cAMP-induced degradation of cyclin D3 through association with GSK-3β

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Summary

In this study we report a new mechanism whereby cyclic AMP (cAMP) regulates the cell-cycle machinery. We demonstrate that elevation of intracellular levels of cAMP promotes degradation of cyclin D3 in proteasomes, and that this occurs via glycogen synthase kinase-3β (GSK-3β)-mediated phosphorylation of cyclin D3 at Thr-283. Elevation of cAMP did not change the subcellular distribution of either cyclin D3 or GSK-3β. However, cAMP promoted the interaction between cyclin D3 and GSK-3β both in vitro and in vivo, indicating that GSK-3β-mediated phosphorylation of cyclin D3 might require the association between the two proteins. These results demonstrate how cAMP enhances degradation of cyclin D3. Furthermore, we provide evidence for a novel mechanism by which GSK-3β might phosphorylate unprimed substrates in vivo.

Key words: cAMP, Cyclin D3, GSK-3β, Degradation

Introduction

Cyclic AMP (cAMP) has been shown to mediate a variety of cellular processes, such as metabolism, cellular growth and differentiation (Shacter et al., 1988). In lymphocytes, physiological stimuli, such as catecholamines and prostaglandin E_2 (PGE_2), induce elevation of intracellular cAMP, which exerts a growth-inhibitory action and leads to accumulation of cells in the G1 phase of the cell cycle (Goodwin and Ceuppens, 1983; Johnson et al., 1981; Kammer, 1988; Simkin et al., 1987). This antiproliferative effect of cAMP has been shown to be mediated through the activity of protein kinase A type I (PKA I) (Skalhegg et al., 1992; Tasken et al., 1994). In a recent report we showed that activation of the cAMP signalling pathway in lymphoid cells led to rapid downregulation of cyclin D3 level and inhibition of cyclin-D3-cyclin-dependent kinase (CDK) kinase activity (Gutzkow et al., 2002; Naderi et al., 2000). The importance of downregulation of cyclin D3 for PKA-mediated growth suppression of T lymphocytes is underscored by the finding that overexpression of cyclin D3 partially alleviates the antiproliferative effect of PKA activity on the Jurkat T cell line (van Oirschot et al., 2001). These findings underline the importance of understanding the mechanisms by which cAMP inhibits the expression of cyclin D3.

D-type cyclins appear to be rate limiting for the progression from G1 into the S phase of the cell cycle (Ando et al., 1993; Musgrove et al., 1994). In response to mitogenic stimulation of quiescent cells, D-type cyclins are induced with delayed early kinetics and assemble into kinase complexes with CDK4 and CDK6 (Sherr, 1993). One major target of cyclin D-CDK4/6 complexes is the retinoblastoma (RB) protein (Weinberg, 1995). RB is expressed in an underphosphorylated active form through much of G1 but, as a result of cyclin-CDK activity, becomes hyperphosphorylated in late G1, resulting in release of sequestered E2F transcription factors (Kaelin, 1999). Owing to short half-lives of both its RNA and its protein, cyclin D rapidly disappears upon removal of mitogens (Matsushime et al., 1992). Furthermore, certain antiproliferative signals, such as transforming growth factor-β, are shown to inhibit the expression of cyclin D1 whereas the ectopic expression of cyclin D1 is reported to lead to shortening of G1 phase (Ko et al., 1995; Musgrove et al., 1994; Quelle et al., 1993). These observations support a role for D-type cyclins as the link connecting extracellular growth-regulatory signals to cell-cycle progression.

Expression of D-type cyclins is regulated by transcriptional and posttranscriptional mechanisms. For instance, activation of the RAS signalling pathway by extracellular mitogens has been shown to promote transcription of cyclin D1 gene (Aktas et al., 1997; Shtutman et al., 1999). In contrast, we have recently shown that activation of cAMP signalling pathway in primary human T lymphocytes reduces the rate of cyclin D3 translation (Naderi et al., 2000). D-type cyclin levels are also subject to posttranslational regulation by the proteasome-mediated protein degradation. For instance, an important mechanism of cyclin D1 regulation is via the proteasome-dependent protein degradation, an event that requires its phosphorylation by glycogen synthase kinase-3β (GSK-3β) enzyme (Diehl et al., 1997; Diehl et al., 1998). Whereas the mechanisms regulating degradation of cyclin D1 have been extensively studied, little is known about the regulation of cyclin D3 degradation. In this report, we show that GSK-3β catalyzes the phosphorylation of cyclin D3 on Thr-283, an event required for its proteasomal degradation, and demonstrate that cyclin D3 associates with

GSK-3β. Importantly, we demonstrate that induction of cAMP signalling pathway promotes association of cyclin D3 with GSK-3β, augments the activity of GSK-3β and increases the turnover of cyclin D3. Our results establish a link between GSK-3β activity and cAMP-mediated degradation of cyclin D3, and reveal a novel mechanism whereby cAMP can promote GSK-3β-mediated phosphorylation of unprimed substrates, such as cyclin D3.

Materials and Methods

Reagents and antibodies

Forskolin and PGE_2 were purchased from Calbiochem. Calf intestinal phosphatase (CIP) was obtained from Promega. 3-isobutyl-1-methylxanthine (IBMX), cycloheximide, LiCl, LLnL, MG-132, NaF, Na_3VO_4 and β-glycerophosphate were from Sigma. Recombinant Tau was purchased from Calbiochem, purified GSK-3β kinase from Upstate, pRSET and pEF1/His expression vectors from Invitrogen. Anti-cyclin D3 (DCS-22; for western blot analysis) was from MBL. Anti-cyclin D3 (AHF-0152; for immunoprecipitation) was purchased from Biosource. Anti-GSK-3β (610201) and anti-AKAP95 (610994) antibodies were obtained from Transduction Laboratories. Anti-GSK-3β (Ser-9; 9336S) antibody was obtained from Cell Signaling. Anti-actin (H-196) antibody was from Sigma. Anti-CDK4 (C-22), anti-CDK6 (C-21) and GST-RB (sc-4112) were obtained Santa Cruz Biotechnology.

Construction of expression vectors

Human cyclin D3 T283A mutant was generated in pD3-H347 plasmid (Xiong et al., 1992) using QuikChange site-diretected mutagensis kit (Stratagene) following the manufacturer's protocol. Full-length cyclin D3 (wt or T283A) cDNA with a *Bam*HI site at the 5′ end and an *Eco*RI site at the 3′ end was obtained using PCR from pD3-H347, and subcloned into the pRSET A vector (for expression in *E. coli*) or the pEF1/His A vector (for expression in Reh). PGEX-4T-1-cyclin D1 vector, expressing the C-terminal 41 amino acids of cyclin D1 with the GST moiety at its N-terminus, was kindly provided by Dr J. Alan Diehl (Diehl et al., 1998). pCMV-HA-ubiquitin plasmid was obtained as a gift from the Dr Dirk Bohmann (Treier et al., 1994).

Purification of recombinant proteins from bacteria

pRSET A-cyclin D3 (wt), pRSET A-cyclin D3 (T283A), or pGEX-4T-cyclin D1 expression vectors were transformed into *E. coli* BL21 (DE3) strain carrying the *pLysS* plasmid. Recombinant N-terminally histidine-tagged cyclin D3 (wt) and cyclin D3 (T283A) [His-cyclin D3 (wt) and His-cyclin D3 (T283A), respectively], or GST-tagged cyclin D1 (GST-cyclin D1) proteins were then expressed by addition of isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM final concentration) to exponentially growing cultures. Purification of the recombinant proteins was performed under denaturing conditions using the Ni-NTA affinity purification system (Qiagen) or Glutathione Sepharose 4B (Amersham) according to the manufacturers' protocol.

Phosphatase treatment

For in vitro dephosphorylation experiments, cells were resuspended in buffer A [50 mM Tris (pH 7.5), 1 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM PMSF, 5 μg/ml leupeptin, 0.25% aprotinin]. The samples were then incubated on ice for 10 minutes with occasional vortexing. After removal of insoluble material by centrifugation at 15,000 ***g***, 50 μg lysates were incubated with 20 U CIP in the presence or absence of protein phosphatase inhibitors (PPI, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_4). After 15 minutes at 30°C, the reactions were stopped by addition of SDS sample buffer, boiled, separated on SDS-PAGE and subjected to immunoblot analysis.

Cell culture and transfections

Purification of resting human B lymphocytes has been described previously (Naderi and Blomhoff, 1999). B cells were cultured in RPMI 1640 medium supplemented with 1% heat-inactivated fetal bovine serum (FBS; GIBCO), 2 mM glutamine, penicillin (125 U/ml) and streptomycin (125 μg/ml) at a density of 2.0×10^6 cells/ml at 37°C in a humidified incubator with 5% CO_2. Cells were stimulated to enter the cell cycle by the addition of the combination of anti-μ [37.5 μg/ml of $F(ab')_2$ fragment of rabbit polyclonal antibodies to human IgM heavy chain; Dako, Copenhagen, Denmark] and 0.005% of formaline-fixed *Staphylococcus aureus* Cowan I (SAC; Sigma). The B-lymphoid precursor cell line, Reh, was originally derived from a patient with acute lymphoblastic leukemia (Rosenfeld et al., 1977) and was kindly provided by Dr M. F. Greaves (Imperial Cancer Research Fund Laboratories, London, UK). Cells were cultured at a density between 0.2×10^6 and 1.0×10^6 cells/ml in RPMI 1640, supplemented with 10% FBS, 2 mM glutamine, penicillin (125 U/ml) and streptomycin (125 μg/ml) at 37°C in a humidified incubator with 5% CO_2. For transfection, Reh cells were washed once in OPTI-MEM and resuspended to a final concentration of 5×10^7 cells/ml. We used 400 μl of cell suspension mixed with 40 μg of the indicated expression vector and performed electroporation at 250 V and 950 μF in a 0.4 cm cuvette. After electorporation, the cells were transferred into RMPI 1640 medium and incubated for 18-20 hours before treatment.

Immunofluorescence microscopy

Cells were cytospun onto poly-L-lysine-coated coverslips. The coverslips were then washed once in cold PBS, fixed in 3% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 for 3 minutes. After washing with PBS, the coverslips were incubated with primary antibody for 1 hour at RT. The coverslips were then washed several times with PBS, and incubated with FITC- or Texas red-conjugated anti-mouse or anti-rabbit secondary antibodies and counterstained with 0.1 μg/ml Hoechst 33342. Observations were made on an Olympus AX70 epifluorescence microscope using a 100× objective, and photographs were taken with a Photonic Science CCD camera and OpenLab software (Improvision).

Subcellular fractionation

Reh cells were resuspended in buffer A [10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM PMSF, 1 mM DTT]. NP-40 (0.05%) was added, and the cells were incubated for 20 minutes on ice. The lysates were then centrifuged for 5 minutes at 200 ***g*** at 4°C, and the supernatant collected (cytosolic fraction). The nuclear fraction was obtained by sonication of the pellet in buffer A. Following analysis for protein content, 50 μg of both fractions were subjected to SDS-PAGE and examined by western blotting.

His-tagged protein pull-down assay

To determine whether His-cyclin D3 (wt) or His-cyclin D3 (T283A) proteins associate with CDK4 or CDK6, Reh cells transfected with His-cyclin D3 (wt) or His-cyclin D3 (T283A) vectors were lysed in buffer N [50 mM Tris (pH 7.5), 300 mM NaCl, 0.1% Triton X-100, 20 mM β-mercaptoethanol, 20 mM imidazole, 10 mM NaF, 10 mM β-glycerophosphate, 0.2 mM PMSF, 10 μg/ml leupeptin, and 0.5% aprotinin]. After removal of insoluble material by centrifugation, lysates containing 750 μg protein were incubated with 10 μl of a 1:1 slurry of Ni-NTA agarose beads for 2 hours at 4°C with rotation. Following incubation, the beads were washed twice with buffer N, and three times with buffer N containing 30 mM imidazole. The beads

were then resuspended in 1× SDS sample buffer, boiled for 10 minutes, fractionated on SDS-PAGE and subjected to immunoblot analysis. To determine whether cyclin D3 forms a complex with GSK-3β in vitro, His-cyclin D3 (wt)- and His-cyclin D3 (T283A)-coated Ni-NTA agarose beads or Ni-NTA agarose beads alone were incubated with 500 μg Reh cell lysates in buffer P (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% NP-40, 20 mM imidazole, 0.5 mM EDTA, 25 mM NaF, 10 mM β-glycerophosphate, 0.5 mM Na_3VO_4, 0.2 mM PMSF, 5 μg/ml leupeptin, 0.25% aprotinin). For in vivo binding assays, Reh cells expressing His-cyclin D3 (T283A) were lysed in buffer P. Cell lysates (750 μg) were incubated with 10 μl of a 1:1 slurry of Ni-NTA agarose beads. After incubation for 2 hours at 4°C, the beads were washed twice with buffer P and three times with buffer P containing 30 mM imidazole. The beads were then resuspended in 1X SDS sample buffer, boiled, separated on SDS-PAGE and subjected to western blot analysis. To determine whether cyclin D3 interacts directly with GSK-3β, Ni-NTA agarose beads bound to 2 μg His-cyclin D3 (wt) protein or Ni-NTA agarose beads alone were incubated with 0.6 μg purified GSK-3β protein in 500 μl buffer P for 2 hours at 4°C. Following incubation, the beads were washed five times with buffer P, and then resuspended in 1X SDS sample buffer, boiled, fractionated on SDS-PAGE and subjected to immunoblot analysis.

Immunoblotting and co-immunoprecipitation

For immunoblotting analysis, cells were lysed in RIPA (radioimmunoprecipitation assay) buffer, and equal amounts of protein were separated by SDS-PAGE followed by transfer to nitrocellulose membranes (Amersham). We detected proteins using standard immunoblotting procedures. For co-immunoprecipitation of cyclin D3 with GSK-3β, cells were resuspended in Triton X-100 lysis buffer [20 mM Tris (pH 7.5), 250 mM NaCl, 0.1% Triton X-100, 10 mM NaF, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.2 mM PMSF, 10 μg/ml leupeptin, and 0.5% aprotinin]. The samples were then incubated on ice and vortexed at 5-minute intervals for 20 minutes. After removal of insoluble material by centrifugation, lysates containing 300 μg protein were immunoprecipitated with 2 μg anti-cyclin D3 for 2 hours at 4°C. The immunocomplexes were then absorbed to 30 μl of 1:1 slurry of protein A-sepharose (Amersham) for 1 hour at 4°C, collected by centrifugation, and washed four times with Triton X-100 lysis buffer. The beads were then resuspended in 1× SDS sample buffer, boiled for 10 minutes, and subjected to western blot analysis. For immunoprecipitation of ubiquitinated cyclin D3, cells were lysed in RIPA buffer containing 10 mM N-Ethylmaleimide. Cell lysates (500 μg) were then subjected to immunoprecipitation with 2 μg anti-cyclin D3 antibody, followed by western blot analysis.

Kinase assays

For detection of GSK-3β activity, cells were resuspended in NP-40 lysis buffer [20 mM Tris (pH 7.5), 10 mM NaCl, 0.5% NP-40, 5 mM DTT, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 0.2 mM PMSF, 5 μg/ml leupeptin, 0.25% aprotinin]. The samples were incubated on ice for 10 minutes with occasional vortexing. After removing the insoluble material by centrifugation, lysates containing 500 μg protein were immunoprecipitated with 2 μg anti-GSK-3β for 2 hours at 4°C. The immunocomplexes were absorbed to 30 μl of 1:1 slurry of protein G-sepharose (Amersham) for 1 hour at 4°C, collected by centrifugation, and washed three times with NP-40 lysis buffer and once with kinase buffer [50 mM Tris (pH 7.5), 12.5 mM $MgCl_2$, 2 mM DTT, 0.2 mM EGTA]. The beads were then resuspended in 20 μl kinase buffer containing 100 μM ATP, 2 μg Tau or GST-cyclin D1, 10 μCi of [γ-^{32}P]ATP per reaction mixture and incubated for 20 minutes at 30°C. Reactions were stopped by addition of 10 μl 3× SDS sample buffer. The ability of GSK-3β to phosphorylate cyclin D3 was determined in 30 μl kinase buffer containing purified GSK-3β and 4 μg bacterially expressed His-cyclin D3. After incubation for 30 minutes at 30°C, the reactions were stopped by addition of 15 μl 3× SDS sample buffer. The samples were boiled for 15 minutes and subjected to SDS-PAGE. Following electrophoresis, gels were stained with Coomassie blue, dried and subjected to autoradiography. For CDK6 kinase assay, Reh cells were resuspended in CDK6 lysis buffer [50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 0.5% NP-40, 1 mM DTT, 10 mM NaF, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.2 mM PMSF, 5 μg/ml leupeptin, 0.25% aprotinin]. The samples were lysed on ice for 20 minutes and the insoluble material was removed by centrifugation. Cell lysates (600 μg) were immunoprecipitated with 2 μg anti-CDK6 antibody for 2 hours at 4°C, followed by incubation with 30 μl of 1:1 slurry of protein A-sepharose for 1 hour at 4°C. The purified immunocomplexes were then collected by centrifugation, washed three times with CDK6 lysis buffer and once with kinase buffer [50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 10 mM DTT]. The beads were then resuspended in 30 μl kinase buffer containing 20 μM ATP, 1 μg GST-RB, 10 μCi of [γ-^{32}P]ATP per reaction mixture and incubated for 30 minutes at 30°C. Reactions were terminated by addition of 15 μl 3× SDS sample buffer. The samples were boiled and subjected to SDS-PAGE. Following electrophoresis, gels were stained with Coomassie blue, dried and subjected to autoradiography.

Northern blot analysis

Total cellular RNA was isolated using RNeasy (Qiagen) as outlined by the manufacturer, and 15 μg RNA per lane was fractionated on a 1.2% formaldehyde/agarose gel. After staining the gel to verify equal loading in each lane, RNA was transferred onto Hybond-N filter (Amersham) in 20× SSC, and crosslinked by UV illumination. The filter was then hybridized with *cyclin D3* cDNA probe following the Amersham Rapid Hybridization Protocol at 65°C in a buffer containing 10% dextran sulphate, 2% SDS, 5× SSPE and 1× Denhardt's solution, washed to a final stringency of 1× SSC, 0.1% SDS and autoradiographed. *Cyclin D3* cDNA probe [*Bam*HI-*Eco*RI fragment of pEF1/His A-cyclin D3 (wt) vector] was labelled with [α-^{32}P]dCTP (Amersham Megaprime Labeling System; Amersham Biosciences) according to the manufacturer's protocol.

Results

Forskolin accelerates cyclin D3 turnover

We have previously shown that forskolin, an activator of adenylyl cyclase, inhibits proliferation of lymphoid cells by decreasing the level of cyclin D3 protein (Naderi et al., 2000). The purpose of the this study was to examine the mechanism by which cAMP promotes downregulation of cyclin D3 level in the B-precursor cell line Reh. To this end, Reh cells were cultured in the presence of 100 μM forskolin for different times and the cells were examined for the expression of cyclin D3 by northern and western blot analysis. Fig. 1A shows that forskolin did not alter the steady state levels of cyclin D3 mRNA. However, exposure of cells to forskolin led to a rapid and marked decrease in the level of cyclin D3 protein (Fig. 1B). In contrast to T lymphocytes (Naderi et al., 2000), forskolin did not significantly affect the translation of cyclin D3 in Reh cells (data not shown). Close examination of the western blot shown in Fig. 1B revealed that both control and forskolin-treated Reh cells expressed a form of cyclin D3 that exhibited a slower electrophoretic mobility. Furthermore, Fig. 1B shows that forskolin induced the level of this slower migrating form of cyclin D3 compared with untreated cells. As phosphorylation generally affects the mobility of proteins in SDS-PAGE, we wished to examine whether the forskolin-

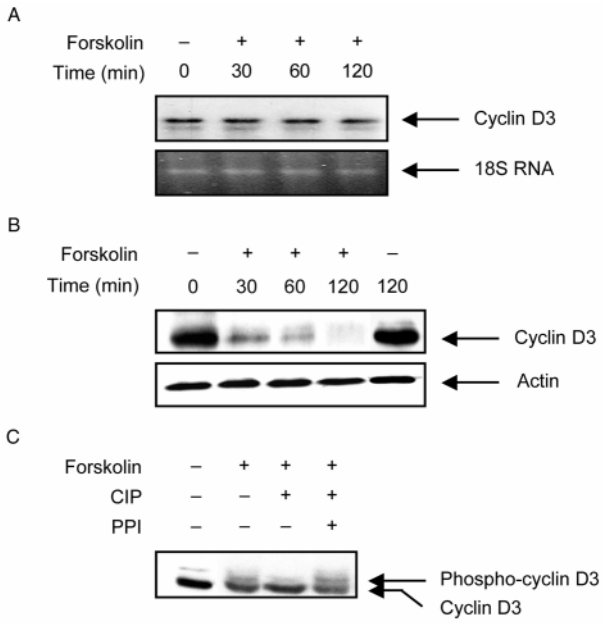

Fig. 1. Regulation of cyclin D3 expression and phosphorylation by forskolin. (A) Reh cells were cultured in the presence or absence of forskolin (100 μM) and harvested at the indicated times. Total RNA was recovered, and 15 μg of the RNA from each sample was analyzed by northern blotting using ^{32}P-labelled cyclin D3 probe as described in Materials and Methods. The ethidium bromide-stained 18S RNA was used as a loading control (lower panel). (B) Reh cells were treated as in A, lysates were prepared and then subjected to immunoblotting with antibodies against cyclin D3 and actin. The immunoblot shown is the representative of five independent experiments. (C) Reh cells were treated with or without forskolin (100 μM) for 1 hour. Cells were lysed in buffer A, and the cell lysates were then treated with CIP in the presence or absence of PPI for 15 minutes at 30°C as described in Materials and Methods. The lysates were then resolved on SDS-PAGE, and cyclin D3 was detected by immunoblotting. The immunoblot shown is the representative of four independent experiments.

induced change in the electrophoretic mobility of cyclin D3 was due to phosphorylation. To do so, lysates from untreated and forskolin-treated cells were treated with calf intestinal phosphatase (CIP). As shown in Fig. 1C, the slower migrating form of cyclin D3 from forskolin-treated cells was sensitive to CIP treatment, indicating that the change in electrophoretic mobility of cyclin D3 was due to phosphorylation.

Phosphorylation plays a critical role in regulation of protein stability (Glickman and Ciechanover, 2002). Forskolin-induced phosphorylation of cyclin D3, therefore, raised the possibility that forskolin might increase the turnover rate of cyclin D3 in Reh cells. To examine this possibility, Reh cells were treated with or without forskolin in the presence of the protein synthesis inhibitor cycloheximide in a time-course experiment and cell lysates were analyzed by immunoblotting. In the absence of forskolin, the half-life of cyclin D3 was estimated to be approximately 25 minutes (Fig. 2A,B). In the presence of forskolin, the half-life of cyclin D3 protein decreased to approximately 15 minutes. Forskolin was also found to induce degradation of cyclin D3 protein in normal human B lymphocytes (Fig. 2C). These results demonstrate that forskolin increases the turnover of cyclin D3, possibly in a phosphorylation-dependent manner.

Forskolin increases the intracellular levels of cAMP through direct activation of adenylyl cyclase. However, forskolin has also been shown to affect certain cellular processes through cAMP-independent mechanisms (Joost and Steinfelder, 1987; Laurenza et al., 1989). To determine if the inhibitory effect of forskolin on the level of cyclin D3 is mediated via cAMP, we treated Reh cells with 3-isobutyl-1-methylxanthine (IBMX) and PGE_2. IBMX induces accumulation of cAMP by inhibiting phosphodiesterases, and PGE_2 increases cAMP levels via stimulation of EP2 and EP4 receptors (Beavo et al., 1970; Beavo and Reifsnyder, 1990; Coleman et al., 1994; Negishi et al., 1993). Reh cells were cultured in the presence of various concentrations of forskolin, IBMX, or PGE_2 for 2 hours and the cells were examined for the expression of cyclin D3 by western blot analysis. As shown in Fig. 3, both forskolin and IBMX decreased the level of cyclin D3 in a dose-dependent manner. Exposure of cells to either 20 μM forskolin or 100 μM IBMX alone had only a slight effect on the level of cyclin D3. However, treatment of cells with a combination of forskolin (20 μM) and IBMX (100 μM), thus increasing cAMP production by stimulating adenylyl cyclase and inhibiting phosphodiesterases, respectively, led to a substantial reduction in the level of cyclin D3. Similarly, treatment of cells with PGE_2 led to reduction of cyclin D3 levels in a dose-dependent manner. Taken together, these observations indicate that the forskolin-induced reduction of cyclin D3 levels in Reh cells is mediated via cAMP.

Forskolin promotes cyclin D3 degradation via a proteasome-dependent pathway

Many cell-cycle proteins, such as cyclins D1, E and A, are degraded by proteasome-mediated proteolysis (Yew, 2001). Therefore, we wished to determine the involvement of proteasome function in degradation of cyclin D3, and whether proteasomal activity is required for forskolin-mediated increase in cyclin D3 turnover. Cells were treated with or without forskolin in the presence of proteasome inhibitors, MG-132 or LLnL, and examined for the level of cyclin D3 protein by western blot analysis. As shown in Fig. 4A, proteasome inhibition led to dramatic stabilization of cyclin D3 in control cells. Furthermore, blocking proteasomal activity resulted in abrogation of forskolin-mediated inhibition of cyclin D3 and led to accumulation of cyclin D3 protein in forskolin-treated cells.

Proteins that are selected for proteasomal degradation are commonly marked by polyubiquitination (Hershko and Ciechanover, 1998; Pickart, 2001). To examine whether forskolin induces ubiquitination of cyclin D3 in intact cells, Reh cells were transfected with HA-tagged ubiquitin expression plasmid. Transfected cells were then treated with forskolin and MG-132 for 2 hours, following which cyclin D3 was immunoprecipitated and immunoblotted using anti-HA antibody to detect ubiquitin-conjugated cyclin D3. Untreated cells contained a small amount of ubiquitinated proteins that precipitated with the anti-cyclin D3 antibody (Fig. 4B). Treatment of cells with forskolin led to reduction of this signal, presumably due to reduced levels of cyclin D3 at the time of analysis. MG-132 alone led to accumulation of ubiquitinated

forms of cyclin D3. Similarly, cotreatment of cells with forskolin and MG-132 dramatically increased the amount of precipitated high molecular weight ubiquitinated cyclin D3. Collectively, these result suggest that: (1) the rate of cyclin D3 degradation in untreated, proliferating cells is regulated by the ubiquitin-proteasomal protein degradation pathway; (2) forskolin accelerates cyclin D3 turnover by inducing its ubiquitination and proteasome-dependent degradation.

Lithium inhibits the forskolin-mediated degradation of cyclin D3

The basal turnover of cyclin D1 is shown to require its phosphorylation by GSK-3β (Diehl et al., 1997; Diehl et al., 1998). We therefore wished to explore the possibility that GSK-3β activity is also required for the basal and/or forskolin-induced degradation of cyclin D3. To this end, Reh cells were treated with or without forskolin in the presence or absence of the GSK-3β kinase inhibitor lithium (Klein and Melton, 1996; Ryves and Harwood, 2001; Stambolic et al., 1996), and examined for the level of cyclin D3 protein by immunoblotting (Fig. 5). Treatment of cells with lithium alone resulted in accumulation of cyclin D3 protein to a level above that present in untreated cells. Interestingly, when administered together with forskolin, lithium led to alleviation of the inhibitory effect of forskolin on cyclin D3 level. Similar results were obtained when cells were treated with another inhibitor of GSK-3β, Kenpaullone (Bain et al., 2003) (data not shown), indicating that the effect of lithium on the level of cyclin D3 is mediated through inhibition of GSK-3β. Taken together, these results indicate that GSK-3β activity is required for both basal and forskolin-induced degradation of cyclin D3.

Phosphorylation of cyclin D3 by GSK-3β

GSK-3β phosphorylates cyclin D1 on Thr-286 (Diehl et al., 1998). This residue is conserved in cyclin D3 protein (Thr-283), and the amino acid sequence surrounding Thr-283 in cyclin D3 shows a high degree of homology with that surrounding Thr-286 in cyclin D1. This observation suggested that similar to cyclin D1, cyclin D3 might be phosphorylated by GSK-3β with Thr-283 as the phospho-acceptor site. To examine this possibility, His-tagged wild-type cyclin D3 [His-cyclin D3 (wt)] was expressed in bacteria, purified by Ni-NTA agarose beads and used as a substrate for purified GSK-3β in an in vitro kinase assay. As shown in Fig. 6A, GSK-3β phosporylated His-cyclin D3 (wt). We found that phosphorylation of His-cyclin D3 (wt) in vitro by GSK-3β reached a stoichiometry of approximately 0.6 mol phosphate per mol of His-cyclin D3 (wt) (data not shown), indicating that GSK-3β effectively phosphorylates cyclin D3 in vitro. To determine whether Thr-283 in cyclin D3 is phosphorylated by GSK-3β, a bacterially produced His-tagged cyclin D3 mutant containing an Ala for Thr-283 substitution [His-cyclin D3 (T283A)] was used as a substrate in an in vitro kinase reaction with purified GSK-3β. Fig. 6B shows that GSK-3β phosphorylated His-cyclin D3 (wt) but not His-cyclin D3 (T283A). Finally, abrogation of His-cyclin D3 (wt) phosphorylation by lithium further confirmed the involvement of GSK-3β in this reaction (Fig. 6B). Taken together, these results indicate that cyclin D3 is a specific substrate for GSK-3β, and map the site of GSK-3β phosphorylation to Thr-283 in cyclin D3.

The T283A mutation renders cyclin D3 resistant to forskolin-induced degradation

The ability of GSK-3β to phosphorylate cyclin D3 at Thr-283 in vitro suggested that forskolin-stimulated degradation of cyclin D3 depends on the integrity of its Thr-283. To investigate this

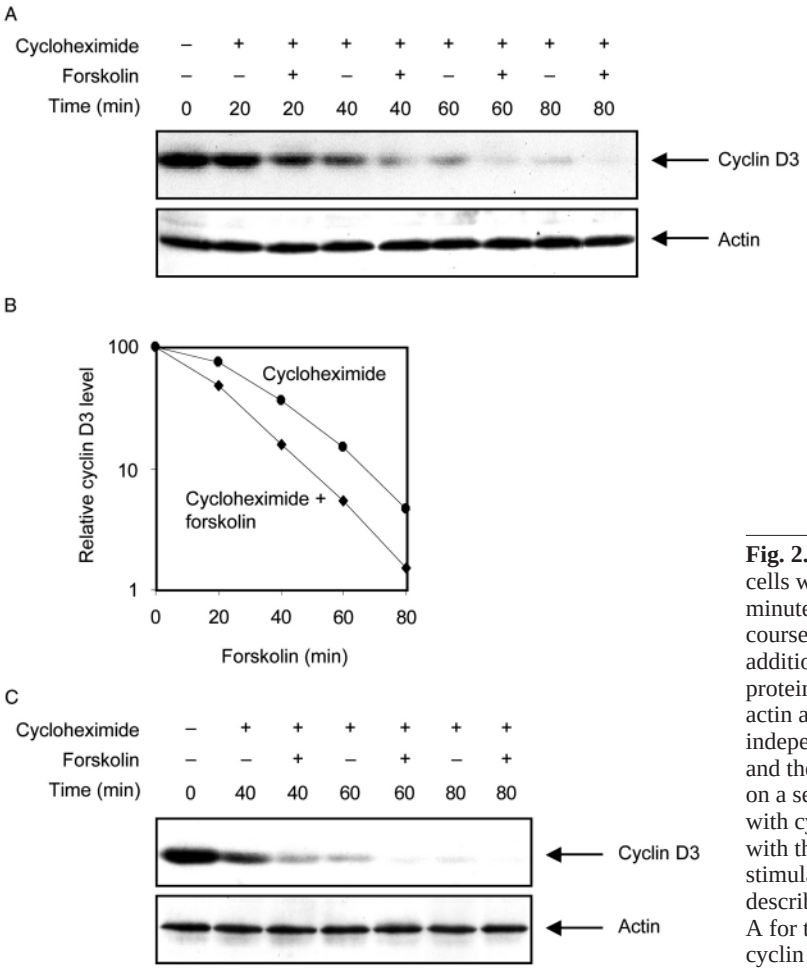

Fig. 2. Forskolin increases the rate of cyclin D3 turnover. (A) Reh cells were pretreated with cycloheximide (25 μg/ml) for 15 minutes followed by forskolin (100 μM) over an 80 minute time course. Cells were harvested at the indicated time points after addition of forskolin, lysates were prepared and equal amounts of protein were analyzed by immunoblotting with cyclin D3 and actin antibodies. The blot shown is the representative of five independent experiments. (B) The immunoblot in A was scanned and the intensity of the protein bands was quantitated and plotted on a semi-log graph with the value obtained for cells not treated with cycloheximide set as 100%. The values were normalized with those of actin. (C) Freshly isolated human B cells were stimulated with a combination of anti-μ and SAC for 32 hours as described in Materials and Methods. Cells were then treated as in A for the indicated times and analysed for the expression of cyclin D3 and actin by western blotting.

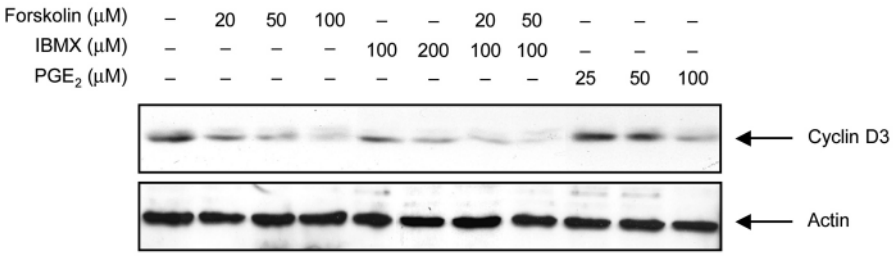

Fig. 3. Forskolin-induced reduction of cyclin D3 levels is mediated via cAMP. Reh cells were treated with the indicated concentrations of forskolin, IBMX or PGE_2 for 2 hours. Following treatment, lysates were prepared, resolved on SDS-PAGE, and then subjected to immunoblotting with cyclin D3 and actin antibodies. The blot shown is the representative of two independent experiments.

possibility, Reh cells were transfected with expression vectors encoding either His-cyclin D3 (wt) or His-cyclin D3 (T283A). Transfected cells were then treated with forskolin and examined for the level of cyclin D3 by western blot analysis. As shown in Fig. 7A, similar to endogenous cyclin D3, the level of His-cyclin D3 (wt) protein was inhibited by forskolin. By contrast, the level of His-cyclin D3 (T283A) remained virtually unchanged after exposure of cells to forskolin. To investigate whether the inability of forskolin to reduce His-cyclin D3 (T283A) levels was due to its stabilization, Reh cells transfected with His-cyclin D3 (wt) or His-cyclin D3 (T283A) vectors were treated with or without forskolin in the presence of cycloheximide and then examined for the expression of cyclin D3 by immunoblotting. His-cyclin D3 (wt) protein exhibited similar basal and forskolin-induced rate of turnover to that of endogenous cyclin D3 (Fig. 7B). In contrast, His-cyclin D3 (T283A) protein was very stable ($t_{½}$~3.5 hours) and forskolin did not affect its stability. Thus the integrity of Thr-283 is required for regulation of basal as well as forskolin-induced rate of cyclin D3 turnover.

Cyclin D3 (T283A) mutant associates with CDK4 and CDK6

Having shown that mutation of Thr-283 to Ala leads to stabilization of cyclin D3, we wished to examine whether cyclin D3 (T283A) could assemble into complexes with CDK4 or CDK6. To do so, we transfected Reh cells with the expression vectors encoding His-cyclin D3 (wt) or His-cyclin D3 (T283A) proteins. Transfected cells were then subjected to immunoprecipitation with antibodies against CDK4 or CDK6, and the immunoprecipitates were examined for bound cyclin D3 to CDK4 or CDK6 by western blot analysis. As shown in Fig. 8A, CDK4 and CDK6 immunocomplexes recovered from cells expressing His-cyclin D3 (wt) or His-cyclin D3 (T283A) contained both His-cyclin D3 and endogenous cyclin D3 proteins, indicating that His-tag or T283A mutation did not prevent the association of exogenous cyclin D3 with CDK4 or CDK6. Furthermore, this analysis revealed that the level of cyclin D3 associated with CDK6 was significantly higher than that bound to CDK4. The higher level of CDK6 expressed in

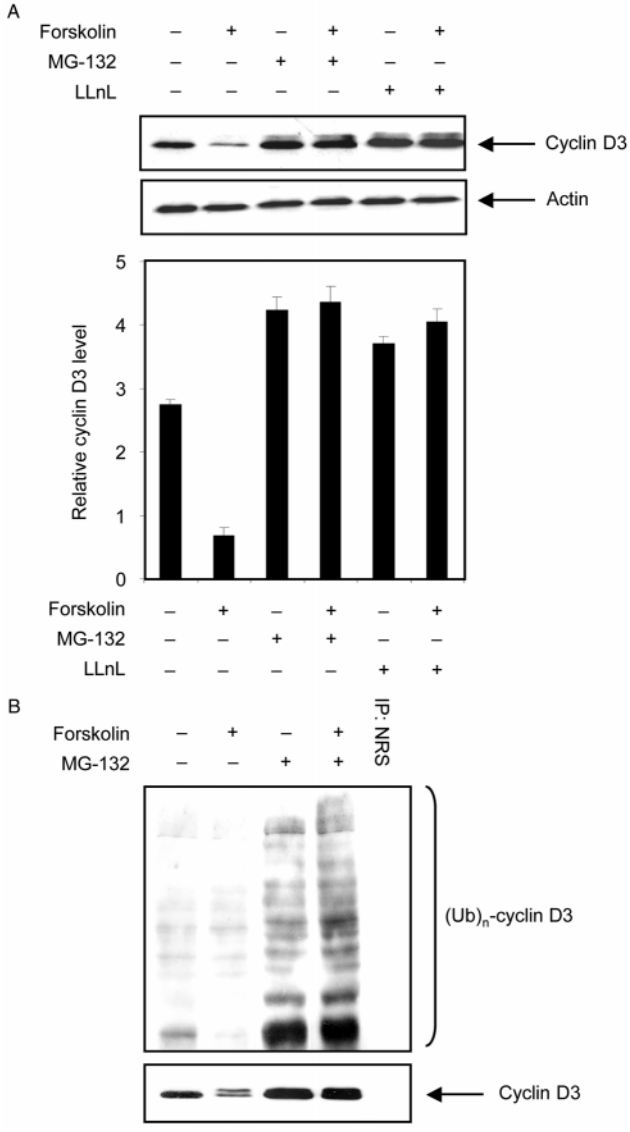

Fig. 4. Forskolin induces degradation of cyclin D3 via the ubiquitin-proteasome pathway. (A) Reh cells were pretreated with the proteasome inhibitors MG-132 (10 μM) or LLnL (100 μM) for 30 minutes before addition of forskolin (100 μM). Cells were harvested at 2 hours after addition of forskolin and total lysates were analyzed with antibodies against cyclin D3 and actin. The blot shown is the representative of four independent experiments. Lower panel, data shown represent the mean ±s.e. of the four independent experiments. The results in each experiment were quantified using a densitometer and the densitometric values of cyclin D3 were normalized with those of actin. (B) Reh cells transfected with HA-Ub vector were exposed to MG-132 (10 μM) for 30 minutes before treatment with 100 μM forskolin for 2 hours. Cells were harvested, whole cell extracts prepared, and immunoprecipitated with anti-cyclin D3 antibodies. The recovered proteins were resolved on SDS-PAGE and then subjected to immunoblotting with anti-HA and anti-cyclin D3 antibodies consecutively. The blot shown is the representative of three independent experiments. IP, immunoprecipitation; NRS, non-immune rabbit serum.

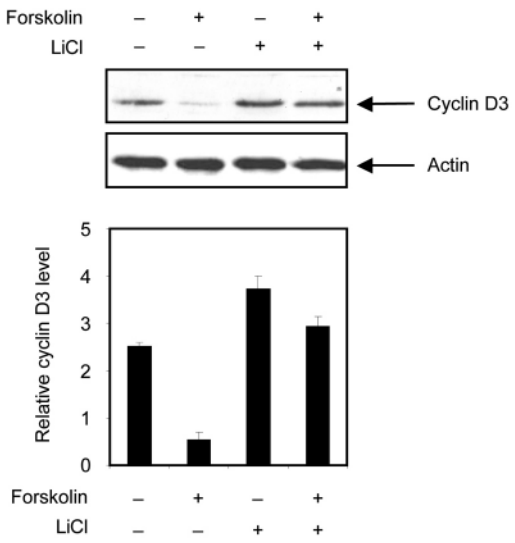

Fig. 5. Effect of GSK-3β inhibition on forskolin-induced degradation of cyclin D3. Reh cells were pretreated with the GSK-3β inhibitor LiCl (20 mM) for 30 minutes before addition of forskolin (100 μM). Cells were harvested at 2 hours after addition of forskolin and total lysates were analyzed with antibodies against cyclin D3 and actin. The blot shown is the representative of four independent experiments. Lower panel, data shown represent the mean ±s.e. of the four independent experiments. The results in each experiment were quantified using a densitometer and the densitometric values of cyclin D3 were normalized with those of actin.

Reh cells compared with that of CDK4 (Fig. 8A) could account for this observation.

To demonstrate further the association of His-cyclin D3 proteins with CDK4 and CDK6, His-cyclin D3 and its associated proteins were isolated with Ni-NTA agarose beads from Reh cell extracts that were transfected with His-cyclin D3 (wt) or His-cyclin D3 (T283A) vectors, and then blotted with antibodies against CDK4 and CDK6. As shown in Fig. 8B, both CDK4 and CDK6 were present in samples prepared from cells transfected with His-cyclin D3 (wt) or His-cyclin D3 (T283) vectors and not in the samples retrieved from mock-transfected cells. Taken together, these results demonstrate that both His-cyclin D3 (wt) and His-cyclin D3 (T283A) proteins can form complexes with endogenous CDK4 and CDK6 in vivo.

Expression of cyclin D3 (T283A) renders Reh cells resistant to forskolin-induced inhibition of CDK6

D-type cyclins assemble with either CDK4 or CDK6 to generate active holoenzymes (Sherr, 1993). This, together with the observed inability of forskolin to induce degradation of cyclin D3 (T283A) mutant, suggested that cells expressing cyclin D3 (T283A) might sustain CDK4 or CDK6 kinase activity after forskolin treatment. To examine this possibility, mock-transfected Reh cells, or cells that were transfected with His-cyclin D3 (wt), or His-cyclin D3 (T283A) vectors were treated with or without forskolin for 2 hours, and then analyzed for CDK6 kinase activity using GST-RB as substrate. CDK6 was chosen because in Reh cells, the major fraction of cyclin D3 was found to be associated with CDK6 (see Fig. 8A,B). In the absence of forskolin, cells expressing His-cyclin D3 (wt) or His-cyclin D3 (T283A) contained higher levels of CDK6 kinase activity compared with mock-transfected cells, possibly as a result of higher level of cyclin D3 (His-cyclin D3 in addition to endogenous cyclin D3) expressed in these cells (Fig. 9C). In the presence of forskolin, CDK6 kinase activity was inhibited in both mock-transfected and cells transfected with His-cyclin D3 (wt). This forskolin-induced reduction of CDK6 kinase activity correlated with the reduction of cyclin D3 levels in complex with CDK6 in these cells (Fig. 9C, lower panel). In contrast, exposure of Reh cells expressing the His-cyclin D3 (T283A) mutant led to reduction in the level of endogenous cyclin D3, and not that of His-cyclin D3 (T283) in association with CDK6. Interestingly, forskolin failed to inhibit CDK6 kinase activity significantly in these cells. Collectively, these results show that: (1) mutation of Thr-283 to Ala does not interfere with the ability of cyclin D3 to activate CDK6 in Reh cells; (2) expression of T283A mutant alleviates the inhibitory effect of forskolin on CDK6 kinase activity.

Subcellular localization of cyclin D3 and GSK-3β

The above results suggested that forskolin-mediated degradation of cyclin D3 might require its phosphorylation by GSK-3β, so it was of interest to examine whether GSK-3β has access to cyclin D3. To do so, we first examined the subcellular localization of cyclin D3 and GSK-3β before and after forskolin treatment. Immunofluorescent staining for GSK-3β showed that GSK-3β was present in both the cytosol and nuclei in untreated cells (Fig. 10A). Furthermore, localization of GSK-3β remained unchanged in cells that were treated with forskolin. To verify the data presented in Fig. 10A, subcellular

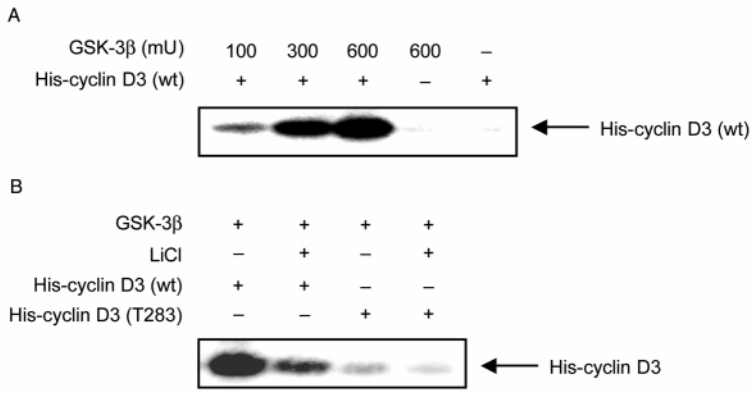

Fig. 6. Phosphorylation of cyclin D3 by GSK-3β. (A) Bacterially produced His-cyclin D3 (wt) (4 μg) was incubated with increasing concentrations of purified GSK-3β in the presence of [γ-^{32}P]ATP for 30 minutes at 30°C. Following incubation, the samples were subjected to SDS-PAGE and autoradiography. One representative experiment of three is shown. (B) Bacterially produced His-cyclin D3 (wt) (4 μg), His-cyclin D3 (T283) (4 μg), and purified GSK-3β (300 mU) were incubated in various combinations as indicated with [γ-^{32}P]ATP in the presence or absence of 50 mM LiCl for 30 minutes at 30°C. The reactions were subjected to SDS-PAGE and autoradiography. One representative experiment of three is shown.

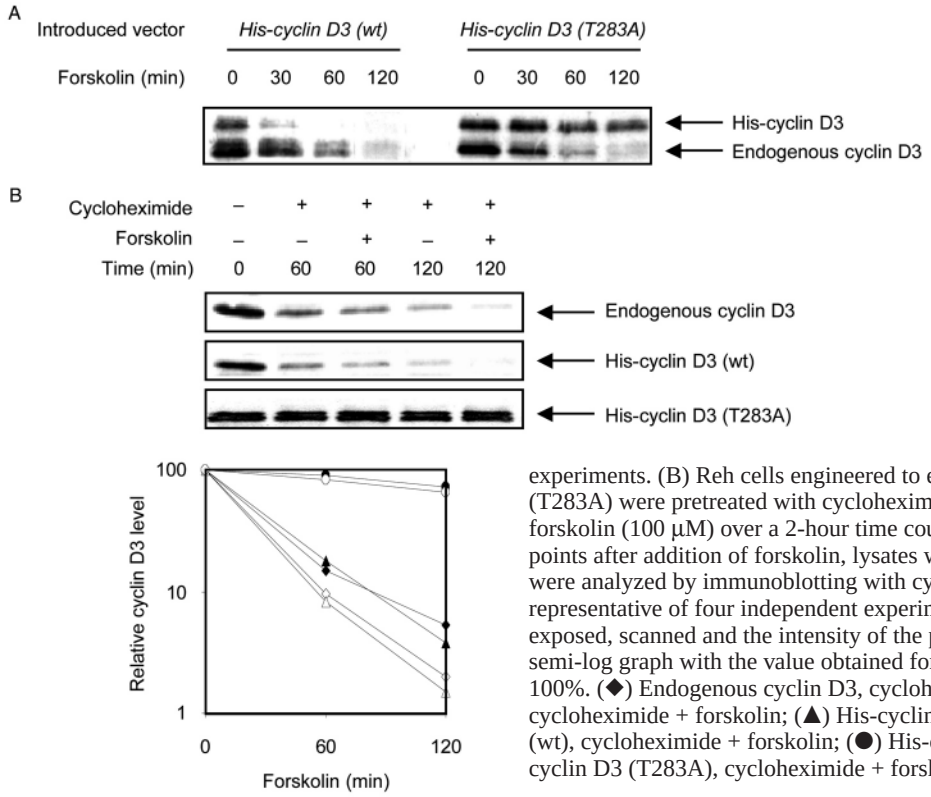

Fig. 7. Regulation of cyclin D3 stability depends on the integrity of Thr-283. (A) Reh cells transfected with His-cyclin D3 (wt) or His-cyclin D3 (T283A) vectors were cultured in the presence or absence of forskolin (100 μM) and harvested at the indicated time points. Total cell lysates were prepared and then subjected to immunoblotting with antibodies against cyclin D3. The immunoblot shown is the representative of three independent experiments. (B) Reh cells engineered to express His-cyclin D3 (wt) or His-cyclin D3 (T283A) were pretreated with cycloheximide (25 μg/ml) for 15 minutes followed by forskolin (100 μM) over a 2-hour time course. Cells were harvested at the indicated time points after addition of forskolin, lysates were prepared and equal amounts of protein were analyzed by immunoblotting with cyclin D3 antibodies. The blot shown is the representative of four independent experiments. Lower panel, the immunoblots were exposed, scanned and the intensity of the protein bands was quantified and plotted on a semi-log graph with the value obtained for cells not treated with cycloheximide set as 100%. (◆) Endogenous cyclin D3, cycloheximide; (◇) endogenous cyclin D3, cycloheximide + forskolin; (▲) His-cyclin D3 (wt), cycloheximide; (△) His-cyclin D3 (wt), cycloheximide + forskolin; (●) His-cyclin D3 (T283A), cycloheximide; (○) His-cyclin D3 (T283A), cycloheximide + forskolin.

fractions of cells that were treated with or without forskolin were subjected to immunoblotting with antibodies against GSK-3β. The purity of cytosolic and nuclear fractions was verified by immunoblotting with actin and AKAP95. Actin is present in the cytosol whereas AKAP95 is a nuclear protein (Collas et al., 1999). As shown in Fig. 10B, GSK-3β was present in both the cytosolic and nuclear fractions, and forskolin treatment of cells did not affect the subcellular distribution of GSK-3β. Examination of the subcellular localization of cyclin D3 revealed that cyclin D3 was predominantly present in nuclei and there was only a small amount in cytosol in unstimulated cells (Fig. 10A). As expected, cells treated with forskolin showed reduced cyclin D3 staining reflecting the increased turnover of cyclin D3 by forskolin. A previous report has shown that phosphorylation of cyclin D1 by GSK-3β redirects cyclin D1 to the cytosol where it is degraded by proteasomes (Diehl et al., 1998). The high degree of homology between cyclin D1 and D3, together with the observation that forskolin-mediated degradation of cyclin D3 requires phosphorylation of cyclin D3 by GSK-3β, suggested that forskolin might induce cytosolic translocation and degradation of cyclin D3. Thus, if even a portion of cellular cyclin D3 undergoes nuclear export before cytosolic degradation after treatment of cells with forskolin, proteasome inhibition should lead to accumulation of cyclin D3 within the cytosol. As shown in Fig. 10A, treatment of Reh cells with the proteasome inhibitor MG-132 led to an increase in cyclin D3 protein levels almost exclusively in the nucleus. Moreover, treatment of cells with forskolin in the presence of MG-132 also induced accumulation of cyclin D3 only within the nucleus. Taken together, these results suggest that: (1) cyclin D3 and GSK-3β colocalize in the nucleus of both untreated and forskolin-treated cells; (2) both basal- and forskolin-mediated degradation of cyclin D3 appears to occur in the nucleus, and is not preceded by translocation of cyclin D3 into cytosol.

In vivo and in vitro interaction between cyclin D3 and GSK-3β

Next, we sought to examine whether cyclin D3 associates with GSK-3β, and whether forskolin treatment of cells affects this association. To do so, we first examined the interaction of cyclin D3 with GSK-3β in intact cells. Cyclin D3 was immunoprecipitated from control or forskolin-treated Reh cells, and the immunprecipitates were then subjected to immunoblot analysis with antibodies against cyclin D3 and GSK-3β. As shown in Fig. 11A, the time and magnitude of changes in the levels of immunoprecipitated cyclin D3 paralleled those of total cyclin D3 (see Fig. 1B). Interestingly, GSK-3β was detected in the cyclin D3 immune complexes retrieved from untreated cells. Furthermore, the level of GSK-3β co-immunoprecipitated with cyclin D3 from lysates of cells treated with forskolin did not follow the level of cyclin D3, but remained essentially constant. Normalization of the densitometric values of GSK-3β with those of cyclin D3 showed that cyclin D3 complexes immunoprecipitated from forskolin-treated cells contained an increased amount of GSK-3β relative to that of the untreated cells. This increase was already apparent at 30 minutes and had reached its maximum of approximately 2.5 fold (by comparison with untreated cells) 120 minutes after forskolin treatment. These results indicate that: (1) cyclin D3 forms a complex with GSK-3β in intact cells at the endogenous level; (2) forskolin promotes the interaction between cyclin D3 and GSK-3β.

To demonstrate further that cyclin D3 associates with GSK-3β, we performed an in vitro pull-down assay. Lysates from cells treated with or without forskolin were incubated with agarose beads containing Ni alone, Ni-His-cyclin D3 (wt), or Ni-His-cyclin D3 (T283A) proteins. The beads were then isolated from the lysates and blotted with antibodies against GSK-3β. As shown in Fig. 11B, GSK-3β immunoreactivity was present in the samples incubated with Ni-His-cyclin D3 (wt), or Ni-His-cyclin D3 (T283A) beads and not in the samples incubated with Ni-beads alone, demonstrating an interaction between cyclin D3 (wt) or cyclin D3 (T283A) with GSK-3β. Moreover, this interaction showed approximately a threefold increase in lysates from forskolin treated cells. Consistent with the observations in intact cells, these results indicate that cyclin D3 (wt) and cyclin D3 (T283A) interact with GSK-3β, and that this interaction is subject to regulation by forskolin.

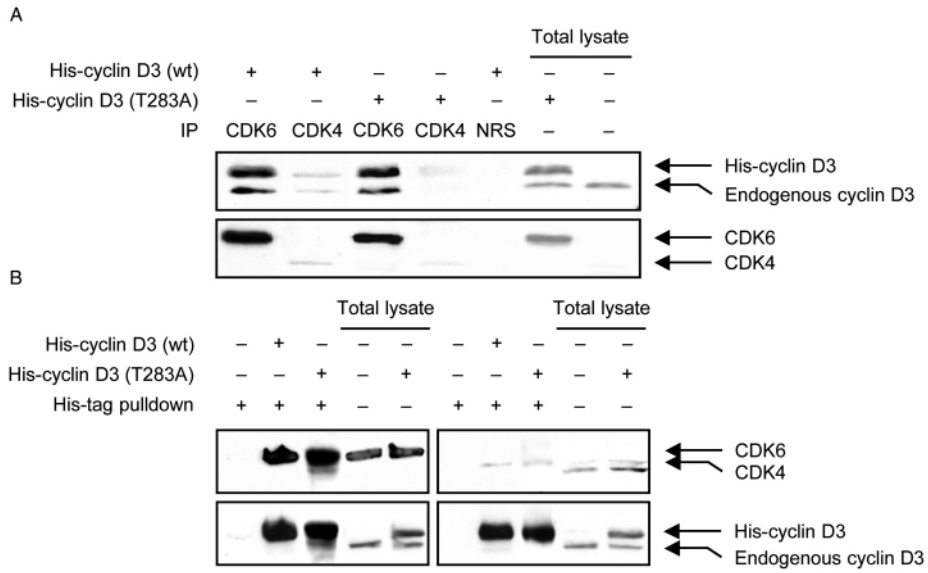

Fig. 8. His-cyclin D3 (T283A) associates with CDK4 and CDK6. (A) Reh cells were transfected with expression vectors encoding His-cyclin D3 (wt) or His-cyclin D3 (T283A). Following transfection, whole cell extracts were prepared and immunoprecipitated (IP) with antibodies against CDK4, CDK6 or with non-immune rabbit serum (NRS). The recovered proteins were resolved on SDS-PAGE and then subjected to immunoblotting with anti-CDK4, CDK6, or cyclin D3 antibodies. As positive control for CDK4, CDK6, and cyclin D3, 50 μg total cell lysates prepared from Reh cells and cells expressing His-cyclin D3 (T283A) were analysed in parallel (total lysate). (B) Reh cells were mock-transfected or transfected with His-cyclin D3 (wt) or His-cyclin D3 (T283A) vectors. Following transfection, whole cell extracts were prepared and incubated with Ni-NTA agarose beads to isolate His-cyclin D3 and its associated proteins. The recovered proteins were then separated by SDS-PAGE and immunoblotted with anti-CDK4, CDK6, or cyclin D3 antibodies. As positive control for CDK4, CDK6, and cyclin D3, 50 μg total cell lysates were prepared from mock-transfected Reh cells, and cells expressing His-cyclin D3 (T283A) were analysed in parallel (total lysates).

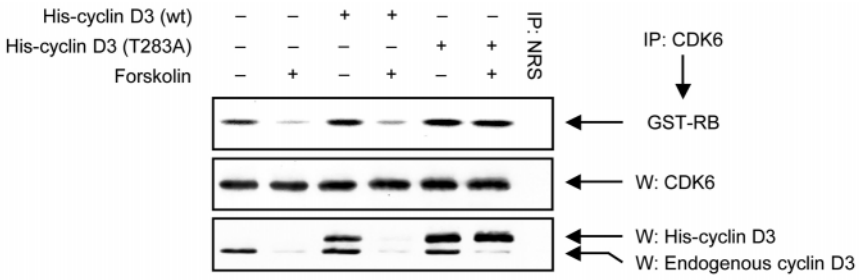

Fig. 9. His-cyclin D3 (T283A) expression abrogates the inhibitory effect of forskolin on CDK6 kinase activity. Mock-transfected Reh cells or cells that were transfected with His-cyclin D3 (wt) or His-cyclin D3 (T283A) vectors were treated with or without forskolin (100 μM). Two hours after treatment cells were harvested and lysed. One aliquot of each whole cell extract (600μg) was immunoprecipitated (IP) with 2 μg anti-CDK6 antibody or non-immune rabbit serum (NRS) and utilized for an in vitro CDK6 kinase assay (upper panel). A second aliquot of each whole cell extract was immunoprecipitated with anti-CDK6 antibody or NRS. The recovered proteins were separated on SDS-PAGE and then subjected to western blotting (W) with CDK6 (middle panel) or cyclin D3 (lower panel) antibodies. The results shown are representative of three independent experiments.

The interaction between cyclin D3 and GSK-3β could be direct or it could occur indirectly through an ancillary protein. To determine whether cyclin D3 could directly interact with GSK-3β, purified GSK-3β was incubated with agarose beads containing Ni alone or Ni-His-cyclin D3 (wt) protein. The beads were then isolated and subjected to western blotting with antibodies against GSK-3β. As shown in Fig. 11C, GSK-3β immunoreactivity was present in the samples incubated with Ni-His-cyclin D3 (wt) and not in the samples incubated with Ni-beads alone. This result indicates that cyclin D3 can interact directly with GSK-3β in vitro.

As shown above, forskolin inhibits the expression of cyclin D3 (wt) but not that of cyclin D3 (T283A). This together with the finding that cyclin D3 (T283A), similar to cyclin D3 (wt), can bind to GSK-3β led us to reason that forskolin should lead to accumulation of cyclin D3 (T283A)-GSK-3β complexes in the cell. To examine this idea, Reh cells transfected with His-cyclin D3 (T283A) vector were treated with or without forskolin. His-cyclin D3 (T283A) and its associated proteins were isolated from cell extracts with Ni-NTA agarose beads, and then blotted with antibodies against cyclin D3 and GSK-3β. As expected, the level of cyclin D3 (T283A) remained unchanged after exposure of cells to forskolin (Fig. 11D). However, the level of GSK-3β co-precipitated with cyclin D3 (T283A) increased after treatment of cells with forskolin for 120 minutes. Densitometric analysis revealed that forskolin induced an approximately threefold increase in the association between cyclin D3 (T283A) and GSK-3β. This result further demonstrates that forskolin stimulates the association between cyclin D3 and GSK-3β in vivo.

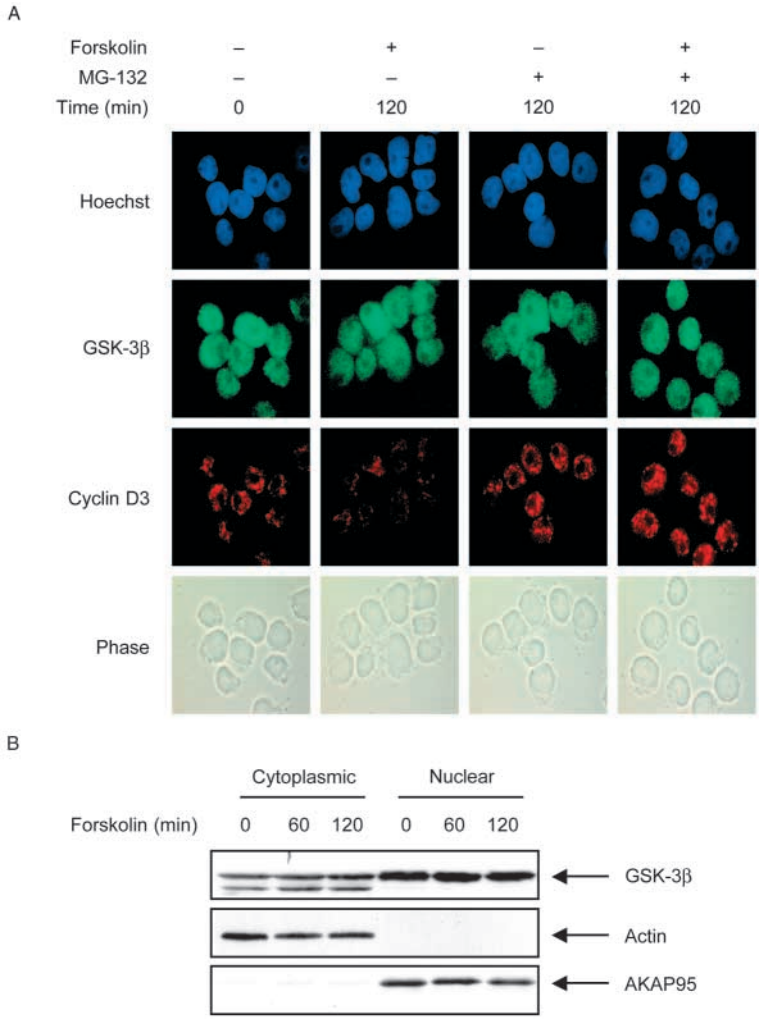

Fig. 10. Analysis of subcellular localization of cyclin D3 and GSK-3β. (A) Reh cells were pretreated with the proteasome inhibitor MG-132 (10 μM) for 30 minutes before addition of forskolin (100 μM). Cells were cytospun onto coverslips at 2 hours after addition of forskolin, fixed in paraformaldehyde, and subjected to immunofluorescence microscopy after staining with cyclin D3 or GSK-3β antibodies. The nuclear and cellular morphologies were visualized by Hoechst staining and phase contrast microscopy, respectively. One representative experiment of four is shown. (B) Reh cells were cultured in the presence or absence of 100 μM forskolin and harvested at the indicated time points. Subcellular fractions were prepared as described in Materials and Methods. Equal amounts of protein were resolved by SDS-PAGE and detected by immunoblotting with antibodies against GSK-3β, actin and AKAP95. The blot shown is the representative of three independent experiments.

Regulation of GSK-3β kinase activity by forskolin

Having shown that cyclin D3 interacts with GSK-3β, and that forskolin stimulates this interaction, we wished to examine whether forskolin affected GSK-3β kinase activity. After treatment of cells with forskolin, GSK-3β was immunoprecipitated and subjected to an in vitro kinase assay with Tau as substrate. Results from five experiments show that forskolin increased the activity of GSK-3β by approximately 25% after 30 minutes (Fig. 12A). Further exposure of cells to forskolin led to a gradual decrease in GSK-3β activity, so that by 120 minutes after forskolin treatment, GSK-3β activity was reduced to approximately 16% above the value found in untreated cells. To confirm the stimulatory effect of forskolin on the activity of GSK-3β in Reh cells, we also measured GSK-3β activity using GST-cyclin D1 as substrate. Densitometric analysis of the result shown in Fig. 12B revealed that forskolin induced the activity of GSK-3β towards GST-cyclin D1 by approximately 30% after 30 minutes. GSK-3β activity gradually decreased with further treatment of cells with forskolin. A similar result was also observed using GST-axin as the substrate for GSK-3β (data not shown). These results indicate that cAMP signalling induces GSK-3β activity in Reh cells and establish a link between increased cyclin D3-GSK-3β interaction, enhancement of GSK-3β activity and degradation of cyclin D3 in cells treated with forskolin.

Finally, because phosphorylation of GSK-3β at Ser-9 has been shown to inhibit its activity (Cross et al., 1995), we also examined the effect of forskolin on phosphorylation state of GSK-3β at Ser-9. As shown in Fig. 12C, immunoblotting with phospho-specific antibodies showed that forskolin had no effect on phosphorylation state of GSK-3β at Ser-9, indicating that forskolin-mediated increase in GSK-3β activity in Reh cells is not mediated through decrease in phosphorylation of GSK-3β at Ser-9.

Discussion

The control of the proliferative capacity of a cell is a tightly regulated process that is governed by intricate interaction of growth promoting and inhibitory signals. Among several physiological signals found to inhibit proliferation of lymphoid cells are agents that increase intracellular levels of cAMP (Goodwin and Ceuppens, 1983; Johnson et al., 1981; Kammer, 1988; Simkin et al., 1987). However, the mechanism by which this second messenger exerts its antiproliferative effect is not fully understood. We have previously shown that elevation of cAMP in lymphoid cells induces dephosphorylation of RB, which in turn leads to sequestration of E2Fs and inhibition of cell cycle progression in late G1 (Christoffersen et al., 1994; Gutzkow et al., 2002). Recently, we proposed a critical role for cyclin D3 in mediating the effect of cAMP in these processes; cAMP was shown to reduce the level of cyclin D3 protein rapidly in primary T lymphocytes and in Jurkat cells leading to reduction of cyclin-CDK4 kinase activity (Gutzkow et al., 2003; Naderi et al., 2000). Furthermore, we showed that in these cells, the cAMP-mediated decline in the level of cyclin D3 protein occurs as a result of inhibition of cyclin D3 translation. In this study, we provide evidence for a new mechanism underlying the regulation of cyclin D3 by cAMP in lymphoid cells. We show that elevation of intracellular cAMP reduces the level of cyclin D3 protein in the B-precursor cell line Reh, as well as primary human B lymphocytes, as a result of decreased protein stability. Furthermore, we show that the proteasome-proteolysis pathway plays an important role in both basal and cAMP-mediated regulation of cyclin D3 protein level. Finally,

we present a model for the mechanism by which cAMP promotes degradation of cyclin D3.

In the majority of examined cases, site-specific phosphorylation is required for targeting proteins for proteasomal degradation (Yew, 2001). Our initial observation of a strong correlation between forskolin-induced phosphorylation of cyclin D3 and its instability is in agreement with these findings, and supports the hypothesis that phosphorylation does have an important role in regulation of cyclin D3 stability. To investigate a possible causal relationship between these two phenomena, we took advantage of the high degree of amino acid homology between cyclin D1 and cyclin

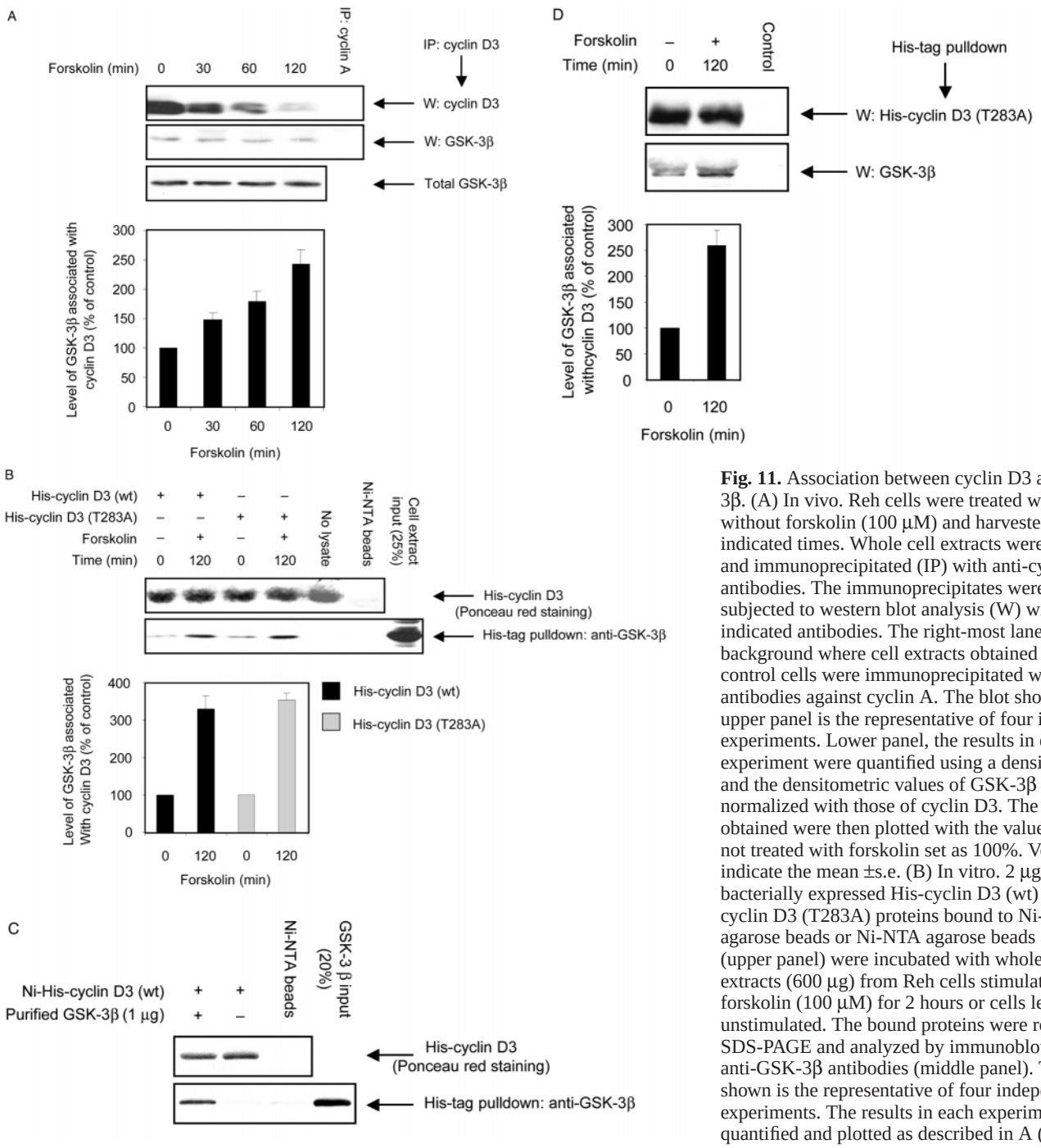

Fig. 11. Association between cyclin D3 and GSK-3β. (A) In vivo. Reh cells were treated with or without forskolin (100 μM) and harvested at the indicated times. Whole cell extracts were prepared and immunoprecipitated (IP) with anti-cyclin D3 antibodies. The immunoprecipitates were then subjected to western blot analysis (W) with the indicated antibodies. The right-most lane shows the background where cell extracts obtained from control cells were immunoprecipitated with antibodies against cyclin A. The blot shown in the upper panel is the representative of four independent experiments. Lower panel, the results in each experiment were quantified using a densitometer and the densitometric values of GSK-3β were normalized with those of cyclin D3. The values obtained were then plotted with the value for cells not treated with forskolin set as 100%. Vertical bars indicate the mean ±s.e. (B) In vitro. 2 μg of bacterially expressed His-cyclin D3 (wt) or His-cyclin D3 (T283A) proteins bound to Ni-NTA agarose beads or Ni-NTA agarose beads alone (upper panel) were incubated with whole cell extracts (600 μg) from Reh cells stimulated with forskolin (100 μM) for 2 hours or cells left unstimulated. The bound proteins were resolved on SDS-PAGE and analyzed by immunoblotting with anti-GSK-3β antibodies (middle panel). The blot shown is the representative of four independent experiments. The results in each experiment were quantified and plotted as described in A (lower panel). (C) 2 μg His-cyclin D3 (wt) protein bound to Ni-NTA agarose beads or Ni-NTA agarose beads alone (upper panel) were incubated with 0.6 μg purified GSK-3β protein for 2 hours. The beads were then subjected to western blot analysis with anti-GSK-3β antibody (lower panel). One representative experiment of three is shown. (D) Reh cells transfected with His-cyclin D3 (T283A) expression vector were treated with 100 μM forskolin or left untreated. His-cyclin D3 (T283A) was precipitated by incubating the cell extracts with Ni-NTA agarose beads and then analyzed by western blot analysis (W) with the indicated antibodies. The right-most lane (control) shows the background where cell extracts obtained from mock-transfected Reh cells were precipitated with Ni-NTA agarose beads. The blot shown is the representative of four independent experiments. The results in each experiment were quantified and plotted as described in A (lower panel).

D3, and the finding that proteasomal degradation of cyclin D1 is facilitated by its GSK-3β-dependent phosphorylation of Thr-286 (Diehl et al., 1998). Thus, we mutated the putative GSK-3β phosphorylation site in cyclin D3 to Ala (T283A). Cyclin D3 (T283A) mutant exhibited a ninefold increase in its stability ($t_{1/2}$ ~3.5 hours) and was resistant to cAMP-mediated degradation. These findings demonstrated a similarity between the mechanisms that regulate stability of cyclin D1 and cyclin D3, and strongly suggested the requirement of a GSK-3β-mediated phosphorylation event for basal, as well as cAMP-induced, degradation of cyclin D3. Several lines of evidence support this suggestion. First, treatment of cells with the GSK-3β inhibitors lithium and Kenpaullone led to accumulation of cyclin D3 in both proliferating and forskolin-treated cells, indicating that GSK-3β activity is required for regulation of basal as well as cAMP-mediated cyclin D3 turnover. Second, purified GSK-3β phosphorylated wild-type cyclin D3 but not cyclin D3 (T283A) mutant. Third, cyclin D3 interacted with GSK-3β, and finally, forskolin promoted the association of cyclin D3 with GSK-3β, and induced the activity of GSK-3β in Reh cells.

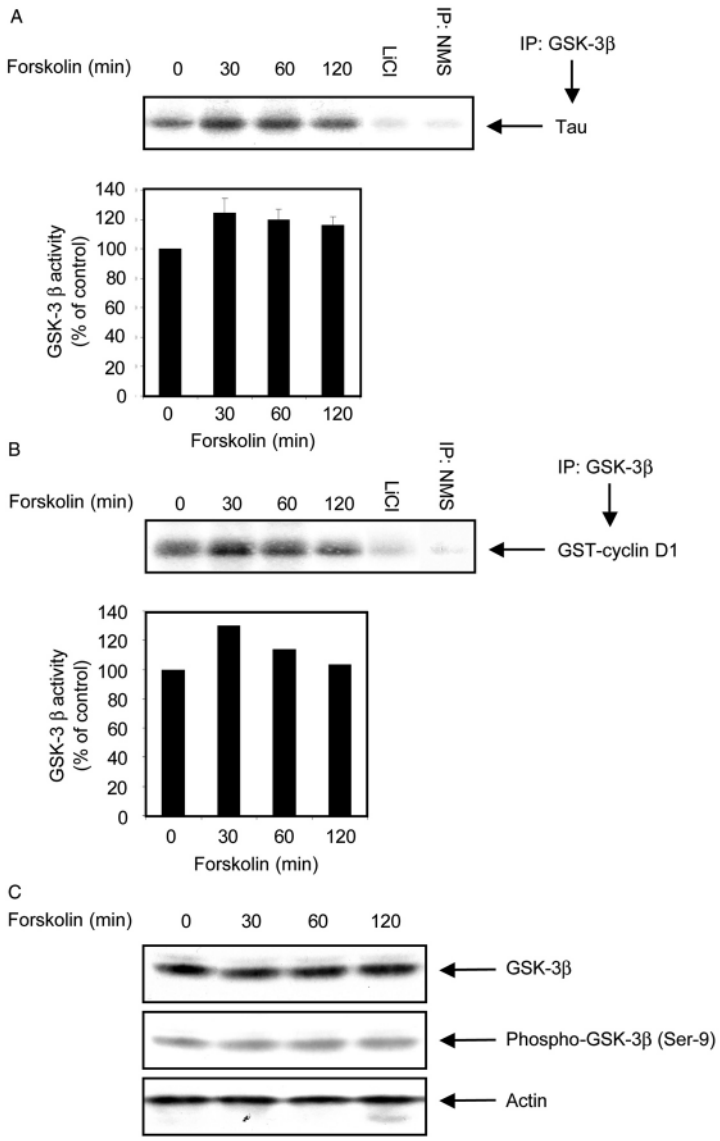

Fig. 12. Regulation of GSK-3β activity by forskolin. (A) Reh cells were treated with or without forskolin (100 μM) and harvested at the indicated times. Whole cell extracts were prepared, immunoprecipitated with GSK-3β antibodies and assayed for activity towards Tau. The specificity of the kinase reactions was examined by assaying the GSK-3β activity recovered from control cells in the presence of 50 μM LiCl, or by immunoprecipitation of control cell extracts with non-immune mouse serum (NMS) (upper panel). The activities were then expressed as a percentage of the activity of GSK-3β recovered from untreated cells (100%) and are mean ±s.e. of five independent experiments (lower panel). (B) Reh cells were treated as in A. Whole cell extracts were prepared, immunoprecipitated with GSK-3β antibodies and assayed for activity towards GST-cyclin D1. The specificity of the kinase reactions was examined as described in A (upper panel). The activities were then plotted with the activity of GSK-3β recovered from control cell set as 100% (lower panel). (C) Reh cells were treated as in A, total cell lysates were prepared and then subjected to immunoblotting with antibodies against GSK-3β, GSK-3β (Ser-9) and actin. The immunoblotblot shown is the representative of four independent experiments.

The data presented here reveal for the first time that cyclin D3 interacts with GSK-3β both in vitro and in vivo, and that this interaction is subject to regulation. The ability of bacterially produced cyclin D3 to interact with purified GSK-3β in vitro suggests a direct interaction between these two proteins. Although a direct interaction between a kinase and its substrate is to be expected, this result does not preclude the possibility that, in vivo, efficient interaction of cyclin D3 with GSK-3β is brought about by an ancillary linker protein. This mode of interaction might have implications for the way by which GSK-3β regulates the phosphorylation of cyclin D3. It is possible that GSK-3β plays an indirect role in phosphorylation of cyclin D3 by facilitating the formation of a multiprotein complex in which cyclin D3 is brought into proximity of a kinase other than GSK-3β. Although we cannot formally exclude this possibility, the observations that GSK-3β can directly bind to, and efficiently phosphorylate cyclin D3 in vitro, suggest that GSK-3β is the enzyme that phosphorylates cyclin D3. Association of cyclin D3 with GSK-3β in a multiprotein complex might also have implications for the way by which GSK-3β acquires its substrate specificity. While the majority of GSK-3β substrates are primed by prior phosphorylation at $n + 4$ (where n is the site of GSK-3β phosphorylation) (Wang and Roach, 1993; Welsh et al., 1997), GSK-3β can also phosphorylate certain substrates in the absence of a priming event. This is exemplified by the ability of GSK-3β to catalyze phosphorylation of unprimed β-catenin in vitro (Thomas et al., 1999), or phosphorylate cyclin D1 (Diehl et al., 1998), or cyclin D3, as shown in this study, at a location in which the n+4 residue is Val. However, GSK-3β has been shown to have 400- to 1000-fold less activity against unprimed substrates than primed proteins, leading to the suggestion that phosphorylation of unprimed substrates by GSK-3β is an in vitro phenomenon that is brought about by high concentrations of enzyme and substrate used in reactions in vitro (Frame et al., 2001; Thomas et al., 1999). However, through formation of protein complexes in vivo, bringing GSK-3β and its unprimed substrate into close proximity could be assumed to facilitate the phosphorylation of the unprimed substrate by GSK-3β.

The in vivo association between cyclin D3 and GSK-3β in the basal state, and the stimulatory effect of forskolin on this interaction, suggest that such a mechanism might indeed be involved in mediating the basal as well as forskolin-induced phosphorylation of cyclin D3 by GSK-3β.

Formation of a multiprotein complex including both cyclin D3 and GSK-3β would also have the potential to insulate the GSK-3β-cyclin D3 pathway from GSK-3β regulators that lie outside this pathway and confer specificity to GSK-3β signal transduction. Insulin and Wnt signalling cascades, both of which utilize GSK-3β as an essential component, provide an example of such regulation. In the Wnt signalling cascade, association of a pool of cellular GSK-3β with β-catenin, axin and adenomatous polyposis coli proteins in a multiprotein complex, referred to as the destruction complex, allows it to phosphorylate β-catenin, thereby leading to its proteasomal degradation (Hart et al., 1998; Hinoi et al., 2000; Ikeda et al., 1998). In response to Wnts, GSK-3β-mediated phosphorylation of β-catenin is inhibited through a mechanism that appears to involve disruption of the destruction complex (Li et al., 1999; Ruel et al., 1999; Willert et al., 1999). In contrast, insulin signalling leads to phosphorylation and inhibition of the population of GSK-3β that is not associated with the destruction complex (Cross et al., 1995; Ding et al., 2000; Yuan et al., 1999). Thus, it appears that sequestration of a fraction of GSK-3β within the destruction complex insulates it from the effect of insulin signalling, and provides the cell with the ability to transduce signals along different pathways without crosstalk or interference.

The cAMP-mediated activation of GSK-3β in Reh cells was unexpected, because a number of recent reports have shown that elevation of intracellular cAMP in other cell types leads to inactivation of GSK-3β by PKA (Fang et al., 2000; Li et al., 2000). One explanation for these contradictory results might be provided by the ability of PKA to phosphorylate GSK-3β at Ser-9. cAMP-induced inactivation of GSK-3β has been shown to occur in HEK 293, NIH 3T3, Rat1 or rat cerebellar granule neurons as a result of PKA-mediated phosphorylation of GSK-3β at Ser-9 (Fang et al., 2000; Li et al., 2000). However, treatment of Reh cells with forskolin did not affect phosphorylation of GSK3β at Ser-9, suggesting that the crosstalk between cAMP and GSK-3β signalling pathways might be cell-type specific. In a recent report, the ability of PKA to inhibit GSK-3β activity was shown to depend on binding of PKA and GSK-3β to AKAP220 protein (Tanji et al., 2002). AKAPs target PKA in close proximity to relevant substrates and thus confer specificity to the cAMP/PKA pathway (Colledge and Scott, 1999; Edwards and Scott, 2000). The inability of cAMP to induce phosphorylation of GSK-3β in Reh cells can therefore be assumed to be owing to a possible lack of AKAP220 expression in Reh cells.

The implication of forskolin-induced increase in GSK-3β activity for forskolin-mediated degradation of cyclin D3 must also be addressed. As shown in this report, GSK-3β can phosphorylate cyclin D3, an event that is required for degradation of cyclin D3. It is therefore possible that the cAMP-mediated induction of GSK-3β activity leads to acceleration of GSK-3β-catalyzed phosphorylation of cyclin D3, thereby leading to its increased rate of degradation. However, we cannot exclude the possibility that cAMP-induced activation of GSK-3β serves an indirect, but regulatory, role in stimulation of cyclin D3 degradation. This idea is based on the observation that GSK-3β activity promotes the formation and stabilization of the destruction complex, which is required for degradation of β-catenin (Jho et al., 1999; Rubinfeld et al., 1996; Salic et al., 2000; Willert et al., 1999). Likewise, if formation of a protein complex containing GSK-3β and cyclin D3 requires the activity of GSK-3β, then an increase in GSK-3β activity would be expected to augment formation of GSK-3β-cyclin D3 complexes, which in turn would lead to an increase in the rate of GSK-3β-mediated phosphorylation of cyclin D3, thereby leading to acceleration of its degradation. Our observation that lithium inhibits the in vivo interaction between cyclin D3 and GSK-3β (data not shown) supports this notion.

In summary, the results of this study reveal a new regulatory mechanism by which cAMP controls the level of a common cell-cycle protein. GSK-3β appears to be a key component of this pathway, and our results further strengthen the proposed role of GSK-3β in regulation of cyclin D expression. In addition, we have also provided evidence for a new mechanism by which cAMP might promote GSK-3β-mediated phosphorylation of unprimed substrates, such as cyclin D3. This mechanism involves cAMP-mediated facilitation of the complex formation between cyclin D3 and GSK-3β. Based on results from our previous, and present studies on cAMP-mediated regulation of cell-cycle progression (Gutzkow et al., 2003; Naderi et al., 2000), it appears that lymphoid cells have developed different mechanisms for cAMP-mediated inhibition of cyclin D3. Taken together, our data underline the importance of both cyclin D3 and cAMP in regulation of lymphoid cell proliferation.

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