Oncogene (2002) 21, 5515–5528

www.nature.com/onc

ORIGINAL PAPERS

Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells

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Mouse embryonic stem (ES) cells are known to express D-type cyclins at very low levels and these levels increase dramatically during *in vitro* and *in vivo* differentiation. Here, we investigate some of the signalling pathways regulating expression of cyclin D1 and progression to S phase, the Ras/Extracellular signal-regulated protein kinase (ERK) pathway and the phosphatidylinositol 3-kinase (PI3-kinase) pathway. We demonstrate that ERK phosphorylation is fully dispensable for the regulation of cyclin D1 level and for the progression from G1 to S phase in ES cells. By contrast, PI3-kinase activity is required for both. Differentiation induced by retinoic acid results in the gain of ERK-dependent control of cyclin D1 expression and of S phase progression. Differentiation is also paralleled by an increase in PI3-kinase activity. This leads (a) to an increase in the p70 S6 kinase-dependent regulation of the steady-state level of cyclin D1, and (b) to a concomitant decrease in the GSK3β-dependent rate of cyclin D1 degradation. Altogether, these multiple pathways account for the dramatic increase in the level of cyclin D1 protein which parallels ES cell differentiation. Our studies suggest that PI3-kinase is an important regulator of the ES cell cycle and that its activity is not regulated by mitogen stimulation.
Oncogene (2002) **21,** 5515–5528. doi:10.1038/sj.onc.1205728

Keywords: embryonic stem cell; cell cycle; cyclin D1; extracellular regulated kinase; phosphatidylinositol 3-kinase

Introduction

Embryonic stem (ES) cells are pluripotential cells derived from the inner cell mass of the blastocyst. ES cells proliferate very actively (generation time <10 h), the S phase of their mitotic cycle is largely predominant, and the G1 phase is no longer than 1.5 h (Savatier *et al*., 1994). ES cells can proliferate indefinitely while retaining pluripotency, and they can form transplantable tumours *in vivo* when they are withdrawn from their normal environment. Altogether, these observations suggest that the control of the ES cell mitotic cycle displays unusual features.

ES cells seem to lack the cyclin-dependent kinase (CDK) 4-associated kinase activity that characterizes all somatic cells. They express low cyclin D1 level, no cyclin D2 and cyclin D3, and they appear to be resistant to the growth inhibitory effect of $p16^{ink4a}$, a cyclin D:CDK4-specific inhibitor (Savatier *et al*., 1996). It is also noticeable that ES cells do not undergo growth arrest in G0 after serum starvation (Schratt *et al*., 2001) and that ES cells which harbour an inactivating mutation of both alleles of the *Grb2* gene proliferate normally and fail to differentiate (Cheng *et al*., 1998a). Taken together, these data strongly suggest that the mechanism which operates in somatic cells, and which transduces signals from tyrosine kinase receptors via the Grb2–Sos–Ras pathway and leads ultimately to control of retinoblastoma protein (Rb) phosphorylation and progression to S phase, is not engaged in ES cells.

An important player in this pathway is cyclin D1. Expression of cyclin D1 is exquisitely regulated by growth factor stimulation. GTP–Ras promotes transcription of the cyclin D1 gene through a cascade of kinases including Raf and the mitogen-activated protein (MAP) kinase ERK (extracellular signal-regulated kinase) to induce the activity of transcription factors (Moodie and Wolfman, 1994; Lavoie *et al*., 1996; Aktas *et al*., 1997). Some of these factors (AP1, ets-2) are directly involved in inducing cyclin D1 expression (Herber *et al*., 1994; Albanese *et al*., 1995; Lavoie *et al*., 1996; Aktas *et al*., 1997; Watanabe *et al*., 1998; Balmanno and Cook, 1999). Expression of cyclin D1 is also regulated by phosphatidylinositol 3-kinase (PI3-kinase)-dependent signalling pathways. On the one hand, PI3-kinase activates the p70 S6 kinase, which in turn up-regulates cyclin D1 translation via phosphorylation of the ribosomal S6 protein (Kawasome *et al*., 1998; Muise-Helmericks *et al*., 1998). On

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Received 11 January 2002; revised 25 April 2002; accepted 7 June 2002

the other hand, PI3-kinase/Akt-dependent signalling phosphorylates glycogen synthase kinase 3β (GSK3β) and suppresses its activity (Sutherland *et al*., 1993). GSK3β phosphorylates cyclin D1 on Thr286 residue thereby triggering translocation of cyclin D1 into the cytoplasm, followed by ubiquitinylation and proteosomal degradation (Diehl *et al*., 1997, 1998). Thus, the PI3-kinase-dependent signalling pathway up-regulates cyclin D1 translation and down-regulates cyclin D1 degradation, both in response to mitogenic stimuli. PI3-kinase-dependent signalling is also known to regulate the level of $p27^{kip1}$, a major inhibitor of G1 CDKs (Sherr and Roberts, 1999; Sun *et al*., 1999; Dijkers *et al*., 2000; Murillo *et al*., 2001; Brennan *et al*., 2002). By virtue of their regulation by Ras and/or PI3-kinase activities, the expression levels of cyclin D1 and of $p27^{kip1}$ are directly responsive to the extracellular growth factor environment.

In an effort to further unravel the mechanisms underlying cell cycle control in mouse ES cells, the role of MEK–ERK and PI3-kinase signalling pathways in the control of the steady-state level of cyclin D1 and of the G1/S transition were investigated.

Results

Even in the presence of saturating concentration of LIF, a small number of ES cells spontaneously differentiate *in vitro*. Therefore, in order to assess the actual level of gene expression and protein activities in self-renewing – undifferentiated – ES cells, we made use of the IOUD2 ES cell line which expresses the *LacZ–neomycin–phosphotransferase* fusion gene (*beta–geo*) driven off the *oct-4* promoter (Dani *et al*., 1998). The promoter of the *oct-4* gene is active in undifferentiated ES cells and it is switched off at the onset of differentiation *in vitro* (Mountford *et al*., 1994). Consequently, all spontaneously differentiating IOUD2 cells are killed by G418, which allows the selection of pure self-renewing ES cell populations.

ES cells do not require ERK activity for progression to S phase

We first examined the steady-state levels of GTP-bound Ras and phosphorylated ERK in undifferentiated and RA-treated IOUD2 cells (Figure 1a). IOUD2 cells express low level of GTP–Ras and this level markedly increases during differentiation induced by RA treatment. Despite low GTP-bound Ras content, the level of phosphorylated ERK is high in undifferentiated IOUD2 cells. Level of phosphorylated ERK decreases abruptly after withdrawal of LIF. Then, it accumulates during RA-induced differentiation, thereby parallels the increase in GTP–Ras level.

Burdon *et al*. (1999) have shown that inhibition of ERK signalling does not impair the propagation of ES cells. In fact, inhibition of ERK signalling stimulates self-renewal and prevents differentiation triggered by the formation of embryoid bodies. We then specifically addressed the question of whether ERK signalling is fully dispensable for progression through the G1 phase and for the G1/S transition in ES cells. IOUD2 cells were cultured in the presence of the specific MEK inhibitor PD098059 (Dudley *et al*., 1995). They were subsequently analysed for growth rate and cell cycle distribution. Treatment of undifferentiated IOUD2 cells for 4 days with 25 μM PD098059 does not result in any significant alteration in their growth rate (Figure 1b), although it induces a minor increase in the proportion of G1 cells at 24 h ($26.6 \pm 2.5\%$ of 2N cells among the PD098059-treated cells compared to $19.4 \pm 0.7\%$ among the control cells, Figure 1c). To demonstrate conclusively that progression through G1 and entry into S phase do not rely on persistent ERK activity, undifferentiated IOUD2 cells were synchronized by colcemid block and mitotic shake-off (Savatier *et al*., 1994, 2002) in the presence or in the absence of MEK inhibitor. Mitotic cells were released from colcemid block, further cultured either in the presence of MEK inhibitor or in vehicle alone, and subsequently analysed for [^{3}H]thymidine incorporation to monitor the onset of DNA replication. The rates of [^{3}H]thymidine incorporation in these two conditions are almost indistinguishable (Figure 1d), which indicates that the transit from M to S phase does not rely on persistent ERK activity in ES cells.

By contrast, treatment of RA-treated IOUD2 cells with MEK inhibitor results in a large increase in the fraction of G1 cells ($74.8 \pm 7.1\%$ of 2N cells among the PD098059-treated cells compared to $50.2 \pm 5.3\%$ among the control cells, Figure 1c) indicating that a large proportion of differentiated cells accumulate in G1 under these conditions. Analysis of growth rates indicates that MEK inhibitor induces a 50% reduction in the number of cells at all time points analysed (Figure 1b).

These data show that ERK activity is dispensable for progression to S phase in ES cells. Dependency on ERK activity resumes during differentiation.

PI3-kinase activity is required for progression to S phase in ES cells

The steady-state levels of PI3-kinase activity and of some downstream components of the PI3-kinase-dependent signalling pathways, namely PKB/Akt, p70 S6 kinase, and GSK3β were examined. Undifferentiated IOUD2 cells express basal level of PI3-kinase activity and this level increases 2.5-fold during RA-induced differentiation (Figure 2a). They also express basal levels of phospho-Akt (Thr308 and Ser473) and phospho-p70 S6 kinase (Thr389) (the active forms of the kinases). Both increase dramatically during RA-induced differentiation (Figure 2b). Differentiation is also accompanied by a moderate increase in the level of phosphorylated (inactive) GSK3β (Ser9). In contrast, levels of total PKB/Akt, total p70 S6 kinase, and total GSK3β remain constant during differentiation. Thus, the ratios of active versus inactive forms of these three kinases are significantly up-regulated during ES cell

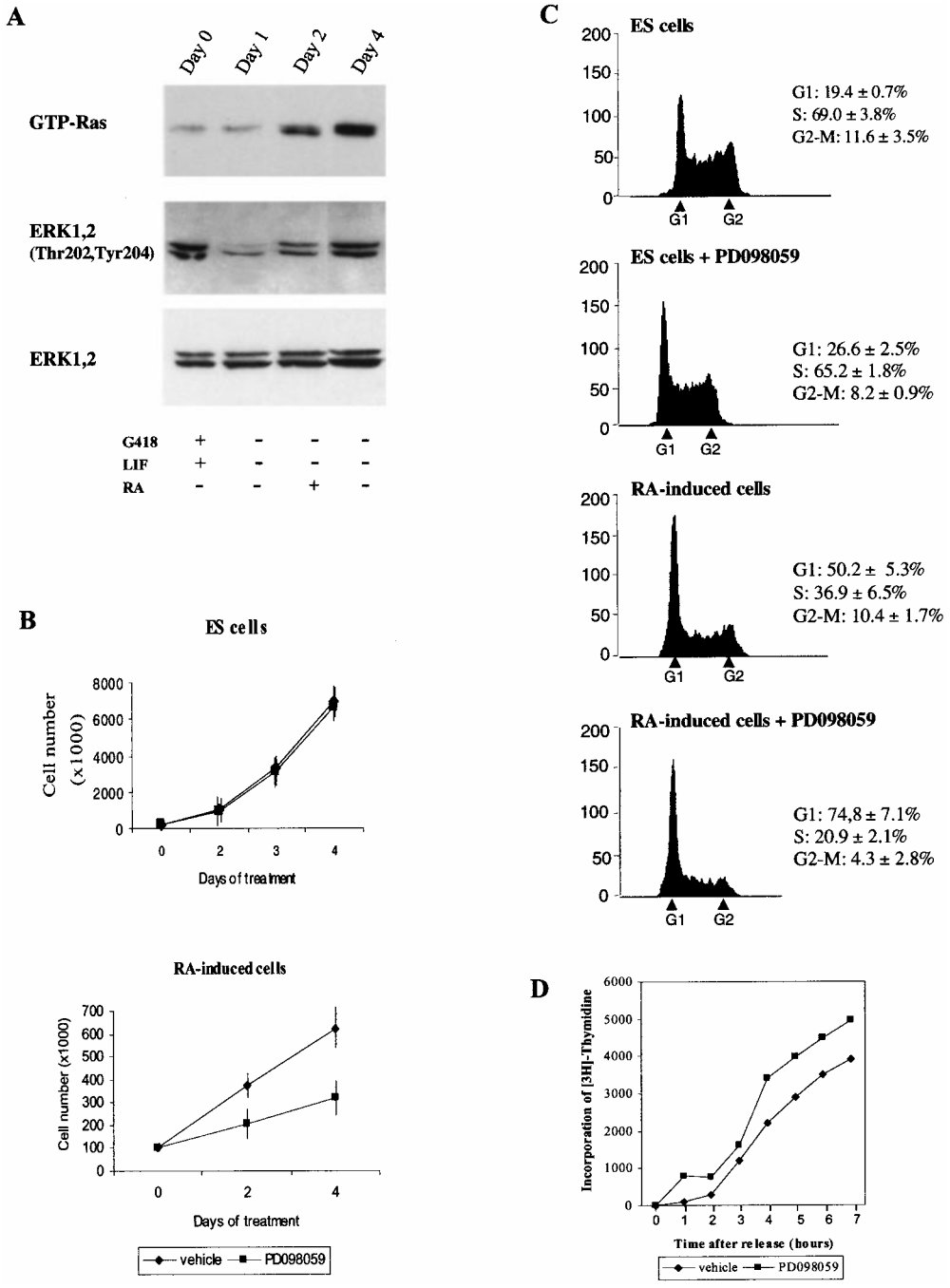

Figure 1 Ras–ERK signalling and progression to S phase in ES cells. (**a**) Analysis of the levels of GTP-bound Ras, phosphorylated ERK (Thr202, Tyr204), and total ERK. Cell lysates were incubated with GST–RBD (ras binding domain of Raf-1) fusion protein to precipitate GTP-activated Ras, or immunoblotted with anti-ERK1,2 or phosphospecific anti-ERK1,2 antibodies. (Day 0): IOUD2 cells, cultured with LIF (and selected in G418); (day 1): IOUD2 cells cultured without LIF for 24 h; (day 2): IOUD2 cells as in day 1, then treated with RA for 24 h; (day 4): IOUD2 cells as in day 1, treated with RA for 48 h then without RA for 24 h. IOUD2 cells at day 0 are undifferentiated ES cells. IOUD2 cells at day 4 are extensively differentiated (less than 1% of undifferentiated stem cells, as evidenced by visual inspection). (**b**) Analysis of growth rates. Undifferentiated IOUD2 cells [day 0 in (**a**)] were cultured with LIF and G418, plus PD098059 inhibitor (or vehicle alone) for 2, 3 and 4 days. RA-treated IOUD2 cells [day 4 in (**a**)] were cultured in medium without RA, plus PD098059 inhibitor (or vehicle alone) for 2, 3 and 4 days. Cells were counted at every time point analysed. Means and standard deviations calculated from three independent experiments are shown. (**c**) Analysis of cell cycle distribution. Undifferentiated IOUD2 cells [day 0 in (**a**)] and RA-induced IOUD2 cells [day 4 in (**a**)] were cultured for 24 h in medium containing PD098059 (or vehicle alone) and analysed for cell cycle distribution. The percentage of cells (means ± s.d.) in the G1, S and G2 phases of the cell cycle were calculated from three independent experiments, using the ModFit LT software. (**d**) Analysis of [^{3}H]thymidine incorporation in undifferentiated IOUD2 cells after synchronization and release from the colcemid block. [^{3}H]thymidine incorporation was measured at the indicated times (0, 1, 2, 3, 4 and 7 h) by incubating 10^5 cells for 30 min with 1 μCi/ml [^{3}H]thymidine before harvesting

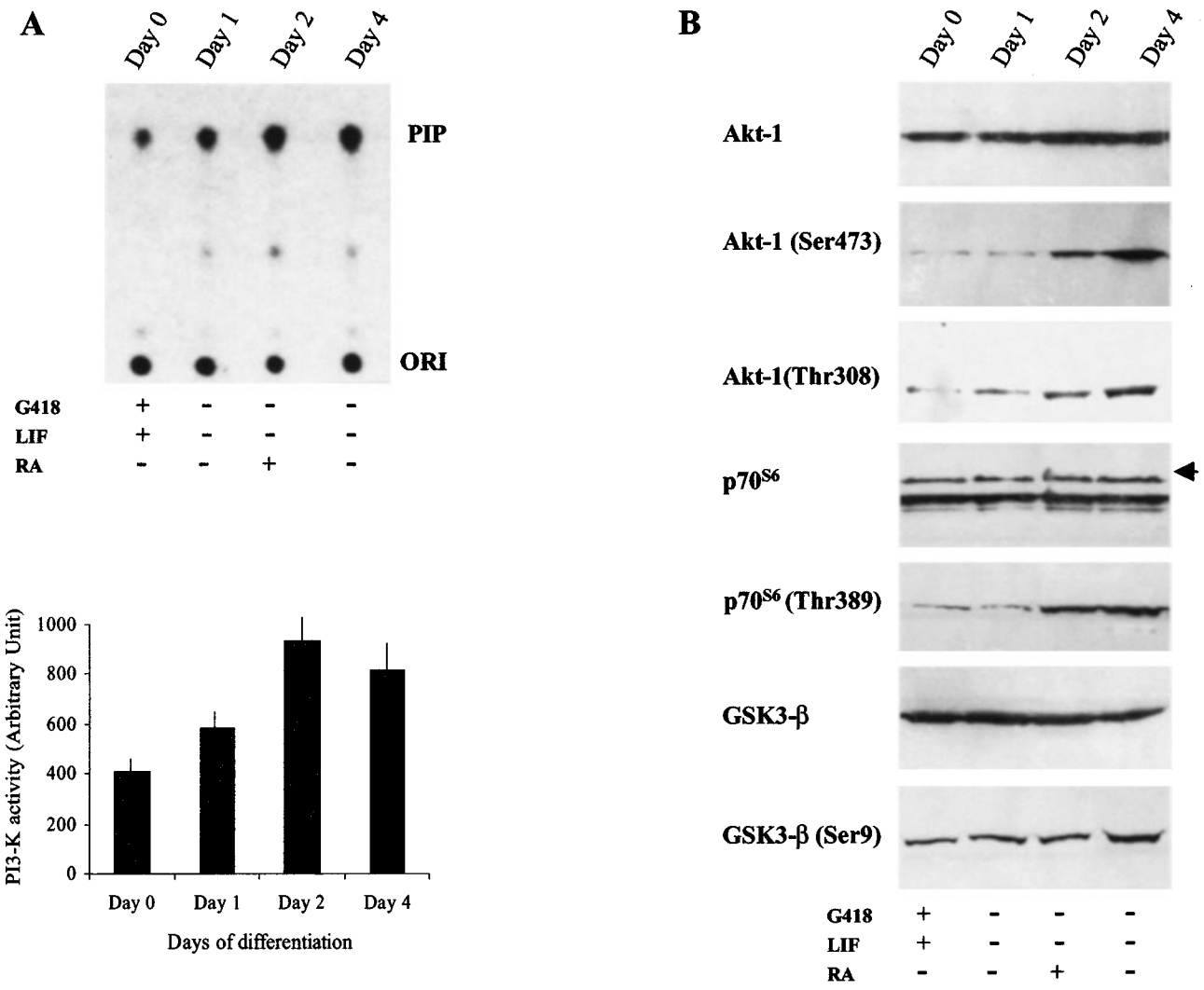

Figure 2 PI3-kinase signalling and progression to S phase in ES cells. (**a**) Analysis of PI3-kinase activity. PI3-kinase was immunoprecipitated from cell lysates with anti-p85 and assayed for kinase activity using phosphatidylinositol. Phosphorylated phosphatidylinositol (PIP) was separated by thin-layer chromatography and detected by autoradiography. Quantification of PIP was performed by means of PhosphoImager. (**b**) Analysis of downstream effectors of PI3-kinase. Protein levels were analysed by direct immunoblotting with specific antibodies to Akt, phospho-aKt(Thr308), p70 S6 kinase, phospho-p70 S6 kinase (Thr389), GSK3β and phospho-GSK3β (Ser9). (**a** and **b**) day 0, day 1, day 2, and day 4 indicate the day of harvesting after the onset of differentiation induced by withdrawal of LIF and treatment with RA as described in the legend to Figure 1

differentiation, most probably as a result of increasing PI3-kinase activity.

We next addressed the question of whether PI3-kinase-dependent signalling is required for progression to S phase in ES cells. IOUD2 cells were cultured in the presence of the specific PI3-kinase inhibitor LY294002 (Vlahos *et al*., 1994). Treatment of undifferentiated IOUD2 cells during 4 days with 25 μM LY294002 almost completely stops the growth of the cell population (Figure 3a). Some cell death is observed in the course of LY294002 treatment, evidenced by the increasing number of floating cells during the time course of the LY294002-treatment. PI3-kinase inhibition is known to induce blockade of anti-apoptotic signalling via Akt/Bad (del Peso *et al*., 1997; Negoro *et al*., 2001). Therefore these cells are likely to be apoptotic cells. To determine if LY294002 induces cell cycle arrest in non-apoptotic cells, the cell cycle distribution of the adherent cell population was examined after 24 h with LY294002. Non-adherent cells were washed out and adherent cells were subsequently harvested. Only 7% of adherent LY294002-treated cells (2% of controlled cells) are positive in the Annexin V binding assay (Van Engeland *et al*., 1996). Moreover, analysis of DNA fragmentation by agarose gel electrophoresis does not reveal any difference between LY294002-treated and control cells (data not shown). Thus, the vast majority (93%) of LY294002-treated cells are non-apoptotic in the experimental conditions used. LY294002 induces accumulation of cells in G1 (45.1 ± 3.6% of 2N cells among the LY294002-treated cells compared to

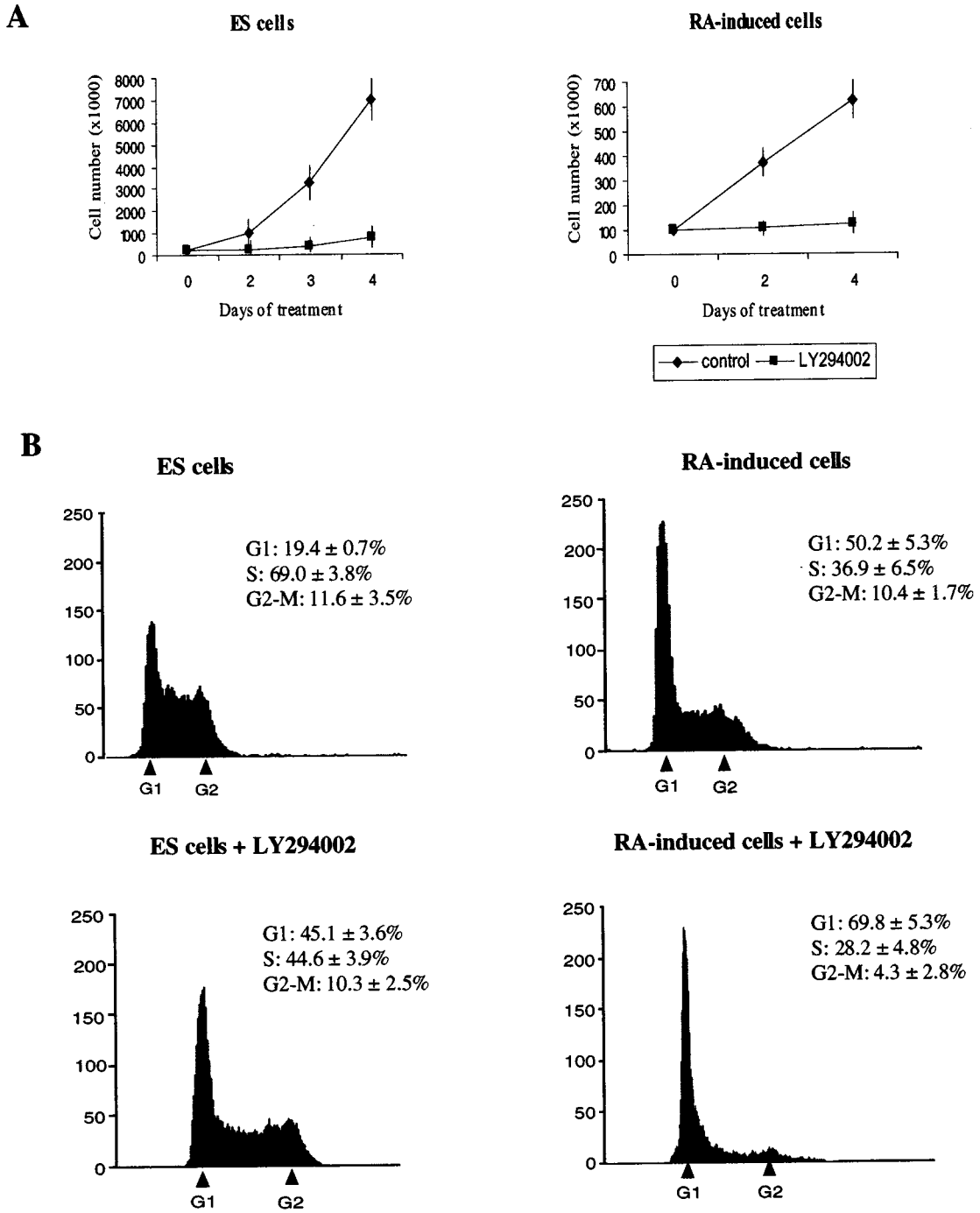

Figure 3 PI3-kinase is essential for progression to S phase in ES cells. (**a**) Analysis of growth rates. Undifferentiated IOUD2 cells (day 0) were cultured with LIF and G418, plus LY294002 inhibitor (or vehicle alone) for 2, 3 and 4 days. RA-treated IOUD2 cells (day 4) were cultured in medium without RA, plus LY294002 inhibitor (or vehicle alone) for 2, 3 and 4 days. Cells were counted at every time point analysed. Means and standard deviations calculated from three independent experiments are shown. (**b**) Analysis of cell cycle distribution. Undifferentiated IOUD2 cells (day 0) and RA-induced IOUD2 cells (day 4) were cultured for 24 h in medium containing 25 μM LY294002 (or vehicle alone) and analysed for cell cycle distribution. The percentage of cells (means$\pm$s.d.) in the G1, S and G2 phases of the cell cycle were calculated using the ModFit LT software

19.4$\pm$0.7% among the control cells, Figure 3b). After wash-out of LY294002 and propagation in inhibitor-free medium for 24 h, the IOUD2 cell population regain the cell cycle distribution characteristic of untreated control cells (data not shown). Taken together, these observations indicate that the growth arrest induced by the inhibitor LY294002 results from reversible blockade of the G1/S transition.

Treatment of RA-induced IOUD2 cells with 25 μM LY294002 has a similar effect both on the accumula-

tion of cells in G1 (69.8±5.3% of 2N cells among LY294002-treated cells compared to 50.2±5.3% among control cells, Figure 3b) and on the growth rate (Figure 3a).

Therefore, these data provide evidence that PI3-kinase signalling is required for progression to S phase both in ES cells and in their RA-induced differentiated derivatives.

Differential contributions of ERK and PI3-kinase signallings to the regulation of cyclin D1 level in ES cells

Cyclin D1 is one of multiple genes whose expression is regulated by the MEK–ERK- and PI3-kinase-dependent signalling pathways (Treinies *et al*., 1999; Marshall, 1999). Undifferentiated IOUD2 express low level of cyclin D1 and this level increases dramatically during differentiation induced by withdrawal of LIF and treatment with RA (Figure 4a). Persistent expression of cyclin D1 in IOUD2 cells, albeit at a very low level, indicates that undifferentiated ES cells do express cyclin D1 protein at basal level. In sharp contrast to the protein data, the steady-state level of cyclin d1 RNA decreases by twofold after withdrawal of LIF and it returns slightly above its original level after RA treatment (Figure 4b). Thus, the increase in cyclin D1 level that occurs during differentiation is likely to result largely from post-transcriptional regulation.

The Ras→ERK pathway is known to play a pivotal role in the regulation of cyclin D1 expression in somatic cells (Lavoie *et al*., 1996; Aktas *et al*., 1997). Therefore, we examined if ERK activity regulates cyclin D1 expression in ES cells. IOUD2 cells were cultured in the presence of the MEK inhibitor. Treatment of undifferentiated IOUD2 cells for 1 h with 25 μM PD098059 almost completely abolishes ERK phosphorylation, but it has no effect on the level of cyclin D1 protein (Figure 5a). In contrast, treatment of differentiated IOUD2 cells (RA-treated) with 25 μM PD098059 reduces both ERK phosphorylation and the level of cyclin D1 protein dramatically. It is worth noting that the steady-state level of cyclin D1 RNA remains unaffected by treatment with MEK inhibitor. Even longer treatments (up to 20 h) with PD098059 fail to reduce the level of cyclin D1 RNA (Figure 5b) in undifferentiated IOUD2 cells, whilst it slightly reduces this level in RA-induced cells. We conclude that synthesis of cyclin D1 is not dependent on persistent ERK activity in ES cells. Dependency on ERK-dependent signalling resumes in differentiating cells, and it takes place largely at the post-transcriptional level.

PI3-kinase-dependent signalling is also involved in the regulation of cyclin D1 protein level in somatic cells. On one hand, the PI3-kinase/Akt pathway phosphorylates and inactivates GSK3β, a positive regulator of cyclin D1 degradation (Diehl *et al*., 1997, 1998). On the other hand, a PI3-kinase-dependent pathway positively controls translation of cyclin D1 mRNA (Muise-Helmericks *et al*., 1998),

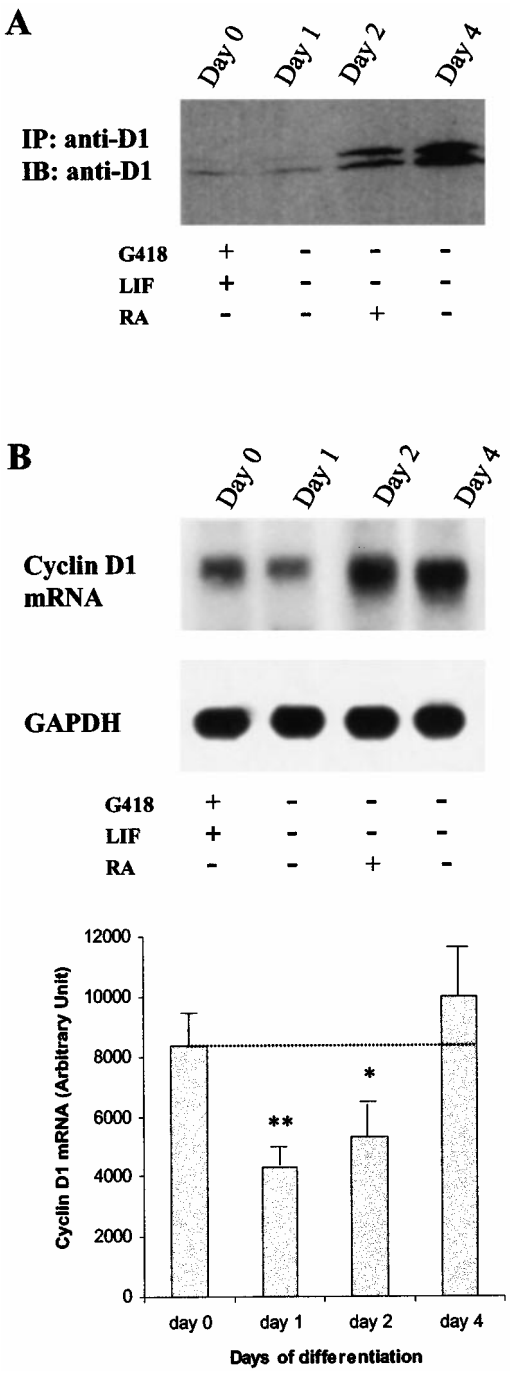

Figure 4 Variations in cyclin D1 RNA and protein levels during ES cell differentiation. (**a**) Cyclin D1 protein level in IOUD2 cells (day 0) and in their differentiated derivatives after withdrawal of LIF (day 1) and treatment with RA (day 2 and 4). Cyclin D1 was immunoprecipitated from 10^6 cells with a polyclonal antibody to cyclin D1 and revealed by immunoblotting with a monoclonal antibody to cyclin D1. (**b**) Cyclin D1 RNA level in IOUD2 cells (day 0) and in their differentiated derivatives after withdrawal of LIF (day 1) and treatment with RA (day 2 and 4). RNA levels were measured by Northern blot hybridization using specific probes for mouse cyclin D1 and GAPDH. Signals were quantified by mean of a PhosphoImager. Upper panel: Northern blot from a representative experiment; lower panel: graph showing the means±s.d. from three independent experiments. Statistical significances were calculated by means of Student *t*-test. *, $P<0.05$; **, $P<0.01$

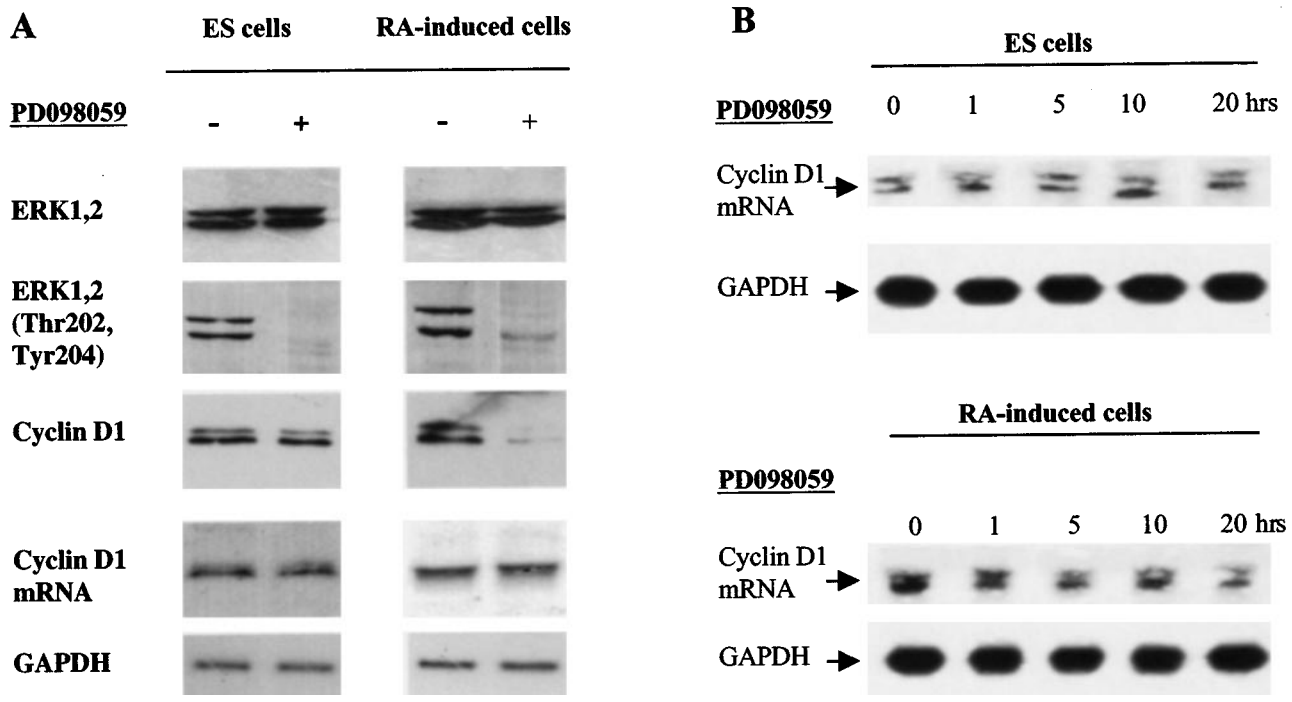

Figure 5 Regulation of cyclin D1 expression by ERK signalling. (**a**) Analysis of cyclin D1 RNA and protein levels in undifferentiated IOUD2 cells (day 0 in Figures 1–3) and RA-treated IOUD2 cells (day 4 in Figures 1–3) after a treatment with 25 μM PD098059, or with vehicle alone, for 1 h. Total cell lysates (40 μg in each lane) were immunoblotted with antibodies to ERK, phosphorylated ERK and cyclin D1. Expression of cyclin D1 mRNA was analysed by Northern blot of total RNA with a mouse cyclin D1 probe. GAPDH probe was used as internal control. (**b**) Cyclin D1 mRNA analysis in undifferentiated IOUD2 cells (day 0 in Figures 1–3) and in RA-treated IOUD2 cells (day 4 in Figures 1–3) after treatment with 25 μM PD098059, or with vehicle alone, for the indicated time

possibly via p70 S6 kinase which regulates phosphorylation of the ribosomal S6 protein (Kawasome *et al*., 1998). The PI3-kinase/p70 S6 Kinase pathway may also play a role in regulating cyclin D1 protein stability (Hashemolhosseini *et al*., 1998). To determine if PI3-kinase-dependent signalling regulates cyclin D1 expression in ES cells, IOUD2 cells were cultured in the presence of the specific PI3-kinase inhibitor LY294002 (Figure 6). Treatment of IOUD2 cells for 1 h with 25 μM LY294002 decreases levels of phosphorylated Akt and phosphorylated GSK3β, but has no detectable effect on level of phosphorylated p70 S6 kinase. PI3-kinase inhibition almost completely abolishes cyclin D1 expression in undifferentiated IOUD2 cells. In RA-induced cells, treatment with PI3-kinase inhibitor leads to complete inhibition of Akt and GSK3β phosphorylation, to partial inhibition of p70 S6 kinase phosphorylation, and abolishes cyclin D1 expression. LY294002 thus appears more efficient at inhibiting phosphorylation of Akt, p70 S6 kinase and GSK3β in RA-induced IOUD2 cells compared to undifferentiated IOUD2 cells. This difference could result from lower levels of phosphorylated isoforms in undifferentiated cells which, consequently, are closer to background levels. In contrast to the protein data, the steady-state level of cyclin D1 RNA remains unaffected by PI3-kinase inhibition in the experimental conditions used. Therefore, these data indicate that expression of cyclin D1 is post-transcriptionally dependent on persistent PI3-kinase activity both in undifferentiated ES cells and in their RA-induced derivatives.

Differential contributions of p70 S6 kinase and GSK3β to the regulation of cyclin D1 protein level

We next determined how PI3-kinase regulates cyclin D1 protein level in ES cells and in their RA-induced derivatives. Cyclin D1 protein level results from a balance between positive regulation by the PI3-kinase/p70-S6-kinase cascade (Muise-Helmericks *et al*., 1998; Hashemolhosseini *et al*., 1998; Grewe *et al*., 1999), and from negative regulation by the PI3-kinase/Akt/GSK3β cascade (Diehl *et al*., 1998). We previously observed that inhibition of PI3-kinase activity by LY294002 down-regulates cyclin D1 expression dramatically in ES cells, whilst the level of phosphorylated p70 S6 kinase remains unchanged (see Figure 6). These data suggest that p70 S6 kinase activity is not critical for regulating the level of cyclin D1 in ES cells. To determine the exact role of p70 S6 kinase activity in the control of cyclin D1 protein level in ES cells, IOUD2 cells were cultured in the presence of rapamycin, an inhibitor of p70 S6 kinase activating phosphorylation on Thr389 (Pearson *et al*., 1995; Brunn *et al*., 1996). Treatment of undifferentiated IOUD2 cells for 2 h with 30 nM rapamycin has little effect on the level of cyclin D1 protein, and this level decreases moderately at 4 h (Figure 7). By contrast, in RA-treated IOUD2 cells,

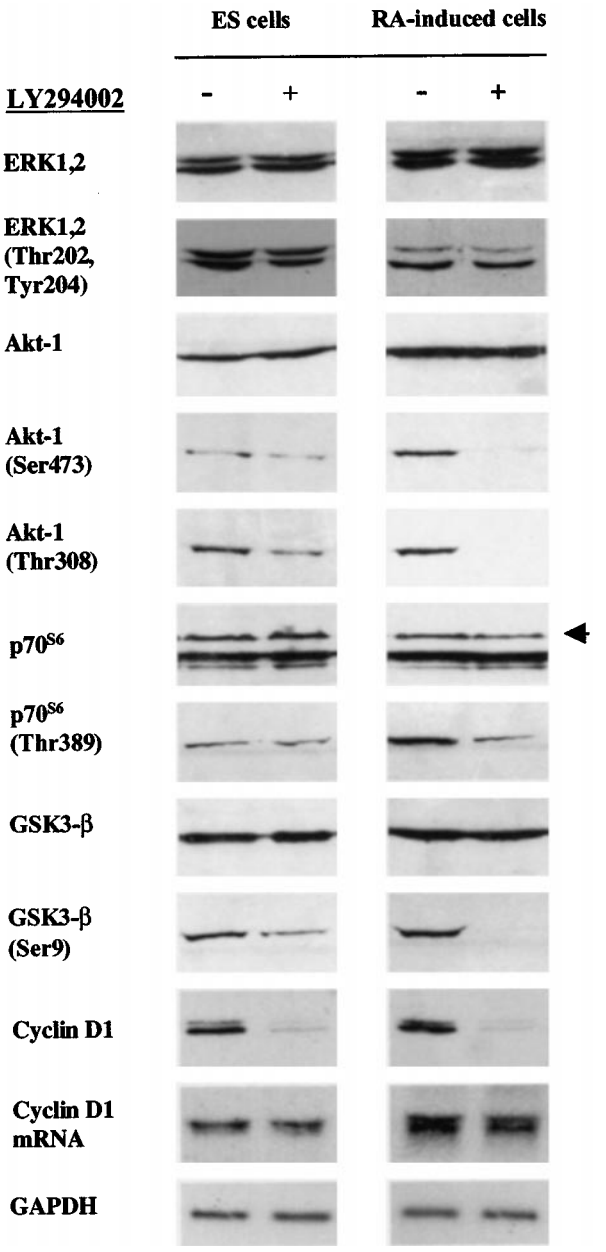

Figure 6 Regulation of cyclin D1 expression by PI3-kinase signalling. Undifferentiated IOUD2 cells (day 0 in Figures 1–3) and RA-treated IOUD2 cells (day 4 in Figures 1–3) were incubated with 25 μM LY294002, or with vehicle alone, for 1 h. Total cell lysates (40 μg in each lane) were immunoblotted with antibodies to ERK, phospho-ERK, Akt, phospho-Akt, p70 S6 kinase, phospho-p70 S6 kinase, GSK3β, phospho-GSK3β, and cyclin D1. Expression of cyclin D1 mRNA was analysed by Northern blot of total RNA with a mouse cyclin D1 probe. GAPDH probe was used as internal control

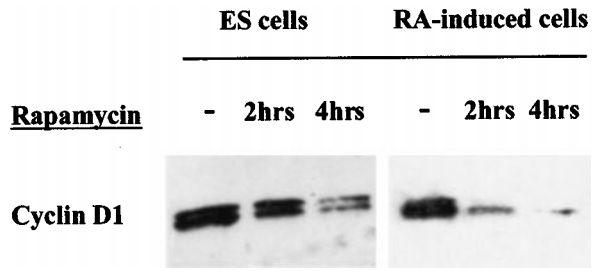

Figure 7 Sensitivity of cyclin D1 protein level to rapamycin. Undifferentiated IOUD2 cells (day 0 in Figures 1–3) and RA-treated IOUD2 cells (day 4 in Figures 1–3) were incubated with 30 nM rapamycin for the indicated times. Expression of cyclin D1 was analysed by immunoblotting of whole cell lysates (40 μg per lane)

Table 1 Cyclin D1 half-time in ES cells and RA-induced cells

	Treatment	*cyclin D1, $t_{1/2}$(min)*
ES cells	–	32±4
	LY294002 (25 M)	11±1
	PD098059 (25 M)	31±1
	LiCl (25 mM)	>180
RA-induced cells	–	82±1
	LY294002 (25 M)	46±4
	PD098059 (25 M)	54±4
	LiCl (25 mM)	>180

Undifferentiated IOUD2 cells and RA-induced IOUD2 cells were labelled with [^{35}S] methionine/cystein and cyclin D1 was detected in Materials and methods. Signals of [^{35}S] cyclin D1 were quantified by ImageQuant software. The mean half-life of cyclin D1 (±s.d.) determined for each treatment was calculated from three independent experiments

cyclin D1 level is dramatically reduced after 2 h incubation in rapamycin and it is virtually abolished at 4 h. Thus, the regulation of cyclin D1 protein level is more sensitive to rapamycin-induced inhibition of p70 S6 kinase in RA-induced cells than in undifferentiated ES cells. Together with our previous observation that the ratio of phosphorylated versus unphosphorylated p70 S6 kinase increases dramatically during ES cell differentiation, these data indicate that p70 S6K activity plays an important role in up-regulating cyclin D1 level during ES cell differentiation.

We next determined the role of GSK3β in negative control of the cyclin D1 protein level in ES cells and in their RA-induced derivatives. GSK3β activity controls phosphorylation of cyclin D1, a prerequisite to nuclear export and proteosomal degradation (Diehl *et al*., 1997, 1998). ES cell differentiation is accompanied by a moderate increase in the level of inactive (phosphorylated) GSK3β (see Figure 2b), leading to suggest that the rate of proteosomal degradation of cyclin D1 decreases as ES cells differentiate. To address this question, the half-life of cyclin D1 was calculated in undifferentiated IOUD2 cells and in their RA-induced derivatives (Table 1). We observed that the half-life of cyclin D1 in undifferentiated ES cells is approximately 2.5 times shorter (32±4 min) than the half-life in RA-treated cells (82±1 min). To determine if the increase in cyclin D1 half-life which parallels RA-induced differentiation results from the increased activity of PI3-kinase (leading to decreased activity of GSK3β, as evidenced in Figure 2b), IOUD2 cells were treated with LY294002. Inhibition of PI3-kinase activity by LY294002 reduces phosphorylation of Akt and GSK3β (see Figure 6), and it induces a 2–3-fold shortening of cyclin D1 half-life (11±1 min in undifferentiated IOUD2 cells and 46±4 min in RA-

treated cells). The reduced phosphorylation of GSK3β in response to PI3-kinase inhibition by LY294002 appears relatively modest. However, only a small fraction (approximately 25%) of the pool of GSK3β is involved in the regulation of nuclear cyclin D1 phosphorylation in fibroblasts (Diehl *et al*., 1998). Thus, a minor alteration in the level of phosphorylated versus unphosphorylated GSK3β is known to have dramatic effects on the rate of cyclin D1 degradation. Whereas inhibition of PI3-kinase by LY294002 decreases cyclin D1 stability, inhibition of GSK3β by 10 mM LiCl (Klein and Melton, 1996; Stambolic *et al*., 1996; Alt *et al*., 2000) induces a >sixfold increase in cyclin D1 half-life (>180 min). We conclude that ES cells are characterized by a higher turnover of cyclin D1 as a result of a relatively low activity of the PI3-kinase/GSK3β signalling pathway.

Regulation of cyclin D1 expression and of PI3-kinase activity by mitogen stimulation

It was previously reported that ES cells do not growth arrest in G0/G1 after serum starvation (Schratt *et al*., 2001). We thus analysed the activity of PI3-kinase and the expression of cyclin D1 in serum-starved IOUD2 cells. Undifferentiated and RA-induced IOUD2 cells were serum starved for 1–12 h. Because serum starvation induces cell death in the RA-treated cell population, murine embryonic fibroblasts (MEF) were used, in addition to RA-treated IOUD2 cells, as a paradigm of differentiated somatic cells. Both in RA-treated IOUD2 cells and in MEF, serum starvation induces progressive down-regulation of PI3-kinase activity (Figure 8a). This down-regulation is more dramatic in MEF (fivefold) than in RA-treated IOUD2 cells (twofold). Cyclin D1 protein level is also decreased in both cell types (Figure 8b). In contrast, neither the activity of PI3-kinase nor the expression of cyclin D1 are affected by serum starvation in undifferentiated IOUD2 cells during the time course of the experiment. Therefore, PI3-kinase signalling does not require persistent serum stimulation in ES cells.

Discussion

Control of G1/S transition is not dependent on ERK signalling in ES cells

We show that neither progression to S phase nor cyclin D1 expression are controlled by ERK activity in self-renewing ES cells. Burdon *et al*. (1999) have shown that inhibition of ERK signalling does not impair the propagation of ES cells. Our observation that neither the growth rate nor the length of the G1 phase are altered in presence of the MEK inhibitor confirms this finding and further demonstrates that ERK signalling is fully dispensable for cell cycle progression in ES cells. Despite low level of GTP–Ras, phosphorylated ERK is relatively abundant in ES cells and its level decreases abruptly after withdrawal of LIF, to increase again after RA-induction. High level of phosphorylated ERK in LIF-stimulated ES cells is likely to result from the heterodimerization of gp130 with the LIF receptor which induces recruitment of the phosphatase SHP-2 and activates MEK signalling (Schiemann *et al*., 1997; Takahashi-Tezuka *et al*., 1998; Burdon *et al*., 1999).

ERK signalling regulates the steady-state level of cyclin D1 at the post-transcriptional level in RA-induced ES cells

Inhibition of ERK signalling has virtually no influence on cyclin D1 mRNA in undifferentiated ES and RA-induced cells, therefore contrasts with the situation observed in many somatic cell types where ERK signalling has been unequivocally implicated in the regulation of cyclin D1 promoter activity (Albanese *et al*., 1995; Watanabe *et al*., 1998; Lavoie *et al*., 1996; Weber *et al*., 1997; Aktas *et al*., 1997; Ramakrishnan *et al*., 1998; Ravenhall *et al*., 2000). In undifferentiated ES cells, high cyclin D1 mRNA level may result from STAT3-mediated (ERK independent) activation of transcription, as it was previously observed in a pro-B cell line (Fukada *et al*., 1998) and in transformed mouse embryonic fibroblasts (Sinibaldi *et al*., 2000). Cyclin D1 protein level is also non-sensitive to MEK inhibition in ES cells. In contrast, after RA-induced differentiation cyclin D1 protein level, but not mRNA level, rises dramatically and becomes strictly dependent on ERK signalling. Thus, a post-transcriptional mechanism which is partially dependent on persistent ERK-signalling up-regulates expression of cyclin D1 during ES cell differentiation. The nature of this mechanism however remains obscure. A comparable phenomenon has been described in airway smooth muscle cells (Ravenhall *et al*., 2000). In these cells, inhibition of ERK signalling by PD098059 prevents stimulation of cyclin D1 expression by low bFGF or low thrombin concentrations, and this inhibition was shown to take place at the protein level. It was also suggested that low mitogen concentrations might negatively regulate cyclin D1 degradation by an inhibitory action of ERK signalling on the proteosome degradation pathway (Stewart *et al*., 1999; Ravenhall *et al*., 2000). In RA-treated ES cells, we observed that cyclin D1 half-life is increased by a factor of 2.5 compared to undifferentiated cells. However, this increase is not dependent on ERK signalling since it is not abolished by PD098059 treatment (Table 1). It is therefore unlikely that an inhibitory activity of ERK signalling on the proteosome degradation pathway could substantially account for the rise in cyclin D1 protein level during ES cell differentiation. Thus, we postulate an as yet unidentified role of ERK signalling in the post-transcriptional control of cyclin D1 synthesis during ES cell differentiation induced by RA.

PI3-kinase signalling plays a critical role in the G1/S transition in ES cells

ES cells express relatively low PI3-kinase activity and this activity progressively increases in the course of

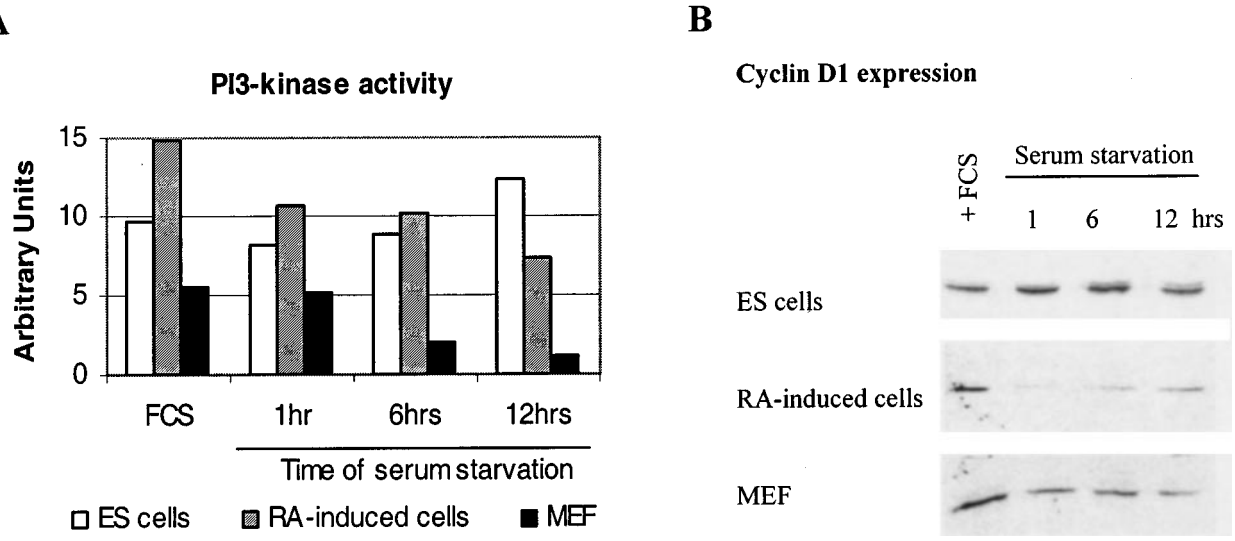

Figure 8 Regulation of cyclin D1 expression and of PI3-kinase activity by mitogens. Undifferentiated IOUD2 cells (day 0 in Figures 1–3), RA-treated IOUD2 cells (day 4 in Figures 1–3), and murine embryonic fibroblasts were cultured in serum-free medium for the indicated times. (**a**) Analysis of PI3-kinase activity. PI3-kinase was immunoprecipitated from cell lysates with anti-p85 and assayed for kinase activity using phosphatidylinositol. Phosphorylated phosphatidylinositol (PIP) was separated by thin-layer chromatography and detected by autoradiography. Quantification of PIP was performed by means of PhosphoImager. A representative experiment, of three, is shown. (**b**) Analysis of cyclin D1 protein level. Total cell lysates (40 μg in each lane) were immunoblotted with antibodies to cyclin D1

RA-induced differentiation. Inhibition of PI3-kinase activity by LY294002 is evidenced by substantial decreases in the steady-state levels of its downstream targets, phospho-Akt (Burgering and Coffer, 1995; Kulik *et al*., 1997), phospho-p70 S6 kinase (Alessi *et al*., 1997; Pullen *et al*., 1998) and phospho-GSK3β (Cross *et al*., 1995). Inhibition of PI3-kinase activity by LY294002 induces a reversible blockade of the G1/S transition. Interestingly, PTEN, a phosphatase which dephosphorylates phosphatidyl Inositol (3,4,5) tri-Phosphates (PIP3) thereby acts as a negative regulator of the PI3-kinase/Akt signalling pathway, was also shown to down-regulate cell cycle progression in ES cells. ES cells lacking PTEN function have an increased PIP3 level, exhibit an increased growth rate and display advanced entry into S phase (Sun *et al*., 1999). Taken together, these data provide conclusive evidence that PI3-kinase signalling is critical for progression to S phase in pluripotent ES cells.

Inhibition of PI3-kinase signalling by LY294002 also increases the rate of apoptosis in ES cells. Apoptosis is evidenced by DNA fragmentation and cell death (data not shown). This suggests, as previously shown in somatic cells (del Peso *et al*., 1997; Negoro *et al*., 2001; Sun *et al*., 1999), that PI3-kinase signalling provides an important cell survival signal in ES cells. Most importantly, the fraction of apoptotic cells were discarded for cell cycle and protein analyses in order to specifically analyse the role of PI3-kinase signalling in the control of the G1/S transition in non-apoptotic cells.

It is noticeable that inhibition of PI3-kinase induces only marginal decrease in levels of phospho-Akt and phospho-p70 S6 kinase in undifferentiated ES cells whereas, in the same experimental conditions, decrease is dramatic in differentiated cells (see Figure 6). This observation suggests that neither Akt nor p70 S6 kinase activity may crucially rely on PI3-kinase to regulate their activity in ES cells. Interestingly ES cells lacking PDK1, an upstream regulator of Akt and p70 S6 kinase and a downstream effector of PI3-kinase, display normal growth although they fail to activate Akt and p70 S6 kinase in response to mitogen stimulation (Williams *et al*., 2000). Taken together, these observations point to the possibility that PI3-kinase may influence ES cell growth through PDK1/Akt- and PDK1/-p70 S6 kinase-dependent pathways.

PI3-kinase signalling plays a critical role in regulating the steady-state level of cyclin D1 protein during ES cell differentiation

Regulation of cyclin D1 mRNA translation is mediated via the PI3-kinase/p70 S6 kinase pathway (Muise-Helmericks *et al*., 1998; Hashemolhosseini *et al*., 1998; Grewe *et al*., 1999), whereas regulation of cyclin D1 protein degradation is mediated via the PI3-kinase/Akt/GSK3β pathway (Diehl *et al*., 1997, 1998). The data reported here provide evidence that both pathways act in opposite directions to regulate the steady-state level of cyclin D1. First, low cyclin D1 level which characterizes ES cells results both from a relatively low rate of PI3-kinase/p70 S6 kinase-dependent synthesis and from a relatively high rate of PI3-kinase/Akt/GSK3β-dependent degradation. Second, the up-regulation of cyclin D1 protein level which takes place during ES cell differentiation results both from an increase in PI3-kinase/p70 S6 kinase-dependent synthesis and from

a concomitant decrease in the rate of PI3-kinase/Akt/GSK3β dependent degradation. Up-regulation of PI3-kinase activity that accompanies differentiation is likely to play a pivotal role in this process.

The alteration in PI3-kinase-dependent regulation of cyclin D1 protein level which is observed during ES cell differentiation is superimposed to the gain of ERK-dependent regulation. A diagram depicting the role of these different pathways in the control of cyclin D1 expression in ES cells and in their RA-induced differentiated derivatives is shown in Figure 9. Inhibition of either pathway fully abolishes expression of cyclin D1, which indicates that the two pathways act synergistically to up-regulate cyclin D1 protein level in response to mitogenic ligand stimulation during ES cell differentiation.

Does the G1/S transition rely on PI3-kinase dependent regulation of cyclin D1 expression in ES cells?

Inhibition of PI3-kinase activity by LY294002 results in a dramatic decrease in cyclin D1 level, and leads to the accumulation of ES cells in the G1 phase. In ES cells lacking the PTEN function ($PTEN^{-/-}$ ES cells), no increase in cyclin D1 expression was observed despite the accelerated G1/S transition (Sun *et al*., 1999). The reason why cyclin D1 protein level is decreased by LY294002 whereas it is not increased by the loss of PTEN function remains to be elucidated. A rate-limiting pathway could prevent the elevation of cyclin D1 level. It is also unclear whether the decreased cyclin D1 level observed in LY294002-treated cells is the cause or the consequence of the G1 arrest. We previously demonstrated that ES cells lack cyclin D : CDK4-associated kinase activity and that they are resistant to the growth-inhibitory activity of $p16^{ink4a}$, a cyclin D : CDK4/6-specific inhibitor. These data led to the proposal that the G1/S transition does not rely on a functional $p16^{ink4a}$→cyclin D : CDK4→RB : E2F pathway in ES cells (Savatier *et al*., 1996, 2002). How does PI3-kinase inhibition induce growth retardation if ES cells lack cyclin D : CDK4-associated kinase activity? First, PI3-kinase signalling is known to be involved in the regulation of $p27^{kip1}$ kinase inhibitor (Li and Sun, 1998; Dijkers *et al*., 2000; Murillo *et al*., 2001; Brennan *et al*., 2002). ES cells lacking the PTEN function display reduced $p27^{kip1}$ level and shortened cell cycle (Sun *et al*., 1999). Conversely, elevated $p27^{kip1}$ level is sufficient to induce G1 arrest (Savatier *et al*., 1996). Therefore, $p27^{kip1}$ level is rate-limiting for cell cycle progression in ES cells. Second, ES cells do contain minute amounts of protein complexes including cyclin D1, CDK4 and $p27^{kip1}$ (data not shown). Disruption of these complexes, as a result of LY294002-induced cyclin D1 degradation, might induce the reshuffling of $p27^{kip1}$ from CDK4 to CDK2 thereby triggering growth-arrest in G1 via inhibition of cyclin E : CDK2 (Sherr and Roberts, 1999).

PI3-kinase does not require serum stimulation for activity in ES cells

The finding that PI3-kinase activity is not dependent on persistent serum stimulation in ES cells allows to reconcile the observation made by Schratt *et al*. (2001). that ES cell proliferation is not dependent on serum factors with our observation that inhibition of PI3-kinase specifically blocks the G1/S transition. An alternative route which could lead to PI3-kinase activation in ES cells is the recruitment of the phosphatase SHP-2 by activated gp130 in response to LIF stimulation. SHP-2 was shown to couple to the scaffold protein Grb2-associated binder protein 1

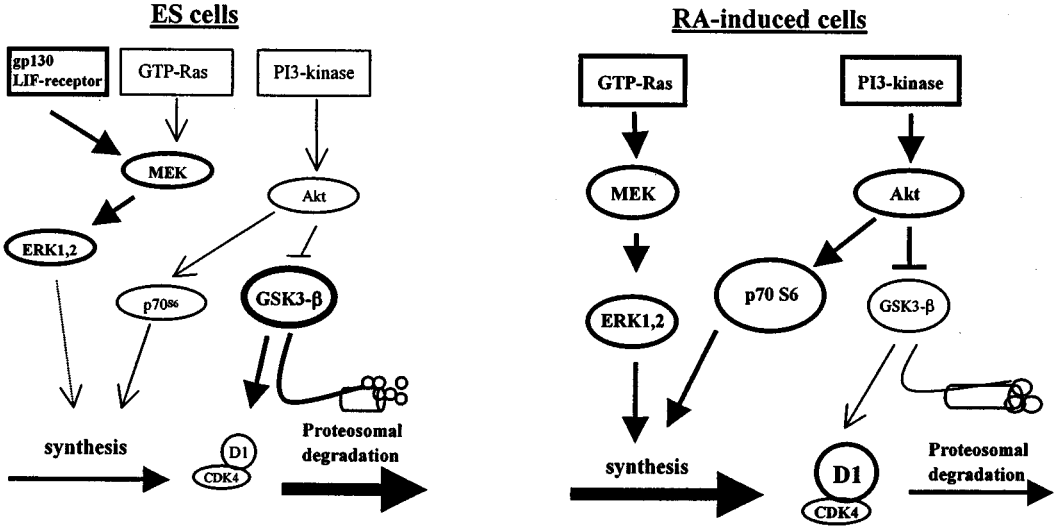

Figure 9 Schematic representation of the contribution of ERK and PI3-kinase signallings in controlling the steady-state level of cyclin D1 during ES cell differentiation induced by RA. Dotted arrows indicate regulatory pathways, which are not functional. Plain arrows indicate functional pathways. Their width reflects the role of a particular pathway in the regulation of cyclin D1 protein level in each cell type

(Gab1) and thereby to activate PI3-kinase in T47D breast cancer cells (Badache and Hynes, 2001). Should this pathway be operative in ES cells, it would connect LIF signalling to the regulation of the mitotic cycle in mouse ES cells.

Materials and methods

Cells, media, and inhibitors

ES cells were cultured in Glasgow's Modified Eagle's Medium (GMEM) supplemented with 15% foetal calf serum, 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, and 1000 U/ml LIF. IOUD2, a feeder-independent ES cell line, was used. IOUD2 cells express the *LacZ-neomycin-phosphotransferase* fusion gene (*beta-geo*) driven off the *oct-4* promoter (Dani *et al*., 1998). They were cultured in the presence of G418 (250 μg/ml) in order to kill spontaneously differentiating cells. G418 was withdrawn from culture medium 4 days prior to inducing differentiation.

Differentiation in monolayer was induced by withdrawal of LIF for 24 h, followed by treatment with retinoic acid (10^{-6} M) for 2 days. At day 3, retinoic acid was withdrawn from the culture medium and cells were further cultured for 1 day. Cells were harvested every 1 or 2 days for analysis. LY242002, PD098059, and Rapamycin were purchased from Calbiochem, Lithium Chloride (LiCl) from Sigma.

Cell synchronization

Confluent cultures were preshaken to remove the loosely attached cells. The medium was then replaced with medium containing 0.06 μg/ml Colcemid (Sigma), with MEK inhibitor or with vehicle alone, and the cells were incubated for 4 h to improve the yield of mitotic cells. The flasks were shaken again and the mitotic cells were collected for use. Collected mitotic cells were washed twice in ice-cold culture medium then cultured in Colcemid-free medium, with MEK inhibitor or with vehicle alone, to allow the cell cycle to proceed (Savatier *et al*., 1994, 2002).

Cell cycle distribution and [^{3}H]thymidine incorporation

For analysis of DNA content, cells were fixed in 70% ethanol, rehydrated in PBS, treated for 30 min with RNase A (1 mg/ml) and for 5 min with propidium iodide (1 μg/ml). Fluorescence intensity was determined by flow cytometry on a Becton Dickinson FACscan. Data acquisition was performed with the CellQuest (Becton Dickinson) software, and the percentages of G1, S and G2 phase cells were calculated with the MODFIT software program. For [^{3}H]thymidine incorporation, cells were incubated in culture medium containing 1 μCi/ml [^{3}H]thymidine (specific activity 25 Ci/mmol) for 30 min. For each sample, 10^5 cells were lysed in 0.1 N NaOH. DNA was precipitated with 10% TCA and counted with scintillation cocktail.

Northern blotting

Total RNAs were extracted using the method described by Chomczynski and Sacchi (1987). For Northern blotting, 10 μg of total RNAs were dissolved in 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 50% formamide, 5% formaldehyde and loaded on a 1% non-denaturing agarose gel. RNAs were transferred to nylon membrane and fixed by UV light. Membranes were hybridized with a [^{32}P]dCTP mouse cyclin D1-specific probe as described previously (Savatier *et al*., 1996).

Western blotting

Cells were lysed in 100 μl RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS), and cell lysates were analysed by Western blotting as described previously (Savatier *et al*., 1996). Antibody to cyclin D1 was from Pharmingen. Antibodies to Akt, phospho-Akt (Thr308), GSK3β, phospho-GSK3β (Ser9), p70 S6-kinase, phospho-p70S6-kinase (Thr389), ERK1,2, phospho-ERK1,2 (Thr202/Tyr204) were from New England Biolabs ('Phospho-Akt pathway sampler', 'PhosphoPlus p70S6 kinase', and 'PhosphoPlus p44/p42 MAP kinase' kits).

Immunoprecipitation

Cells were lysed in 50 mM HEPES pH 7.5, 150 mM NaCl, 4 mM EGTA, 1 mM EDTA, 1 mM NaF, 0.1% Tween-20, 1 mM sodium orthovanadate, 4 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and cocktail of protease inhibitors (Roche). Cyclin D1 was immunoprecipitated using a polyclonal antibody (Santa Cruz, C-20) (3 μg) bound to protein A-sepharose (25 μl). Immune complexes bound to protein A-sepharose were collected, then analysed by SDS polyacrylamide gel electrophoresis and Western blotting using a monoclonal antibody (DCS-6, Pharmingen).

GTP-Ras assay

GTP-activated Ras was quantified by virtue of its specific interaction with the ras binding domain (RBD) of Raf kinase (de Rooij and Bos, 1997). Expression of GST–RBD fusion protein in the ML21 strain of *E. coli* was induced by 1 mM IPTG, and the fusion protein was purified on glutathione–agarose beads. Cells were lysed in 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA and cocktail of protease inhibitors (Roche). Cell lysates were incubated with purified GST–RBD protein pre-bound to glutathione agarose for 30 min at 4°C. Bound proteins were eluted with SDS–PAGE sample buffer, resolved on 15% SDS–PAGE and analysed by Western blotting using monoclonal antibody to Ras (Clone Ras-10) from Upstate Biotechnology.

PI3-kinase assay

Cells were lysed in 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM PMSF and 1 mM Na_2VO_4, and PI3-kinase was immunoprecipitated with rabbit polyclonal antibody to p85 (Upstate Biotechnology). Immune complexes bound to protein A-sepharose were washed twice in phosphate buffer saline (PBS) pH 7.4, 1% NP40, 200 μM Na_2VO_4, twice in 0.5 M LiCl, 0.1 M Tris–HCl pH 7.4, and twice in 10 mM Tris–HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 200 μM Na_2VO_4. They were incubated with 10 μg of phosphatidylinositol (Sigma) and 10 μCi [^{32}P]ATP in kinase buffer (40 mM HEPES pH 7.4, 78 μM ATP, 20 mM $MgCl_2$) for 15 min at 30°C and the reaction was stopped with 250 μl of 1N HCl. After extraction with chloroform : methanol (1 : 1), the [γ^{32}P]phosphatidylinositols were separated by thin layer chromatography and detected by autoradiography.

Determination of the half-life of cyclin D1

Cells were metabolically labelled with 200 μCi/ml [^{35}S]-Methionine and [^{35}S]-Cystein (Redivue L-[^{35}S] *in vitro* cell labelling mix, Amersham) for 20 min. Chase was performed by culturing cells in cold medium for the indicated times. Cells were lysed in 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 0.4 mM NaF, 1 mM DTT and cocktail of protease inhibitors. Cyclin D1 was immunoprecipitated with an equal mixture of two monoclonal antibodies to mouse cyclin D1 (HD11 and 72-13G from Santa Cruz). Proteins were resolved by SDS–PAGE and cyclin D1 was quantified on a PhosphoImager.

Acknowledgements
We are grateful to Dr Austin Smith for the gift of IOUD2 cells and to Dr Johannes Bos for the gift of GST–RBD cDNA. This work was supported by research grants from Association pour la Recherche sur le Cancer (Nos. 5571, 7602 and 5738) and from Comités Départementaux de l'Yonne, de l'Isère et du Rhône de la Ligue Contre le Cancer. L Jirmanova is a recipient of a fellowship from the Fondation pour la Recherche Médicale (FRM).

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