Calcineurin Regulates Cyclin D1 Accumulation in Growth-stimulated Fibroblasts

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Calcium (Ca^{2+}) and calmodulin (CaM) are required for progression of mammalian cells from quiescence into S phase. In multiple cell types, cyclosporin A causes a G_1 cell cycle arrest, implicating the serine/threonine phosphatase calcineurin as one Ca^{2+} /CaM-dependent enzyme required for G_1 transit. Here, we show, in diploid human fibroblasts, that cyclosporin A arrested cells in G_1 before cyclin D/cdk4 complex activation and retinoblastoma hyperphosphorylation. This arrest occurred in early G_1 with low levels of cyclin D1 protein. Because cyclin D1 mRNA was induced normally in the cyclosporin A-treated cells, we analyzed the half-life of cyclin D1 in the presence of cyclosporin A and found no difference from control cells. However, cyclosporin A treatment dramatically reduced cyclin D1 protein synthesis. Although these pharmacological experiments suggested that calcineurin regulates cyclin D1 synthesis, we evaluated the effects of overexpression of activated calcineurin on cyclin D1 synthesis. In contrast to the reduction of cyclin D1 with cyclosporin A, ectopic expression of calcium/calmodulin-independent calcineurin promoted synthesis of cyclin D1 during G_1 progression. Therefore, calcineurin is a Ca^{2+} /CaM-dependent target that regulates cyclin D1 accumulation in G_1 .

INTRODUCTION

Calcium (Ca^{2+}) and its major intracellular receptor calmodulin (CaM) are essential for proliferation. During reentry from a quiescent state, mammalian cells required Ca^{2+} and CaM during at least two distinct points, in early G_1 and close to the G_1 /S boundary (Boynton *et al.*, 1977; Hazelton *et al.*, 1979; Chafouleas *et al.*, 1984). However, neither the crucial relevant downstream cell cycle targets of Ca^{2+} /CaM-dependent pathways nor the Ca^{2+} /CaM-dependent proteins that mediate those pathways have been well characterized in mammalian cells, despite the extensive progress that has been made in understanding the regulation of cell cycle progression in recent years.

Experimental evidence from both unicellular eukaryotes and mammalian cells supports the hypothesis that the protein phosphatase 2B calcineurin is one Ca^{2+} /CaM-dependent enzyme required for G_1 progression. Calcineurin is a heterodimer composed of a catalytic subunit, calcineurin A, and a Ca^{2+} -binding regulatory subunit, calcineurin B (Klee *et al.*, 1998; Aramburu *et al.*, 2000). In *Aspergillus nidulans*, the calcineurin A gene was essential and its disruption led to an early cell cycle arrest (Rasmussen *et al.*, 1994). When calcineurin A expression was repressed using an inducible promoter, the majority of cells arrested in G_1 , with some cells arrested in G_2 and M phases (Nanthakumar *et al.*, 1996). In *Saccharomyces cerevisiae*, none of the three genes encoding calcineurin subunits (*CNA1*, *CNA2/CMP2*, and *CNB1*) were essential (Cyert *et al.*, 1991; Cyert and Thorner, 1992). However, deletion of the calcineurin subunit genes reduced the ability of cells to recover upon release from α -factor-mediated

arrest (Cyert *et al.*, 1991; Cyert and Thorner, 1992; Withee *et al.*, 1997).

In mammalian systems, calcineurin is well known for its essential role during the initial activation and proliferation of quiescent T lymphocytes after T cell receptor engagement (Cardenas and Heitman, 1995). Although the T cell represents a very specialized system of reentry from quiescence, cyclosporin A has antiproliferative effects in a variety of cells, including adenocarcinoma cell lines, lymphoma and leukemia cell lines, keratinocytes, fibroblasts, and smooth muscle cells (Furue *et al.*, 1988; Sharpe and Fisher, 1990; Thyberg and Hansson, 1991; Richter *et al.*, 1995; Tomono *et al.*, 1996). Where investigated, the cell cycle arrest induced by cyclosporin A was in G_1 , although numerous, distinct mechanisms have been proposed. Two recent studies have implicated calcineurin function in the regulation of the transcription, and therefore expression, of both cyclin D and cdk4. Although cyclosporin A reduced the levels of cyclin D1 mRNA in pancreatic acinar cells (AR42J), mouse embryonic fibroblasts lacking calcineurin A α had higher amounts of cdk4 mRNA and protein (Baksh *et al.*, 2002; Schneider *et al.*, 2002). Together, these results suggest that calcineurin function promotes cyclin D1 expression and inhibits cdk4 expression. Because both cyclin D and cdk4 are transcriptionally induced during G_1 , these disparate results initially seem incompatible and may be due to cell type-specific differences.

In mammalian cells, D type cyclins act as growth factor sensors, regulate the pathway leading to retinoblastoma protein (pRb) hyperphosphorylation, and are often implicated in oncogenesis (Sherr, 1996; Ortega *et al.*, 2002). On mitogenic stimulation, the D type cyclins accumulate and assemble with cdk4/6 to form active kinase complexes in mid- G_1 , which preferentially phosphorylate pRb. Hyperphosphorylation of pRb leads to the release of the E2F family of transcription factors, which act to stimulate a number of genes required for S phase (Nevins *et al.*, 1997b). Therefore,

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D type cyclins are critical for passage through the restriction point, a defined time in G_1 in which cells become independent of mitogenic stimuli and commit to entry into S. One hypothesis is that to become transformed, human cells must subvert the cyclin D/cdk4/Rb pathway by one of several methods: overexpression of cyclin D1 or cdk4, loss of expression of cdk4 inhibitors (p15/p16 family), or loss of retinoblastoma gene expression (Sherr, 1996).

Because normal diploid fibroblasts are unlikely to harbor disruptions in either cell cycle or Ca^{2+} /CaM-dependent pathways that frequently occur during immortalization and transformation, we have investigated the role of calcineurin during reentry in WI-38 cells. We found that cyclosporin A arrested WI-38 cells early in G_1 with low levels of cyclin D1 protein similar to the recent results from pancreatic acinar cells (Schneider *et al.*, 2002). In contrast to that study, we did not observe an effect on the accumulation of cyclin D1 mRNA. Rather, cyclosporin A reduced the synthesis of cyclin D1 in WI-38 cells with little change in its protein stability. Opposite to this cyclosporin A result, expression of Ca^{2+} /CaM-independent calcineurin A, in which the C-terminal Ca^{2+} /CaM-binding domain has been removed, promoted cyclin D1 synthesis, supporting the idea that calcineurin activity positively regulates cyclin D1 protein translation during G_1 progression. Therefore, we propose one function of Ca^{2+} /CaM-dependent pathways during early G_1 progression is to activate calcineurin, which in turn, promotes cyclin D1 translation.

MATERIALS AND METHODS

Cell Culture and Reagents

WI-38 fibroblasts were obtained from the Coriell Cell Repository (National Institute on Aging, Repository #AG06814F) at passage 14. Cells were grown according to their guidelines in Eagle's minimal essential medium (MEM) with Earle's salts supplemented with MEM nonessential amino acids, MEM vitamins, L-glutamine, and 20% fetal bovine serum (FBS). For experiments, cells were plated at ~5000 cells/cm² and grown overnight at 37°C and 5% CO₂. To arrest cells with low serum, they were washed two times with Puck's solution followed by the addition of media containing 0.2% FBS for 26–32 h. To arrest WI-38 cells in mitosis, cells were serum starved as described above, serum stimulated for 18 h, and then media containing 0.5 µg/ml nocodazole were added for 6 h. To arrest WI-38 cells in S phase, cells were serum starved as described above, serum stimulated for 10 h, and then media containing 2 µM hydroxyurea were added for 10 h.

All cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). FBS was obtained from Sigma-Aldrich (St. Louis, MO), Invitrogen, or Hyclone Laboratories (Logan, UT), and each time a new lot of serum was used, cells were tested to ensure similar growth parameters. Cyclosporin A and MG-132, from Calbiochem (San Diego, CA), and nocodazole, from Sigma-Aldrich, were dissolved in dimethyl sulfoxide. W-13, from RBI/Sigma (Natick, MA) or Seikagaku America (Rockville, MD), and hydroxyurea, from Sigma-Aldrich, were dissolved in sterile water.

Flow Cytometry and 5-Bromo-2-deoxyuridine (BrdU) Incorporation

For flow cytometry, $0.5\text{--}1.0 \times 10^6$ cells were trypsinized, pelleted by centrifugation, and resuspended in 200 µl of phosphate-buffered saline (PBS) and 800 µl of ethanol. Cells were fixed overnight at -20°C and resuspended in 0.5–1.0 ml of DNA Prep (Beckman Coulter, Fullerton, CA). Samples were analyzed using an EPICS Profile II and EXPO software analysis program (Beckman Coulter).

To assay BrdU incorporation by immunofluorescence, cells were plated onto coverslips or glass chamber slides (BD Biosciences, San Jose, CA) and pulse labeled with 10 µM BrdU for 30 min. Cells were fixed with cold methanol for 5 min at 4°C followed by permeabilization with 0.25% Triton X-100 in PBS for 5 min. Samples were denatured with 2 N HCl for 30 min, washed once with 0.1 M Na₂B₄O₇ and twice with PBS, and blocked with 1% bovine serum albumin in PBS for 30 min. Anti-BrdU (Beckman Coulter) was diluted to a final concentration of 1 µg/ml in blocking solution and incubated overnight at 4°C. Samples were washed three times with PBS, followed by incubation for 1 h with goat anti-mouse fluorescein isothiocyanate (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in blocking solution. Samples were then washed three times with PBS, incubated with 4,6-dia-

midino-2-phenylindole (DAPI) (5 mg/ml stock) diluted 1:10,000 in PBS for 5 min, and mounted under coverslips by using a solution of 25 mg/ml triethylenediamine in 50% glycerol and 50% PBS. For each sample, 500–700 cells were counted at random, and S-phase percentage was determined by dividing the number of BrdU-positive nuclei by DAPI-positive nuclei.

Assays of Cdk Activity

Cdk2 assays were carried out based on protocols described by DeGregori *et al.* (1995) and Sheaff (1997). Cells were lysed in Cdk2 immunoprecipitation (IP)/radioimmunoprecipitation buffer, and protein concentration was determined using DC protein assay kit (Bio-Rad, Hercules, CA). For each sample, 500 µg of total protein was immunoprecipitated using 2 µg of anti-cdk2 (M2; Santa Cruz Biotechnology) and 10 µl of protein A-Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ). The final kinase reaction was carried out in 50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 10 µM ATP, 2.5 µCi per reaction of [γ -³²P]ATP (Amersham Biosciences), and 5 µg per reaction of histone H1 (Roche Diagnostics, Indianapolis, IN). Samples were incubated at 30°C for 30 min followed by the addition of SDS-PAGE sample buffer to stop the reaction after which they were boiled and the proteins separated by electrophoresis. The amount of ³²P-labeled histone H1 was evaluated by autoradiography and quantified by PhosphorImager analysis.

Cdk4 kinase assays were carried out based on protocols described by DeGregori *et al.* (1995) and Phelps and Xiong (1997). Cells were lysed in Cdk4 IP Buffer and protein concentration was determined using DC protein assay kit (Bio-Rad). For each sample, 500 µg of total protein was immunoprecipitated using 2 µg of anti-cdk4 (C-22 or H-22; Santa Cruz Biotechnology) and 10 µl of protein A-Sepharose 4 Fast Flow (Amersham Biosciences). The final kinase reaction was carried out in 50 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β -glycerophosphate, 0.1 mM Na₂VO₄, 1 mM NaF, 20 µM ATP, 2.5 µCi per reaction of [γ -³²P]ATP (Amersham Biosciences), and 2 µg/reaction GST-Rb 769–921 (Santa Cruz Biotechnology). Samples were incubated at 30°C for 30 min and processed as described above for the cdk2 assays.

Western Analyses

After lysis in Cdk4 IP buffer, samples were assayed for protein concentration, and 50–100 µg of protein (depending on assay) was separated by SDS-PAGE. For most Western blots, proteins were separated by 10–12% SDS-PAGE, but for pRb analysis, 6% SDS-PAGE was used to ensure separation of the phosphorylated forms. After gel electrophoresis, proteins were transferred to Immobilon P (Millipore, Bedford, MA) based on the manufacturer's recommendations for the electrophoretic transfer apparatus (Bio-Rad). Membranes were blocked for 1–2 h in blocking solution (Tris-buffered saline with 0.1% Tween 20, 5% dry milk, and 0.5% fish gelatin). Primary and secondary antibodies were diluted at 1:1000 (primary) or 1:5000 (secondary) in blocking solution and incubated either at 4°C overnight or at room temperature for 60 min with rocking. Signal was detected chemiluminescently with enhanced chemiluminescence (Amersham Biosciences). Primary antibodies included anti-Rb (C-19; Santa Cruz Biotechnology), anti-cyclin D1 (H-295; Santa Cruz Biotechnology), anti-cyclin D1 (UBI, Waltham, MA), anti-cdk4 (C-22; Santa Cruz Biotechnology), anti-cdk4 (H-22; Santa Cruz Biotechnology), anti-cdk2 (M2; Santa Cruz Biotechnology), anti-cyclin E (M-20; Santa Cruz Biotechnology), anti-cyclin A (C-19; Santa Cruz Biotechnology), anti-calcineurin A (BD Biosciences), and anti-calcineurin B (Sigma-Aldrich). The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse (Jackson ImmunoResearch laboratories, West Grove, PA).

Northern Analysis

Total RNA was extracted from tissue culture cells by using Ultraspec RNA (Biotecx Laboratories, Houston, TX). Total RNA concentrations were determined, and Northern analysis was performed as described by Wu and colleagues (Ribar *et al.*, 2000). Equal amounts (15 µg) of total RNA were separated by formaldehyde gel electrophoresis and transferred to a Zeta-Probe membrane (Bio-Rad). A 900-base pair fragment of the mouse cyclin D1 cDNA was amplified by polymerase chain reaction (PCR) and randomly labeled with ³²P-dCTP (Amersham Biosciences). Northern blots were evaluated by autoradiography and quantified by PhosphorImager analysis. Membranes were routinely stripped and reprobed with GAPDH as a loading control.

Metabolic Labeling

The metabolic labeling of proteins is based on standard protocols for adherent cells (Ausubel, 2001; Querido *et al.*, 2001). Because only Sigma-Aldrich provides MEME without methionine/cystine, WI-38 cells were thawed into Sigma-Aldrich, rather than Invitrogen, MEME with the usual supplements. Cells were serum stimulated with media lacking methionine/cystine and 20% FBS for 5–6 h. The media were removed and replaced with a small volume (2.5 ml/100-mm dish) of MEME without methionine/cystine and containing ³⁵S EasyTag express protein labeling mix (PerkinElmer Life Sciences, Boston, MA) at a final concentration of ~0.5 mCi of [³⁵S]methionine/cystine mix per 100-mm dish. After a 2-h incubation, the labeling media were removed, cells

Figure 1. Synchronous cell cycle reentry of WI-38 fibroblasts. (A) DNA profiles of reentry. WI-38 fibroblasts were growth arrested in low serum and then, stimulated to reenter the cell cycle by the readdition of growth media. Cells were harvested at several times after serum addition (T = 0 to T = 24), fixed, stained with propidium iodide, and analyzed by fluorescence-activated cell sorting analysis. Each histogram plots cell count versus DNA content. In the histogram, the first peak represents cells in G₀/G₁ with 2N DNA content and the second peak represents cells in G₂/M with 4N DNA content. Cells traversing S phase are between the two peaks with DNA content ranging from 2N to 4N. (B) Cyclin and calcineurin expression during reentry. Cell lysates were separated by SDS-PAGE and analyzed for the expression of the cyclins (D1, E, and A) and calcineurin A by Western blotting.

were washed with PBS, and then lysed in 1 ml of Cdk2 IP buffer and frozen with liquid N₂. For immunoprecipitation, lysates were thawed and cleared by centrifugation. Cleared WI-38 lysate was incubated with 2–4 μ g of anti-cyclin D1 (M-20; Santa Cruz Biotechnology), and 10–20 μ g of packed resin for 2–4 h at 4°C. The resin was washed extensively, and proteins were separated by SDS-PAGE. The amount of ³⁵S-labeled cyclin D1 was determined by autoradiography and quantified by PhosphorImager analysis.

Adenoviral Infection of Cells

All adenoviruses were generated using the AdEasy system first described by He and colleagues and now available from Qbiogene (formerly Quantum Biotechnologies) (He *et al.*, 1998). The methods used to generate these viruses were based on the initial description of the system, the Web site with updated protocols, and sequence data (<http://www.coloncancer.org/adeasy/protocol.htm>), and the protocols published by Qbiogene. WI-38 cells were routinely infected as described by others (DeGregori *et al.*, 1995; He *et al.*, 1998; Cook, *et al.*, 2002).

The cDNAs encoding wild-type mouse calcineurin A and calcineurin B, as well as catalytically inactive calcineurin A (H151Q) were gifts of Joseph Heitman (Duke University, Chapel Hill, NC). Wild-type calcineurin A and calcineurin B were amplified by PCR and subcloned into pAdTrack-CMV. The Ca²⁺/CaM-independent form of calcineurin A (1–397) was generated by PCR to introduce a stop codon and subcloned into pAdTrack-CMV. All pAdTrack-CMV vectors containing the calcineurin cDNAs were verified by sequencing. For recombination, the pAdTrack-CMV vectors were linearized with *Eco*RI for calcineurin A and *Pme*I for calcineurin B.

RESULTS

Cyclosporin A Inhibits Cell Cycle Progression in WI-38 Cells

To investigate the effects of cyclosporin A on G₁ progression, we first characterized the cell cycle arrest and release profile

of mammalian fibroblasts. Subconfluent human WI-38 fibroblasts were arrested by serum starvation and stimulated to reenter the cell cycle with the readdition of serum. Greater than 90% of cells arrest in G₀/G₁ as determined by following DNA content by flow cytometry (Figure 1A). The cells synchronously entered S phase ~16–20 h after serum stimulation as determined by monitoring either DNA content by flow cytometry (Figure 1A) or BrdU incorporation (our unpublished data). Additionally, we examined several cell cycle markers to biochemically characterize the arrest and release profile of these cells. The expression of the cyclins correlated with reentry as cyclin D expression preceded that of cyclin E which, in turn, preceded cyclin A (Figure 1B). We also evaluated calcineurin A expression by Western blotting and found it was expressed in serum-starved cells and remained constant as cells entered G₁. Finally, CaM expression, as determined by radioimmunoassay (our unpublished data), was similar to previously published results for early passage WI-38 cells (Brooks-Frederich *et al.*, 1993).

We next determined the effects of cyclosporin A, a calcineurin inhibitor, and W-13, a CaM antagonist, on cell cycle progression after serum readdition to starved WI-38 cells. Both cyclosporin A and W-13 dramatically reduced the number of cells entering S phase at 18 h after serum stimulation as shown by the DNA profiles in Figure 2A. These results were confirmed using BrdU incorporation as a measure of DNA synthesis (our unpublished data). Next, we evaluated whether the cyclosporin A-induced cell cycle ar-

Figure 2. Cell cycle inhibition in G_1 by cyclosporin A. (A) DNA profiles of reentry with W-13 and cyclosporin A. Serum-starved WI-38 cells were stimulated with growth media in the presence of the vehicle dimethyl sulfoxide, 15 $\mu\text{g}/\text{ml}$ W-13, or 25 μM cyclosporin A. Cells were harvested at 18 h after serum addition and analyzed by fluorescence-activated cell sorting analysis (FACS), with each histogram plotting cell count versus DNA content. (B) DNA profiles of release from S phase. WI-38 cells were arrested in early S phase with hydroxyurea and then released into fresh media. Then, cells were harvested at 6 and 12 h for FACS analysis. (C) DNA profiles of release from M phase. WI-38 cells were arrested in M phase with nocodazole and then released into fresh media. Then, cells were harvested at increasing times after the removal of nocodazole for FACS analysis. (D) DNA profiles of release from the cyclosporin A G_1 arrest. Serum-starved WI-38 cells were stimulated with growth media in the presence of cyclosporin A, followed by release into fresh media. Cells were harvested at 12 and 20 h for FACS analysis. (E) Time course of cyclosporin A addition. Serum-stimulated WI-38 cells, treated with cyclosporin A, were pulse labeled with BrdU for 30 min and harvested at 18 h after serum addition. Cells were counted at random for BrdU incorporation, and S phase percentage was determined by dividing the number of BrdU-positive cells by the total number of cells, as determined by DAPI nuclear staining.

rest was G_1 selective or whether cyclosporin A inhibited progression through other cell cycle phases. WI-38 fibroblasts were arrested in early S phase by using hydroxyurea and then released into fresh media to allow synchronous progression through S and G_2 /M phases. Cyclosporin A had no effect on the ability of cells to progress through S and G_2 /M phases as shown in Figure 2B. Next, we examined whether the cyclosporin A arrest was specific to reentry from growth arrest or also occurred during G_1 after entry from mitosis. WI-38 fibroblasts were arrested in metaphase by using nocodazole and then released into fresh media to allow synchronous progression through mitosis and into G_1 . Whereas cyclosporin A had no effect on the ability of cells to exit mitosis and enter G_1 , it prevented cells from entering S and G_2 /M phases (Figure 2C). These results demonstrate that the cyclosporin A arrest is specific to G_1 and that this G_1 arrest occurs no matter whether cells are entering G_1 from quiescence or from mitosis.

To evaluate which G_1 pathways cyclosporin A affects, we questioned whether the cyclosporin A arrest occurred in early or late G_1 . First, we asked whether the cyclosporin A arrest was reversible and if so, how long did it take cells to enter S phase? We found the cyclosporin A arrest was reversible, but cells released from cyclosporin A took nearly 20 h to enter S phase after drug removal (Figure 2D). Because this was similar to the time needed to enter S from serum starvation, we reasoned cyclosporin A arrested cells in very early G_1 . However, we could not rule out the possibility that it may have taken several hours to completely remove cyclosporin A from its targets within the cell. To further evaluate this question, we added the cyclosporin A at increasing times after serum addition and determined the amount of BrdU incorporation at peak S phase, ~18 h for WI-38 cells (Figure 2E). Cyclosporin A was most effective at preventing S-phase entry when added at the same time as serum. Because cyclosporin A arrested WI-38 cells in early G_1 progression, calcineurin may represent one essential target of Ca^{2+} /CaM in G_1 .

Cyclosporin A Prevents pRb Phosphorylation by Inhibiting Cyclin D1 Accumulation

To examine the biochemical nature of the cyclosporin A arrest point, we next evaluated the activation status of two G_1 cdk, cdk2 and cdk4. Cdk2 kinase complexes were immunoprecipitated and assayed for activity by using histone H1 as a substrate. In control cells, cdk2 activity occurred by 16 h after serum addition and was dramatically increased at 20 h (Figure 3A). Cells treated with cyclosporin A demonstrated no cdk2 activity at either 16 or 20 h. Because there was such little activity, we chose not to evaluate the relative contributions of cyclin E/cdk2 and cyclin A/cdk2 to the total cdk2 activity. Because cdk4 activity is required before cdk2 activity during normal G_1 progression, we analyzed cdk4 activity by immunoprecipitating cdk4 kinase complexes followed by kinase assays by using GST-Rb as an *in vitro* substrate. Although control cells demonstrated cdk4 activity at 18 h after serum addition, cells treated with cyclosporin A had no activity (Figure 3B). As an *in vivo* monitor of cdk4 activity, we analyzed the phosphorylation status of endogenous pRb protein by Western analysis (Figure 3C). Serum-starved cells have primarily a single immunoreactive band, corresponding to the hypophosphorylated form of pRb. At 18 h after stimulation, pRb became hyperphosphorylated, as demonstrated by reduced mobility, and the total amount of protein increased. Cyclosporin A-treated cells seemed similar to serum-starved cells with pRb exclusively in the hypophosphorylated form.

A major rate-limiting step in cdk4 activation during G_1 is the accumulation of cyclin D, and its expression is strictly dependent on the presence of growth factors (Sherr, 1996). Although cyclins D1, D2, and D3 form complexes with both cdk4 and cdk6, we examined the most abundant complex in WI-38 cells, cyclin D1 bound to cdk4 (Parry *et al.*, 1999). In WI-38 cells, cyclosporin A prevented cyclin D1 protein accumulation (Figure 3D). Because cyclin D accumulation is tightly controlled at the level of transcription, translation, and ubiquitin-mediated proteolysis, we first examined the level of cyclin D1 mRNA. Whereas serum-starved WI-38 cells expressed low levels of cyclin D1 mRNA, it was dramatically induced by 4 h after serum addition (Figure 3E). Next, we examined cyclin D1 mRNA at 4 h after serum addition in the presence of cyclosporin A and found similar levels in both treated and untreated cells (Figure 4F). Because cyclin D1 mRNA was up-regulated in cyclosporin A-treated cells, we concluded that cyclosporin A either prevented efficient translation of cyclin D1 mRNA or accelerated cyclin D1 protein degradation.

Cyclosporin A Inhibits the Synthesis of Cyclin D1

To examine both the synthesis of cyclin D1 and its half-life, we used [35 S]methionine/cystine metabolic labeling of cells, followed by cyclin D1 immunoprecipitation. After a 2-h labeling period in mid- G_1 , we found cyclosporin A-treated cells had dramatically less labeled cyclin D1 (Figure 4A). This effect seemed selective for cyclin D1 because we found similar labeling of total cell extracts. Additionally, we detected no reduction in the labeling of cdk4 (our unpublished data), which is also translationally regulated (Ewen *et al.*, 1995). On average, we found that the amount of labeled cyclin D1 in cells treated with cyclosporin A was ~40% that of untreated cells (Figure 4B). In WI-38 cells, the half-life of cyclin D was ~25 min, which was similar to the half-life of endogenous cyclin D1 in U2OS cells (Russell *et al.*, 1999). Although cyclosporin A did not shorten the half-life, we were concerned that the low level of labeled cyclin D1 may affect the half-life determination (Figure 4C). One possibility was that cyclin D1 was being rapidly degraded during the 2-h labeling period and that the reduction in cyclin D1 reflected accelerated degradation rather than reduced synthesis during the labeling period. To further evaluate this question, we used a proteasome inhibitor, MG-132, during the labeling period to determine whether inhibition of degradation affected the labeling of cyclin D1. As expected, in untreated cells, MG-132 treatment increased the amount of labeled cyclin D1 twofold (Figure 4D). In contrast, MG-132 had little effect on the amount of labeled cyclin D1 in cyclosporin A-treated cells. Together, these results implied that cyclosporin A reduced cyclin D1 levels due to decreased translation rather than increased degradation.

Ca^{2+} /CaM-independent Calcineurin A Promotes Cyclin D1 Synthesis

Although these pharmacological studies suggested a role for calcineurin in cyclin D1 synthesis, we sought to implicate calcineurin more directly. An adenoviral-mediated transgene expression system was chosen to introduce calcineurin and its mutants into WI-38 cells. The primary advantage of this system is the ability of adenovirus to infect nearly 100% cells in a serum-starved condition (Nevins *et al.*, 1997a). In addition to using wild-type calcineurin A, two mutants were used: 1) truncated calcineurin A (1–397), which is Ca^{2+} /CaM-independent; and 2) full-length calcineurin A containing the H151Q mutation, which is inactive and acts as a dominant negative in some cases (Shibasaki and McKeon,

Figure 3. Inhibition of pRb phosphorylation and cyclin D1 accumulation by cyclosporin A. (A) Cdk2 IP kinase assays. Serum-starved WI-38 cells were stimulated with growth media in the presence or absence of cyclosporin A and then harvested at 16 and 20 h. Cdk2 complexes were immunoprecipitated from cell lysates followed by an in vitro kinase assay with histone H1 as a substrate. (B) Cdk4 IP kinase assays. For cdk4 assays, cells were harvested at 18 h and then cdk4 complexes were immunoprecipitated and assayed for activity by using GST-pRb CT as a substrate. As a measure of nonspecific activity in the immunoprecipitation, extracts were immunoprecipitated in the presence of the cdk4 peptide (used to generate the immunoprecipitating cdk4 antibody) that prevents cdk4 immunoprecipitation and demonstrates minimal activity against the GST-pRb substrate. (C) pRb Western analysis. WI-38 lysates were separated by SDS-PAGE, and pRb was detected by Western blotting. Hypophosphorylated pRb migrates as a single band, whereas hyperphosphorylated pRb migrates with a reduced mobility shift. (D) Cyclin D1 and cdk4 Western analyses. The expression of cyclin D1 and cdk4 were determined at 18 h after serum addition by Western blotting. (E) Time course of cyclin D1 mRNA accumulation. Equal amounts of total RNA (15 μ g) from cells at 1.5 and 4 h after serum addition were subject to Northern analysis by using a radiolabeled probe from mouse cyclin D1. (F) Cyclin D1 mRNA accumulation with cyclosporin A. Equal amounts of total RNA from cells at 4 h after serum stimulation, with and without cyclosporin A, were subject to Northern analysis for cyclin D1 expression.

1995; Mondragon *et al.*, 1997; Sun *et al.*, 1998). For all three calcineurin A constructs, coinfection with calcineurin B was required for calcineurin A expression (Figure 5A). Although we wanted to test whether the inactive calcineurin A acted as a dominant negative and mimicked the results of cyclosporin A, we were unable to express calcineurin in serum-starved cells and only began to detect expression between 7 and 10 h after serum addition (Figure 5B). Because cyclosporin A did not effectively inhibit reentry when added at 8 h after serum stimulation, we reasoned it was highly unlikely that the inactive calcineurin A would have any effects due to its delayed and low level expression in G_1 . Although Ca^{2+} /CaM-independent calcineurin A also demonstrated delayed expression, we reasoned that even low levels of this Ca^{2+} /CaM-independent construct would have

effects on cyclin D1 in mid- G_1 due to its constitutive activity. Therefore, we examined the synthesis of cyclin D1 by metabolic labeling of cells infected with calcineurin 1–397 plus calcineurin B versus control cells infected with green fluorescent protein (GFP) plus calcineurin B. In the presence of Ca^{2+} /CaM-independent calcineurin A, the initial labeling of cyclin D1 was greater than with GFP (Figure 5C). The average amount of newly synthesized cyclin D1 in the presence of calcineurin A 1–397 was ~250% that of control samples (Figure 5D). Therefore, the effect of Ca^{2+} /CaM-independent calcineurin A overexpression was opposite to the cyclosporin A effect on cyclin D1 synthesis, providing additional evidence that calcineurin A was the target of cyclosporin A in this Ca^{2+} /CaM-dependent pathway that regulates cyclin D1 translation in G_1 .

Figure 4. Reduction of cyclin D1 synthesis by cyclosporin A. (A) Cyclin D1 labeling in the presence of cyclosporin A. Serum-stimulated WI-38 cells, in the presence or absence of cyclosporin A, were pulse labeled with ³⁵S-EasyTag methionine/cystine (PerkinElmer Life Sciences) for 2 h between 6 and 8 h after serum addition. Endogenous cyclin D1 was immunoprecipitated, followed by separation of proteins by SDS-PAGE. (B) Half-life determination of cyclin D1. After the 2-h labeling, the media were removed and replaced with fresh media containing methionine/cystine for 40 min. PhosphorImager analysis was used to quantify the amount of ³⁵S-labeled cyclin D1, and the results are graphed as percentage of remaining versus time. CsA represents cells in the presence of cyclosporin A, and No Tx represents cells in the absence of cyclosporin A. (C) Inhibition of cyclin D1 synthesis by cyclosporin A. The amount of labeled cyclin D1 was determined in three independent experiments, with the cyclosporin A-treated samples expressed as a percentage of untreated samples, which was set to 100%. (D) Cyclin D1 labeling in the presence of MG-132. WI-38 cells were pulse labeled as described above in the presence of 10 μ M MG-132. Cyclin D1 immunoprecipitates were separated by SDS-PAGE.

DISCUSSION

Here, we demonstrate that cyclosporin A induces a reversible G₁ arrest in diploid human fibroblasts (WI-38 cells). Although numerous studies have documented the antiproliferative

effects of cyclosporin A in a variety of cell types, only a small subset of those studies have characterized the cell cycle arrest point induced by cyclosporin A. Where the nature of the cyclosporin A arrest has been evaluated biochemically, two themes have surfaced. First, cyclosporin A caused an elevation of p21 levels mediated via transforming growth factor- β in both adenocarcinoma cell lines and human T cells (Hojo *et al.*, 1999; Khanna and Hosenpud, 1999). Second, the cyclosporin A arrest was characterized by a loss of G₁/S cyclin expression in other cell types (Tomono *et al.*, 1998; Schneider *et al.*, 2002). In human fibroblasts, we found that cyclosporin A had no effect on p21 levels (our unpublished data) but effectively blocked the accumulation of cyclin D1 protein. Similarly, Schneider *et al.* (2002) found that cyclosporin A prevented cyclin D1 accumulation in pancreatic acinar cells without any change in p21 levels. In contrast to this cyclosporin A-induced reduction in cyclin D1, Tomono and colleagues found normal levels of cyclin D1, but reduced cyclins E and A, in Swiss 3T3 fibroblasts (Tomono *et al.*, 1998; Schneider *et al.*, 2002). Together, these results raise the question why cyclosporin A causes a loss of cyclin D1 in some cells, but not others. One possibility is the degree of immortalization and/or transformation of the cells, because Swiss 3T3 cells are immortalized, whereas WI-38 cells are not. It is possible that some mechanisms that regulate the level of cyclin D1 in normal cells are lost during immortalization and/or transformation. This idea is supported by the fact that tumor cell lines have dramatically different rates of cyclin D1 degradation, suggesting that one or more fundamental pathways regulating cyclin D1 turnover has been lost during tumorigenesis in some cell lines (Russell *et al.*, 1999). If the regulation of cyclin D1 mRNA accumulation and protein degradation is altered in some human tumor cells, it follows that the regulation of cyclin D1 translation may also be perturbed in some tumors.

Whereas cyclosporin A reduced cyclin D1 levels in both pancreatic acinar cells and WI-38 cells, the mechanisms responsible for cyclin D1 reduction are clearly distinct. In contrast to the result in pancreatic acinar cells in which cyclin D1 mRNA was reduced, we found no change in the levels of cyclin D1 mRNA between untreated and cyclosporin A-treated cells (Schneider *et al.*, 2002). Rather, using metabolic labeling of endogenous cyclin D1 in WI-38 cells, we show the primary effect of cyclosporin A to be a reduction in the amount of newly synthesized cyclin D1 protein with minimal alteration in the degradation of cyclin D1.

To support our pharmacological studies with cyclosporin A that were suggestive of a role for calcineurin in cyclin D1 translational regulation, we examined the effects of adenoviral expression of calcineurin A mutants (constitutively active, Ca²⁺/CaM-independent, or catalytically inactive, "dominant-negative"). As reported previously in other cell types, we found calcineurin A was expressed only when calcineurin B was coexpressed (Shibasaki and McKeon, 1995). However, none of the calcineurin A constructs were expressed in the serum-starved state and protein only began to accumulate several hours after serum stimulation. This result seems to be selective for calcineurin A because we have expressed numerous other adenovirally encoded proteins in WI-38 cells in the serum-starved state without difficulty.

Because we could not express the catalytically inactive calcineurin A to high levels in early G₁, we felt it would be unlikely to act as a dominant-negative mimicking the results found with cyclosporin A. On the other hand, we reasoned that even a low expression of constitutively active, Ca²⁺/CaM-independent calcineurin A may have effects because it

Figure 5. Promotion of cyclin D1 synthesis by expression of Ca^{2+} /CaM-independent calcineurin A. (A) Coexpression of calcineurin A and calcineurin B. Subconfluent WI-38 cells were infected with Ad-calcineurin A (wild-type, 1-397, and H151Q), with and without coinfection of Ad-calcineurin B, at multiplicities of infection of 100. Western analysis was performed using anti-calcineurin A and anti-calcineurin B. (B) Calcineurin A expression during reentry. Serum-starved WI-38 cells were infected with Ad-calcineurin A (1-397, H151Q) in the presence of Ad-calcineurin B. After 18 additional hours of serum starvation, cells were released into growth media and harvested at increasing times. Calcineurin A expression was determined by Western blotting. (C) Cyclin D1 labeling in the presence of calcineurin A overexpression. Serum-starved WI-38 cells were infected with either Ad-GFP or Ad-calcineurin A 1-397, both in the presence of Ad-calcineurin B. Cells were serum stimulated and pulse-labeled with ^{35}S -EasyTag methionine/cystine as described in text. (D) Promotion of cyclin D1 synthesis by calcineurin A overexpression. The amount of ^{35}S -labeled cyclin D1 was determined for three independent experiments.

would raise the level of calcineurin activity in the cell. Indeed, ectopic expression of Ca^{2+} /CaM-independent calcineurin A stimulated the synthesis of cyclin D1, assessed by metabolic labeling of cells in mid- G_1 . Therefore, this result supported our hypothesis that calcineurin was the target of cyclosporin A in WI-38 cells and may act to regulate the accumulation of cyclin D1 by regulating its translation.

Although the effects of cyclosporin A and calcineurin A are related to cyclin D1 translation in WI-38 cells, the regulatory mechanisms of cyclin D1 translation have not been extensively characterized. One potential mechanism might involve the cap-binding protein, eIF-4E, which has been shown to regulate cyclin D1 accumulation. Overexpression of eIF-4E-stimulated cyclin D1 protein accumulation in the absence of mRNA accumulation in NIH-3T3 cells (Rosenwald *et al.*, 1993). Whereas the effect eIF-4E on cyclin D1 accumulation was presumed to be specific, eIF-4E regulates the translation of a number of mRNAs, particularly those characterized by long, highly structured, G/C-rich 5'-untranslated regions (Gray and Wickens, 1998; McKendrick *et al.*, 1999). However, even though cyclin D1 has a G/C-rich

5'-untranslated region, no studies to date have examined this region with regard to translational regulation. Although we have not found evidence that cyclosporin A globally affects translation, it is possible that cyclosporin A affects the translation of a subset of mRNAs whose translation is dependent on eIF-4E. On the other hand, the relationship between eIF-4E and cyclin D1 may be more selective because additional studies have suggested that eIF-4E overexpression augmented the transport of cyclin D1 mRNA from the nucleus to cytoplasmic polysomes (Rosenwald *et al.*, 1993; Rosenwald *et al.*, 1995; Rousseau *et al.*, 1996).

Another mitogenic signaling pathway that has been reported to regulate cyclin D1 translation is the phosphatidylinositol-3 kinase (PI-3K) pathway. In MCF10A cells, the initial induction of cyclin D1 protein after mitogenic stimulation began before mRNA accumulation (Muise-Helmicks *et al.*, 1998). This result suggested that cyclin D1 translation was regulated by growth factors independent of its mRNA induction. The authors found that PI-3K inhibitors prevented cyclin D1 accumulation and that transfection of activated AKT into serum-starved cells prevented the nor-

mal down-regulation of cyclin D1 levels. We examined AKT phosphorylation after serum stimulation and found no difference in the induction of AKT phosphorylation in cyclosporin A-treated and untreated cells, suggesting that the PI-3K and AKT signaling pathway was activated normally in the presence of cyclosporin A (our unpublished data). However, the previous study addressed the block in cyclin D1 accumulation by herbimycin A, an ansamycin antibiotic (Muise-Helmericks *et al.*, 1998). These antibiotics target the chaperone Hsp90 and therefore lead to the degradation of a number of proteins, including many protein kinases. Muise-Helmericks and colleagues believe the primary effect of herbimycin A was on the PI-3K/AKT pathway because mitogen-activated protein kinase inhibitors had no effect on cyclin D1 in these cells. However, their results with herbimycin A are interesting because calcineurin activity was stimulated by Hsp90 in vitro and calcineurin A coimmunoprecipitated with Hsp90 from cell extracts (Someren *et al.*, 1999). Together, these results raise the provocative question of whether the herbimycin A effect on cyclin D1 translation might actually be mediated through Hsp90 regulation of calcineurin activity in vivo.

On mitogenic stimulation, inhibition of Ca^{2+} /CaM arrests cells at two points, early after mitogenic stimulation and later, near the G_1 /S boundary. Because cyclosporin A arrests cells very early in G_1 and prevents cyclin D1 accumulation, this cyclosporin A arrest point coincides with the early G_0 / G_1 requirement for Ca^{2+} /CaM, suggesting that calcineurin represents an initial Ca^{2+} /CaM target enzyme during G_1 and is required for the proper accumulation of cyclin D1.

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