Calcineurin Regulates Cyclin D1 Accumulation in Growth-Stimulated Fibroblasts

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Abstract

Calcium (Ca\textsuperscript{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\textsubscript{1} cell cycle arrest, implicating the serine/threonine phosphatase calcineurin as one Ca\textsuperscript{2+}/CaM-dependent enzyme required for G\textsubscript{1} transit. Here we show, in diploid human fibroblasts, that cyclosporin A arrested cells in G\textsubscript{1} prior to cyclin D/cdk4 complex activation and retinoblastoma (pRb) hyperphosphorylation. This arrest occurred in early G\textsubscript{1} with low levels of cyclin D1 protein. Since 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\textsubscript{1} progression. Therefore, calcineurin is a Ca\textsuperscript{2+}/CaM-dependent target that regulates cyclin D1 accumulation in G\textsubscript{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 G1 and close to the G1/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 G1 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 G1, with some cells arrested in G2 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 following 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 wide 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 G1 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. While cyclosporin A reduced the levels of cyclin D1 mRNA in pancreatic acinar cells (AR42J), mouse embryonic fibroblasts (MEFs) lacking calcineurin Aα had higher amounts of cdk4 mRNA and protein (Baksh et al., 2002; Schneider et al., 2002). Taken together, these results suggest that calcineurin function promotes cyclin D1 expression and inhibits cdk4 expression. Since both cyclin D and cdk4 are transcriptionally induced during G1, 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). Upon mitogenic stimulation, the D type cyclins accumulate and assemble with cdk4/6 to form active kinase complexes in mid-G1, 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, D type cyclins are critical for passage through the restriction point, a defined time in G1 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).

Since normal diploid fibroblasts are unlikely to harbor disruptions in either cell cycle or Ca2+/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 G1 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 Ca2+/CaM-independent calcineurin A, in which the C-terminal Ca2+/CaM-binding domain has been removed, promoted cyclin D1 synthesis, supporting the idea that calcineurin activity positively regulates cyclin D1 protein translation during G1 progression. Therefore, we propose one function of Ca2+/CaM-dependent pathways during early G1 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 MEM with Earle’s salts supplemented with MEM non-essential amino acids, MEM vitamins, L-glutamine, and 20% fetal bovine serum (FBS). For experiments, cells were plated at approximately 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 hours. To arrest WI-38 cells in mitosis, cells were serum starved as above, serum stimulated for 18 hours, and then, media containing 0.5 µg/ml nocodazole was added for 6 hours. To arrest WI-38 cells in S phase, cells were serum starved as above, serum stimulated for 10 hours, and then, media containing 2 µM hydroxyurea was added for 10 hours.

All cell culture media and supplements were purchased from Gibco-BRL. FBS was obtained from Sigma, Gibco-BRL, or Hyclone, 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, and nocodazole, from Sigma, were dissolved in DMSO. W-13, from RBI/Sigma or Seikagaiku, and hydroxyurea, from Sigma, were dissolved in sterile water.

Flow cytometry and BrdU incorporation

For flow cytometry, 0.5-1.0 x 10⁶ cells were trypsinized, pelleted by centrifugation, and resuspended in 200 µl PBS and 800 µl ethanol. Cells were fixed overnight at -20°C and resuspended in 0.5-1.0 ml DNA Prep (Beckman/Coulter).
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 (Becton Dickinson) and pulse-labeled with 10 μM BrdU for 30 minutes. Cells were fixed with cold methanol for 5 minutes at 4°C followed by permeabilization with 0.25 % Triton X-100 in PBS for 5 minutes. Samples were denatured with 2N HCl for 30 minutes, washed once with 0.1 M Na₂B₄O₇ and twice with PBS, and blocked with 1% BSA in PBS for 30 minutes. 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 one hour with goat anti-mouse FITC (Santa Cruz) diluted 1:200 in blocking solution. Samples were then washed three times with PBS, incubated with DAPI (5 mg/ml stock) diluted 1:10,000 in PBS for 5 minutes, and mounted under coverslips 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. and Sheaff (DeGregori et al., 1995; Sheaff, 1997). Cells were lysed in Cdk2 IP/RIPA Buffer and protein concentration was determined using DC Protein Assay Kit (Bio-Rad). For each sample, 500 μg total protein was immunoprecipitated using 2 μg anti-cdk2 (M2, Santa Cruz) and 10 μl Protein A Sepharose 4 Fast Flow (Amersham). The final kinase reaction was carried out in 50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM DTT, 10 μM ATP,
2.5 μCi per reaction of [γ-32P] ATP (Amersham), and 5 μg per reaction histone H1 (Roche). Samples were incubated at 30°C for 30 minutes 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. and, Phelps and Xiong (DeGregori et al., 1995; 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 total protein was immunoprecipitated using 2 μg anti-cdk4 (C-22 or H-22, Santa Cruz) and 10 μl Protein A Sepharose 4 Fast Flow (Amersham). The final kinase reaction was carried out in 50 mM HEPES pH 7.5, 10 mM MgCl2, 1 mM DTT, 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), and 2 μg per reaction GST-Rb 769-921 (Santa Cruz). Samples were incubated at 30°C for 30 minutes and processed as described above for the cdk2 assays.

**Western Analyses**

Following lysis in Cdk4 IP buffer, samples were assayed for protein concentration and 50-100 μg of protein (depending on assay) were 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 in order to ensure separation of the phosphorylated forms.

Following gel electrophoresis, proteins were transferred to Immobilin P (Millipore) based on the manufacturer’s recommendations for the electrophoretic transfer apparatus (Bio-Rad). Membranes were blocked for 1-2 hours in Blocking Solution (TBS 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 minutes with rocking. Signal was
detected chemiluminescently with ECL (Amersham). Primary antibodies included anti-Rb (C-19, Santa Cruz), anti-cyclin D1 (H-295, Santa Cruz), anti-cyclin D1 (UBI), anti-
cdk4 (C-22, Santa Cruz), anti-cdk4 (H-22, Santa Cruz), anti-cdk2 (M2, Santa Cruz), anti-
cyclin E (M-20, Santa-Cruz), anti- cyclin A (C-19, Santa Cruz), anti-calcineurin A (BD
Biosciences), and anti-calcineurin B (Sigma). The secondary antibodies were HRP-
conjugated goat anti-rabbit or anti-mouse (Jackson).

Northern Analysis

Total RNA was extracted from tissue culture cells using Ultraspec RNA (Biotecx Laboratories). Total RNA concentrations were determined and northern analysis was
derformed as described by Wu and colleagues (Wu, 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 bp fragment of the mouse cyclin D1 cDNA
was amplified by 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 re-probed with GAPDH as a loading
control.

Metabolic Labeling

The metabolic labeling of proteins is based on standard protocols for adherent
cells (Ausubel 2001; Querido, Blanchette et al. 2001). Since only Sigma provides
MEME without methionine/cystine, WI-38 cells were thawed into Sigma, rather than
Invitrogen, MEME with the usual supplements. Cells were serum stimulated with media lacking methionine/cystine and 20% FBS for 5-6 hours. The media was removed and replaced with a small volume (2.5 ml per 100 mm dish) of MEME without methionine/cystine and containing $^{35}$S EasyTag express protein labeling mix (Perkin Elmer) at a final concentration of $\sim 0.5$ mCi $^{35}$S methionine/cystine mix per 100 mm dish. Following a two-hour incubation, the labeling media was removed, cells were washed with PBS, and then, lysed in 1 ml Cdk2 IP buffer and frozen with liquid N$_2$. For immunoprecipitation, lysates were thawed and cleared by centrifugation. Cleared WI-38 lysate was incubated with 2-4 $\mu$g anti-cyclin D1 (M-20, Santa Cruz), and 10-20 $\mu$g packed resin for 2-4 hours at 4°C. The resin was washed extensively and proteins separated by SDS-PAGE. The amount of $^{35}$S-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 (He, Zhou et al. 1998). The methods used to generate these viruses were based on the initial description of the system, the website with updated protocols and sequence data (http://www.coloncancer.org/adeasy/protocol.htm), and the protocols published by Qbiogene (formerly, Quantum Biotechnologies). WI-38 cells were routinely infected as described by others (DeGregori, Leone et al. 1995; He, Zhou et al. 1998; Cook, Park 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. Wild type
calcineurin A and calcineurin B were amplified by PCR and subcloned into pAdTrack-CMV. The Ca^{2+}/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 EcoRI for calcineurin A and PmeI 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 re-addition 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 approximately 16-20 hours after serum stimulation as determined by monitoring either DNA content by flow cytometry (Figure 1A) or BrdU incorporation (our unpublished results). 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 results), 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 following serum readdition to starved WI-38 cells. Both cyclosporin A and W-13 dramatically reduced the number of cells entering S phase at 18 hours 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 results). Next, we evaluated whether the cyclosporin A
induced cell cycle arrest was G₁ selective or if cyclosporin A inhibited progression through other cell cycle phases. WI-38 fibroblasts were arrested in early S phase using hydroxyurea and then, released into fresh media to allow synchronous progression through S and G₂/M phases. Cyclosporin A had no effect on the ability of cells to progress through S and G₂/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 G₁ following entry from mitosis. WI-38 fibroblasts were arrested in metaphase using nocodazole and then, released into fresh media to allow synchronous progression through mitosis and into G₁. Whereas cyclosporin A had no effect on the ability of cells to exit mitosis and enter G₁, it prevented cells from entering S and G₂/M phases (Figure 2C). These results demonstrate that the cyclosporin A arrest is specific to G₁ and that this G₁ arrest occurs no matter if cells are entering G₁ from quiescence or from mitosis.

In order to evaluate which G₁ pathways cyclosporin A affects, we questioned if the cyclosporin A arrest occurred in early or late G₁. First, we asked if 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 hours to enter S phase following drug removal (Figure 2D). Since this was similar to the time needed to enter S from serum starvation, we reasoned cyclosporin A arrested cells in very early G₁. 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 following serum addition and determined the amount of BrdU incorporation at peak S phase, about 18 hours for WI-38 cells (Figure 2E). Cyclosporin A was most effective at preventing S
phase entry when added at the same time as serum. Since cyclosporin A arrested WI-38 cells in early G₁ progression, calcineurin may represent one essential target of Ca²⁺/CaM in G₁.

_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 G₁ cdk’s, cdk2 and cdk4. Cdk2 kinase complexes were immunoprecipitated and assayed for activity using histone H1 as a substrate. In control cells, cdk2 activity appeared by 16 hours after serum addition and was dramatically increased at 20 hours (Figure 3A). Cells treated with cyclosporin A demonstrated no cdk2 activity at either 16 or 20 hours. 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. Since cdk4 activity is required prior to cdk2 activity during normal G₁ progression, we analyzed cdk4 activity by immunoprecipitating cdk4 kinase complexes followed by kinase assays using GST-Rb as an _in vitro_ substrate. Although control cells demonstrated cdk4 activity at 18 hours 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 hours after stimulation, pRb became hyperphosphorylated, as demonstrated by reduced mobility, and the total amount of protein increased. Cyclosporin A treated cells appeared similar to serum starved cells with pRb exclusively in the hypophosphorylated form.
A major rate-limiting step in cdk4 activation during G1 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). Since 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 hours after serum addition (Figure 3E). Next, we examined cyclin D1 mRNA at 4 hours after serum addition in the presence of cyclosporin A and found similar levels in both treated and untreated cells (Figure 4F). Since cyclin D1 mRNA was upregulated 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 two-hour labeling period in mid-G1, we found cyclosporin A treated cells had dramatically less labeled cyclin D1 (Figure 4A). This effect appeared selective for cyclin D1 since we found similar labeling of total cell extracts. Additionally, we detected no reduction in the labeling of cdk4 (our unpublished results), 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 about 40% that of...
untreated cells (Figure 4B). In WI-38 cells, the half-life of cyclin D was about 25 minutes, 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 two-hour 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 if inhibition of degradation affected the labeling of cyclin D1. As expected, in untreated cells, MG-132 treatment increased the amount of labeled cyclin D1 two-fold (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, 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 if 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 hours after serum addition (Figure 5B). Since cyclosporin A did not effectively inhibit reentry when added at 8 hours 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 G1. 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 G1 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 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 about 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 G1.
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 wide 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 point has been evaluated biochemically, two themes have appeared. First, cyclosporin A caused an elevation of p21 levels mediated via TGFβ in both adenocarcinoma cell lines and human T cells (Hojo et al., 1999; Khanna and Hosenpad, 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 results) but effectively blocked the accumulation of cyclin D1 protein. Similarly, Schneider and colleagues also found that cyclosporin A prevented cyclin D1 accumulation in pancreatic acinar cells without any change in p21 levels (Schneider et al., 2002). 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, as Swiss 3T3 cells are immortalized while 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, Ca\(^{2+}\)/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 since we have expressed numerous other adenovirally encoded proteins in WI-38 cells in the serum starved state without difficulty.
Since 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, Ca$^{2+}$/CaM-independent calcineurin A may have effects since it 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-G1. 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 of 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’ UTRs (Gray and Wickens, 1998; McKendrick et al., 1999). However, even though cyclin D1 has a G/C rich 5’ UTR, 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.
as 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 PI-3K pathway. In MCF10A cells, the initial induction of cyclin D1 protein following mitogenic stimulation began prior to mRNA accumulation (Muise-Helmericks 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 normal down-regulation of cyclin D1 levels. We examined AKT phosphorylation following 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 results). 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 since MEK inhibitors had no effect on cyclin D1 in these cells. However, their results with herbimycin A are interesting since calcineurin activity was stimulated by Hsp90 in vitro and calcineurin A coimmunoprecipitated with Hsp90 from cell extracts (Someren et al., 1999). Taken 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.

Upon mitogenic stimulation, inhibition of Ca\(^{2+}/\)CaM arrests cells at two points, early after mitogenic stimulation and later, near the G\(_1\)/S boundary. Since 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.
Acknowledgments

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Figure Legends

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 re-addition 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 FACS 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, A) and calcineurin A by western blotting.

Figure 2: Cell cycle inhibition in G₁ 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 DMSO, 15 µg/ml W-13, or 25 µM cyclosporin A. Cells were harvested at 18 hours after serum addition and analyzed by 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 hours 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 G₁ 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 hours for FACS analysis. 

E) **Timecourse of cyclosporin A addition.** Serum stimulated WI-38 cells, treated with cyclosporin A, were pulse-labeled with BrdU for 30 minutes and harvested at 18 hours 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.

**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 hours. Cdk2 complexes were immunoprecipitated from cell lysates followed by an *in vitro* kinase assay using histone H1 as a substrate. 

B) *Cdk4 IP kinase assays.* For cdk4 assays, cells were harvested at 18 hours and then, cdk4 complexes were immunoprecipitated and assayed for activity using GST-pRB CT as a substrate. As a measure of non-specific 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, while 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 hours after serum addition by
western blotting. **E) Timecourse of cyclin D1 mRNA accumulation.** Equal amounts of total RNA (15 µg) from cells at 1.5 and 4 hours after serum addition were subject to northern analysis 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 hours after serum stimulation, with and without cyclosporin A, were subject to northern analysis for cyclin D1 expression.

**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 $^{35}$S-EasyTag methionine/cystine (Perkin Elmer) for 2 hours between 6 and 8 hours after serum addition. Endogenous cyclin D1 was immunoprecipitated, followed by separation of proteins by SDS-PAGE. **B) Half-life determination of cyclin D1.** Following the two-hour labeling, the media was removed and replaced with fresh media containing methionine/cystine for 40 minutes. PhosphorImager analysis was used to quantify the amount of $^{35}$S-labeled cyclin D1 and the results are graphed as percent 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 percent 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 above in the presence of 10 µM MG-132. Cyclin D1 immunoprecipitates were separated by SDS-PAGE.
**Figure 5: Promotion of cyclin D1 synthesis by expression of Ca^{2+}/CaM-independent calcineurin A.**

* A) *Co-expression 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 MOIs 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 earlier.  

* D) Promotion of cyclin D1 synthesis by calcineurin A overexpression.* The amount of ^{35}S-labeled cyclin D1 was determined for three independent experiments.
References


Figure 1

A)

B) 0 4 8 12 16 20 24 Time (hrs)

Cyclin D1

Cyclin E

Cyclin A

Calcineurin A
Figure 2

A) Vehicle W13 CsA

B) HU T=6 CsA T=12 CsA

C) NOC T=2 CsA T=10 CsA T=13 CsA T=16 CsA

D) T=18 CsA T=12 Post CsA T=20 Post CsA

E) 

S Phase (%) Time of CsA Addition

0 10 20 30 40 50 60

T=0 T=4 T=8 T=12
Figure 3

A) IP: cdk2

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$^{32}$P Histone H1

B) IP: cdk4

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$^{32}$P GST-pRb CT

C) pRb-P

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pRb

D) Cyclin D1

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Cdk4

E) Cyclin D1

| Time (hrs) | 0  | 1.5 | 4  |

F) Cyclin D1

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Figure 4

A) $^{35}$S Cyclin D1

B) 

C) 

D) $^{35}$S Cyclin D1
Figure 5

A) 

IB: CnA

Endogenous CnA
CnA (full length)
CnA (1-397)

IB: CnB

CnB
Ad-CnB

Ad-CnA wt
Ad-CnA 1-397
Ad-CnA H151Q

B) 

T=0  T=4  T=7  T=10

CnA (full length)
CnA (1-397)
Ad-CnA

C) 

35S Cyclin D1
35S GFP

Ad-GFP 1-397
Ad-CnA 1-397

D) 

Percent

Ad-GFP + Ad-CnB
Ad-CnA 1-397 + Ad-CnB