MAPK was required to phosphorylate S75 and S123 on Cdc25A and induce its degradation. Others have shown that, in response to DNA damage, phosphorylation of S75, followed by phosphorylation of S82 and S88, leads to β TrCP-dependent ubiquitination and degradation of Cdc25A (Donzelli et al., 2004). The kinase that phosphorylates Cdc25A at S82 and S88 is unknown, and it may be active in cytokine-deprived lymphocytes in addition to p38 MAPK as we have shown. In other cell types, others have shown that mutation of S75 alone was sufficient to confer stability to Cdc25A (Goloudina et al., 2003; Busino et al., 2004), however we found only a partial effect, whereas mutation of both S75 and S123 conferred a full stabilizing effect (Fig. 7). Mutation of both sites on Cdc25A also restored proliferation to cells deprived of cytokines (Fig. 6), whereas single mutations had little effect (not depicted), providing the strongest support for the importance of the stability of this phosphatase in regulating lymphocyte proliferation.

We have shown that cytokine withdrawal from lymphocytes results in two distinguishable responses, one after the other. First, cells undergo cell cycle arrest; second, they undergo apoptotic cell death. These two processes, cell cycle arrest and apoptosis are not only distinguishable kinetically and mechanistically, they also appear to require different levels of cytokine receptor occupancy, i.e., a low concentration of the cytokine is sufficient to protect from cell death but is insufficient to induce cell division (unpublished data). These concentration effects of cytokines, low dose inducing survival, high dose inducing division, presumably account for these two homeostatic activities of IL-7 in the peripheral immune system (Khaled and Durum, 2002). Thus, when lymphocyte density is at its maximum, cells would consume the available IL-7 as rapidly as it is synthesized and each cell would encounter just

enough IL-7 to protect from cell death but not enough to proliferate. When the lymphoid compartment is relatively empty, IL-7 would be sufficiently abundant to drive proliferation as well as survival. Our studies with lymphoid cell lines and primary cells suggest that, in vivo, this homeostatic proliferation of lymphocytes may be regulated by p38MAPK and Cdc25A and further studies to test this hypothesis are underway.

Materials and methods

Cell lines, cells, and treatments

The IL-7-dependent cell line, D1, was established from CD4⁺CD8⁻ mouse thymocytes isolated from a p53^{-/-} mouse as previously described (Kim et al., 2003). The IL-3-dependent cells are murine pro-B cell line, FL5.12A (Khaled et al., 2001b). Pharmacological inhibitors of p38 MAPK, PD169316, and SB202190 (Calbiochem), MEK1 inhibitor, PD98059 (Calbiochem), JNK Inhibitor II (Calbiochem), Cdk2 Inhibitor II (Compound 3; Calbiochem), and Cdc25 inhibitor (NSC 95397; Sigma-Aldrich) were made as 20 mM stocks in DMSO. Inhibition of Chk1 was achieved by introducing chemically synthesized small interfering RNA (Santa Cruz Biotechnology, Inc.) using a lipid reagent, *Trans-IT* TKO (Mirus) following the manufacturer's protocol. Cells were treated as described in figure legends. Lymph node cells from 12-wk-old C57Bl/6 mice were isolated by mechanical teasing and placed in culture, 5×10^6 cells/ml, in the presence of 100 ng/ml IL-7 (Peprotech) and 0.25 ng/ml Con A (Sigma-Aldrich) from 48 h before IL-7 withdrawal.

Plasmids, site-directed mutagenesis, and transfections

For dominant negative inhibition of p38 MAPK, pCMV-Flag-p38 (agf; a gift from R. Davis, University of Massachusetts Medical Center, Worcester, MA) was used to transfect cells. Overexpression of cyclin D1 was achieved with pCMV-HA-cyclin D1 (a gift from P. Kalds, National Cancer Institute at Frederick). The pCMV-HA-Cdc25 WT plasmid (a gift from J. Bartek, Danish Cancer Society, Copenhagen, Denmark) was used to generate mutants pCMV-HA-Cdc25 S75A and/or S123A, as previously described (Goloudina et al., 2003). For expression in the D1 cells, the 1.6-kb EcoRI fragment from either pCMV-HA-Cdc25 WT or pCMV-HA-Cdc25 S75,123A mutant was subcloned into pcDNA 6/V5-HisB (Invitrogen) for selection with Blastocidin. Cells were transfected by electroporation (BTX model 830) using standard methodologies. Before electroporation, cells were incubated in a hypoosmolar electroporation buffer (Eppendorf) to slightly swell the cells and improve transfection efficiency. Primary lymphocytes were isolated from lymph nodes as described, treated with IL-7 and Con A for 48 h, and transfected with *Trans-IT* Transfection reagent (Mirus) following the manufacturer's guidelines. Transfected cells (4–6%) were selected by GFP coexpression and assayed for cell cycling and DNA synthesis after 48 h with or without IL-7.

Cell cycle analysis and BrdU incorporation

For cell cycle analysis, DNA content was measured by propidium iodide (PI) or 7AAD staining. Cells, withdrawn from cytokine and/or treated with pharmacological inhibitors (as described in figure legends), were placed in detergent buffer (Kim et al., 2003) with 50 µg/ml Rnase (Invitrogen) at a concentration of $1-2 \times 10^6$ cells/ml with an equal volume of PI (50 µg/ml) or 7AAD. Samples were mixed and incubated at RT for 1 h before analysis, then assayed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest software. Listmode data was acquired and analyzed using ModFit LT software (Verity), excluding apoptotic cells and cell aggregates. DNA synthesis was assessed by BrdU incorporation, using FITC-anti-BrdU or PE-anti-BrdU antibody for detection, and 7AAD staining for DNA content with a commercially available kit following the manufacturer's instructions (BD Biosciences). BrdU incorporation in primary T cells was measured using an anti-BrdU-PE-labeled antibody, gating on the GFP-positive cells.

Immunoblotting and RPA

For detection of Cdc25A, cell lysates and the making of specific antibodies have been previously described (Goloudina et al., 2003). For analysis of phospho-Rb and phospho-Cdk2, nuclear cell lysates were made using a modified Dignam protocol containing phosphatase inhibitors and protease inhibitors (Dignam et al., 1983; Trede et al., 1993; Khaled et al., 1999a). Cells lysates were resolved by SDS-PAGE and immunoblotted. For detection of Cdc25A, mAbs were used (Ab-3, NeoMarkers). For de-

tection of the phosphorylated form of Rb, cyclin D3, and Cdk4, specific antibodies were used: a rabbit polyclonal for detecting phospho-Rb (Serine 780; Cell Signaling Technology) and mouse monoclonals for detecting cyclin D3 and Cdk4 (Cell Signaling Technology). Cdk2 was detected using a rabbit pAb (M2; Santa Cruz Biotechnology, Inc.). To detect the phosphorylated forms of Cdk2 and Cdk1, blots were probed for phospho-Cdks (Tyr15) with a rabbit pAb to phospho-Cdk1 (Tyr15; Cell Signaling Technology), which can also detect Cdk2 when catalytically inactivated by phosphorylation at Tyr15, and for phospho-Cdk (Thr160) with a rabbit pAb specific for Cdk2 and Cdk1 when activated and phosphorylated at this site (Cell Signaling Technology). Appropriate secondary rabbit or mouse antibodies cross-linked to HRP (Cell Signaling) were used for detection. ECL (Pierce Chemical Co.) was used for visualization following the manufacturer's protocol. For detection of mRNA levels for Cdks and cyclins, RPA was performed with BD Riboquant multi-probe kits, mCC-1, mCYC-1, and mCYC-2 (BD Biosciences) following the manufacturer's guidelines. For measurement of p38 activity a commercially available p38 nonradioactive kinase assay kit (Cell Signaling) was used following the manufacturer's protocol. Chk1 protein levels were determined by immunoblotting nuclear and cytosolic lysates with a specific antibody (sc8408; Santa Cruz Biotechnology, Inc.) as described above for Rb, and detection of γ -tubulin with a specific antibody (Sigma-Aldrich) was used as a loading control for nuclear lysates.

Online supplemental material

Fig. S1 is an example of IL-3 driven progression through S phase as demonstrated by BrdU incorporation. Fig. S2 demonstrates that p38 MAPK activity increases rapidly after cytokine withdrawal, whereas the protein levels of CHK1 decline. Fig. S3 shows that overexpression of cyclin D1 fails to restore S phase progression in cells deprived of IL-3. Fig. S4 displays the gating used to select and examine GFP/Cdc25-DP double transfected primary lymphocytes. Fig. S5 demonstrates that overexpression of BCL-XL, though able to protect cells from apoptosis due to IL-3 withdrawal, maintains cells in a shrunken, nonproliferative state. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200409099/DC1>.

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