Cell Cycle Studies Based Upon Quantitative Image Analysis

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Abstract

When cell cycle studies are performed following cell cycle synchronization, it is possible that critical properties of an actively cycling cell will be overlooked. For this reason past studies have not revealed critical aspects of cell cycle control; such as how a cell determines when to exit the cell cycle, or how rapidly it should cycle. To address these challenging questions we have developed a procedure to quantitate fluorescent stains in a monolayer culture, where nuclear fluorescence and cell cycle history can be assessed with accuracy on a cell by cell basis. The cell cycle position of each cell can be determined by analyzing DNA and BrdU levels. The behavior of cells in a given cell cycle position can then be studied by quantitating up to two other stained markers. When the microinjection of siRNA, neutralizing antibodies, and expression plasmids are coupled with quantitative image analysis, these cell cycle studies can be conducted following alterations in the expression levels of selected cellular targets. With these techniques we have discovered critical aspects of cell cycle control; including how cyclin D1 levels vary through the cell cycle, the molecular mechanisms governing these changes, and the biological implications of changes in cyclin D1 concentration in various cell cycle stages. Our studies with cyclin D1, coupled with similar studies of p27Kip1, form the basis of an entirely new model of cell cycle control proposed here. This model explains how cell cycle progression is terminated, and how the length of the cell cycle is regulated.

Key terms

cyclin D1; cell cycle; quantitative image analysis; p27Kip1

CYCLIN D1 and p27Kip1 (p27) occupy a unique position among cell cycle control proteins in that they are regulated by the extracellular signaling environment of the cell, and in turn serve to control the activity of the cell cycle control machinery (1). These two molecules, therefore, play critical roles in relaying proliferative information into the cell. Cyclin D1 levels are regulated by a wide range of stimuli, but most importantly by proliferative signaling through Ras. Regulation at the level of transcription and translation together with mRNA and protein stability are each important. Phosphorylation of Thr286, however, has been recognized as being particularly important in determining the stability of cyclin D1, and thereby its expression level (2). This phosphorylation has been reported to be controlled by glycogen synthase kinase 3 (GSK3) (3), which is in turn regulated by the phosphatidylinositol-3 kinase/AKT pathway and therefore by proliferative signaling. Further studies suggest that regulation involves transport into and out of the nucleus through the cell cycle (4), while the overall expression level of cyclin D1 has not generally been believed to vary significantly through the cell cycle.

Once formed, cyclin D1 functions by binding cyclin dependent kinase 4 (CDK4) to form a kinase for the retinoblastoma protein (Rb). Upon phosphorylation by cyclin D1/CDK4, the Rb protein ceases to be an inhibitor for the E2F transcription factor, which then is able to direct production of proteins involved in cell cycle
progression and DNA synthesis (5). There is evidence that cyclin D1 functions primarily to phosphorylate Rb, although it is also reported to titrate p27 away from CDK2 to allow for the initiation of DNA synthesis (6).

The above studies provide a compelling and simple explanation for the cell cycle control properties of cyclin D1. This simple explanation, however, fails to answer some critical questions related to cell cycle control. Even though up to 30% of breast tumors have elevated cyclin D1 expression, this is not a highly common event in tumor formation. Why is cyclin D1 not more commonly induced to high levels in tumors? This question might be answered by the fact that cyclin D1 has also been observed under a number of circumstances to be inhibitory for growth (7,8), an observation not well explained by the above biochemical studies. Moreover, while we understand how the elevation of cyclin D1 expression is critical for progression from quiescence into S phase, we have little understanding of the potential role of cyclin D1 in the regulation of cell cycle progression once the cell has entered the cell cycle. As the following discussion will show, the simple model for cyclin D1 activity described above is correct only in general details.

Our technical approach utilizes cells remaining attached to a coverslip throughout the analysis. This makes it possible to combine quantitative image analytical procedures, microinjection, and time-lapse analyses of the same cells. Importantly, quantitative analysis of high resolution microscopic images of attached cells makes it possible to analyze fluorescence levels specifically of the nucleus, with minimal interference from cytoplasmic contamination or background light scatter. Individual cells can be followed in time-lapse to determine their proliferative history prior to analysis. Finally, the microinjection of appropriate materials can enhance or reduce the expression of selected cellular proteins, allowing an analysis of their functions in cells from specific cell cycle stages. In this way our approach is fundamentally different from flow cytometric analysis, where critical quantitative analysis of cellular fluorescence markers is performed upon detached cells (9,10). With our approach we show that the regulation of cyclin D1 through the cell cycle is much more detailed than previously understood; and have answered the critical questions discussed above concerning the roles of cyclin D1 and p27 in cell cycle control.

**Time-lapse, Microinjection, and G2 Phase**

**Technical Approach**

The technique of single cell microinjection involves physically inserting a fine glass capillary (0.5 microns O.D.) into either the nucleus or cytoplasm of monolayer cells. Injections vary in size but range near 5% of the total cell volume. No long-term adverse effects from the microinjection itself have been observed, although mitosis may be delayed for 1–2 h. Proteins, DNA expression plasmids, and siRNA are routinely injected and behave as expected almost immediately within the recipient cell.

Time-lapse movies consist of a timed series of still images viewed in sequence. A CCD camera can be controlled by a public domain software program such as NIH Image, Image J, or Sion Image; or by a variety of commercial programs to collect sequential images. Light is shuttered between images to reduce the amount of light falling upon living cells, particularly for fluorescence time-lapse. The movie is viewed as the computer displays sequential images. The environment of the cells during time-lapse is critical. Several commercial environmental chambers are available, but we have found that a simple bell jar covered with an optical glass plate placed over the cells upon a rigorously controlled heating stage works well. It is necessary to have a gentle but constant flow of moist, 5% CO₂ in air pass over the cells. We ensure that the medium does not evaporate by placing on the heating stage a donut shaped reservoir filled with water placed around the plate of cells being observed. To avoid condensation on the bell jar a heating strip is placed around the outside of the upper portion of the bell jar. Under these conditions cells can be viewed for many days, and grow at a rate similar to parallel plates in an incubator.

**Biological Application**

Our first challenge was to determine exactly when in the cell cycle of an actively growing cell it makes the commitment to continue cycling. We first followed cells in time-lapse for 20 h to determine the age of each cell, or the time since passing through mitosis. Previous studies with time-lapse and BrdU labeling indicated the normal lengths of each cell cycle period following mitosis in these cells. The cells were removed from time-lapse observation, and those within an area designated by a circle drawn on the back of the cover slip were microinjected with anti-Ras antibody (which took only 15 min). Cells were immediately returned to time-lapse observation and followed for another 30 h. The age of each cell at time of injection could then be compared to the effects of the anti-Ras antibody, which has been shown to completely and immediately neutralize all Ras activity within the cell. The results of this analysis were totally unexpected. All injected cells divided exactly one time following these injections. For comparison, microinjection of anti-cyclin D1 antibody blocked cell cycle progression if introduced at any time during G1 phase (11). This experiment indicated that no matter when anti-Ras is introduced into the cell, the cell progresses through the cell cycle to G2 phase, where the absence of Ras activity directs it to enter quiescence immediately following the upcoming mitosis (see Fig. 1). Previous to these studies G2 phase had been considered merely a period of preparation for mitosis. This experiment was the first to demonstrate the critical role played by G2 phase in cell cycle control, but other studies to be mentioned below substantiate this observation.

**Quantitative Image Analysis and Cyclin D1 Expression**

**Technical Approach**

The next challenge was to determine how proliferative signaling during G2 phase is able to promote a commitment...
to continued proliferation. To address this question, we utilized the quantitative image analysis technique recently developed in the laboratory. All digital images contain a wealth of quantitative data, but this information must be extracted carefully. The brightness of illumination across a microscopic field varies depending upon the configuration of the microscope, the type and age of the bulb, and the position of filters. The first step in quantitating a microscopic image is to correct for this uneven illumination. The Metamorph program and the Image J public domain software program allow for this correction based upon a shading image taken of a sample in which fluorochrome is evenly distributed on the slide. For example, a fluorescent solution can be placed underneath a cover slip and photographed for the shading image. Once corrected for uneven illumination, it is next necessary to identify each individual cell or nucleus. This is accomplished by analyzing a shaded image of DAPI stained nuclei, or cells uniformly stained. Objects stained positively with predetermined characteristics are identified by threshold analysis, followed by the creation of a mask image with open areas in the location of each object of interest (Fig. 2). These open areas are used by the program for quantitative image analysis of companion images of different fluorochromes (Fig. 2). Each measured image must also be corrected for shading, and obviously must be in exact register with the mask image. Leica, for example, has a filter cube tray designed to ensure that the images taken with each filter are in exact register. There is an option in the Metamorph program to adjust the position of individual images, but this feature is time consuming and impractical for routine analysis. The CCD camera used must be highly sensitive and monochromatic (12).

In this way, the cell cycle stage of individual cells can be determined by the amount of DNA determined from DAPI fluorescence, and from the incorporation of BrdU stained with a specific antibody detection procedure (Boehringer, Mannheim). The cell cycle stages of each cell can then be directly related to the fluorescence staining of up to two other markers. Careful correlations between staining intensity and Western analysis must be utilized with each antibody to ensure that staining is proportional to protein concentration within the cell, and to verify that the antigen is not masked during different cell cycle positions or other conditions. With this approach

**Figure 1.** Ras is required during G2 phase for continued proliferation. In the above model, small round circles indicate the time of microinjection of anti-Ras antibody into cells in different cell cycle positions. The line extending from these small circles indicates the progression of the cell through the cell cycle after injection. Only when a cell passes through the shaded area of ras requirement is cell cycle progression affected. After passing through this cell cycle period, the cell completes mitosis and enters quiescence. In this way, each injected cells passes through mitosis exactly one time following injection of anti-Ras antibody. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

**Figure 2.** Digital image analytical technique: For digital image analysis, separate images of a given field of cells are taken with filter sets to visualize each individual fluorochrome. In this figure, images of DAPI stained DNA, and fluorescent antibody markers for BrdU (CY5), cyclin D1 (CY3), and phosphorylated cyclin D1 (CY2) were also taken, and are shown following shading correction. The DAPI image was utilized for generation of the masked image also shown. In this image, the position of each individual nucleus is identified and the number indicated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
Biological Application

When cells were stained for cyclin D1 and subjected to the above image analytical procedure, we obtained a clue as to how Ras activity during G2 phase promotes a commitment to continued proliferation. Cyclin D1 levels were high in G2 and G1 phase nuclei, but extremely low in S phase cells (Fig. 3). A review of the past literature has provided some rational for this cell cycle expression characteristic. Cyclin D1 has been observed to block DNA synthesis by virtue of its ability to bind PCNA (13,14). It is also critical to emphasize that while cell cycle expression characteristics of cyclin D1 are most apparent when studied by quantitative image analysis, biochemical studies have confirmed this expression pattern (15).

Careful quantitation of the cytoplasm of these cells failed to reveal any indication that cyclin D1 had been exported to the cytoplasm during S phase (12). Previous uncertainties regarding this conclusion might have resulted from the difficulty of determining the overall fluorescent intensity of the cytoplasmic region, particularly when the staining intensity of the nucleus varies dramatically. Without quantitative data, which takes into account the area of the nucleus and its thickness, the generally greater background staining of the cytoplasm can readily be misunderstood to account for export of nuclear cyclin D1 into the cytoplasm. For biological considerations, however, the distinction between these two possibilities is critical. As will be shown later, the suppression of cyclin D1 during S phase plays an essential regulatory function. It is clearly much different to simply export a protein from the nucleus, compared to actually degrading that protein. Thus, quantitative image analysis provided an understanding of the regulation of cyclin D1 through the cell cycle.

Manipulations of Cellular Proteins

Technical Approach

When neutralizing antibodies, recombinant proteins, siRNA, or DNA plasmids are microinjected, the expression/activity of endogenous proteins can be altered. Quantitative image analysis then allows a determination of the extent of this alteration, and an identification of the positively microinjected cells. Importantly, it is possible to quantitatively determine the extent of endogenous protein ablation or stimulation on a cell by cell basis, and relate this to the behavior of each cell. In many cases, it is also possible to directly measure alterations in the activity of a protein by analyzing phosphorylation or the behavior of a substrate.

Biological Application

The above results suggest that Ras activity during G2 phase might be involved in promoting the elevation of cyclin D1, which in turn signals the continuation of proliferation. Two experiments were next performed to validate this proposal. First, anti-Ras antibody was microinjected into cycling cells, which were then fixed and stained at various times thereafter for the cell cycle distribution of cyclin D1. The injected antibody was shown to work immediately following injection by its ability to block migration and activation of ERK (16). We found that the anti-Ras antibody blocked elevation of cyclin D1 during G2 phase several hours before the levels of cyclin D1 in G1 phase were affected (12). It was apparent that the maintenance of cyclin D1 during G1 phase was largely Ras-independent, while the elevation of cyclin D1 during G2 phase was highly dependent upon continued Ras activity. Next, we microinjected oncogenic Ras protein, previously shown to actively transform NIH3T3 cells and function in all cell cycle stages. Once again, the cells were fixed and stained for cyclin D1 at various times following injection. We found that the levels of cyclin D1 were first elevated only in G2 phase cells (16). With time, as these cells cycled into G1 phase, the levels of cyclin D1 in G1 phase cells increased. In both cases, the manipulation of cellular Ras activity confirmed that the increase in cyclin D1 levels during G2 phase are absolutely dependent upon Ras activity, and that it is only in this cell cycle period that Ras has the ability to increase cyclin D1 levels, at least initially. These observations serve as strong support to our model, in which Ras induces cyclin D1 levels during G2 phase in order to promote continued cell cycle progression.

As a final confirmation of this model, we microinjected a plasmid able to drive the expression of cyclin D1. Injected cells were then followed in time-lapse to determine their proliferative behaviors, and stained to determine cyclin D1 levels. Serum was removed from the cultures following cyclin D1 plas-
mid injection. We found that in those cells which expressed exogenous cyclin D1 near or slightly above endogenous levels, the cells continued to proliferate even in the absence of serum (17). Thus, if cyclin D1 is artificially elevated during G2 phase, the requirement for growth factors is dramatically reduced.

These data, therefore, confirm our model of cell cycle control. This model represents a total departure from the conclusions reached on the basis of traditional biochemical observations. Importantly, our model answers the questions raised above regarding the inhibitory activity and tumor expression characteristics of cyclin D1. First of all, we now understand why high cyclin D1 levels are inhibitory for growth, as its expression during S phase blocks DNA synthesis. Second, we have formulated a model to explain why this control mechanism is of value to the organism. If a mutation were to promote high cyclin D1 expression, it might well promote entry into S phase independently of extracellular signaling. Such high levels of cyclin D1, however, would most likely overcome the ability of the cell to degrade it, resulting in cell cycle blockage during S phase. Thus, the cell has ensured the normal expression of this critical cell cycle regulatory protein by giving it both positive and negative proliferative activity in different cell cycle stages (18).

**Fluorescence Time-lapse and Thr286 Phosphorylation**

**Technical Approach**

In order to understand how cyclin D1 levels are reduced during S phase, we utilized fluorescence time-lapse analyses. As indicated above, time-lapse analyses are limited by the amount of light falling upon a cell. For phase-contrast cell cycle studies this is not generally a problem, but for fluorescence studies, where the intensity of light is high, it is a critical limitation. We have addressed the problem by utilizing a highly sensitive, back-thinned CCD camera cooled to \(-40^\circ\text{C}\). Even better results could be obtained with even greater cooling. A plasmid expressing the cyclin D1 sequence linked to the green fluorescent protein (GFP) sequence was introduced into NIH3T3 cells at 2 \(\mu\text{g}/\text{ml}\). This low DNA concentration was required to ensure that the expression of the exogenous protein remained in the range of endogenous protein levels, and therefore did not block proliferation. Alternatively, clones of NIH3T3 cells with stable expression of this plasmid, or a plasmid with Thr286 mutated to alanine, were selected. We found that exposures of 50–100 ms every hour allowed normal growth of cells. To limit background fluorescence, the culture medium was prepared without phenol red or riboflavin, and supplemented with charcoal/dextran absorbed fetal calf serum (HyClone, Logan Utah). Filter wheels containing excitation and emission filter sets were employed to allow observations of different fluorochromes during a single experiment, but were found to be a problem since the filter positions varied from observation to observation making shading corrections difficult. We were limited, therefore, to observation of a single fluorochrome, together with phase contrast images in a single experiment. To ensure that the focus remained constant throughout the experiment, we utilized indexed alignment tools, and at times auto-focus procedures at selected times during the analysis. The camera, filters, and focus were controlled by Metamorph software. A particular concern in these types of experiments, however, is the fact that the expression of an exogenous plasmid varies with time in individual cells. Thus, in the quantitative time-lapse analyses as described in the following, large numbers of cells had to be analyzed to make definite conclusions.

**Biological Application**

Mutations were introduced into a variety of sites in the cyclin D1 gene shown by others to be phosphorylated or otherwise involved in protein metabolism. Each of these mutants together with the wild type sequence were analyzed quantitatively through the cell cycle in fluorescence time-lapse. Fluorescence from the wild type cyclin D1-GFP declined during S phase as expected. Mutation of Thr286 (but no other site tested) produced a protein that even at low levels failed to decline during S phase (19). We conclude that phosphorylation of Thr286 is critical for the decline in cyclin D1 during S phase.

**Analysis of Multiple Markers Confirms Phosphorylation of Thr286**

**Technical Approach**

As indicated, up to four markers can be simultaneously quantitated for each cell in a given experiment. It is, of course, necessary that each cell occupy exactly the same position within each image, so that the quantitation can be compared between images (see Fig. 2). It is also critical to select appropriate filter sets to minimize fluorescence contamination between fluorochromes. We have routinely utilized CY2, CY3, CY5, and DAPI, with filters designed by Chroma. There is some limited overlap between channels, but this can be compensated for if necessary by determining the extent of this overlap with a singly stained sample, and then making mathematical corrections in the final results accordingly. In practice, we were able to adjust the strength of each stain to the extent that negligible overlap between filters was observed.

**Biological Application**

To confirm that phosphorylation of Thr286 is responsible for the suppression of cyclin D1 levels during S phase, we obtained an antibody against cyclin D1 phosphorylated on Thr286 from the M. Garrett laboratory (currently available commercially). Cells were stained with antibodies against cyclin D1 and phospho-cyclin D1 (19). This experiment, which has been repeated many times, consistently demonstrated that the extent of phosphorylation of cyclin D1 is significantly increased during S phase (Fig. 4). To extend this observation, cells were treated with MG132 to block proteasomal degradation. Following such treatments, the amounts of total cyclin D1 were observed to increase with time in a uniform manner through the cell cycle (19). This not only indicated that the degradation of cyclin D1 is largely responsible for the maintenance of its levels in actively cycling cells, but also that the decline during S phase is primarily the result of protein degradation. Moreover, this result indicated that the rate of
cyclin D1 synthesis is essentially constant through the cell cycle, since once proteasomal degradation was blocked, the amount of protein increased in all cell cycle phases at the same rate. Interestingly, however, we found that when serum was removed from an NIH3T3 culture, or when anti-Ras antibody was microinjected, cyclin D1 mRNA was reduced in all cell cycle phases simultaneously. The elevation of cyclin D1 during G2 phase, therefore, is due not only to the reversal of Thr286 phosphorylation, but to the stabilization of cyclin D1 mRNA by proliferative signaling (20).

**Identification of the Cyclin D1 Kinase**

With the above descriptions, we have reviewed the technical aspects of each of the novel procedures utilized in our studies. The following discussions will, therefore, focus upon the use of these and other techniques to further explore cell cycle regulation. Our first goal was to identify the kinase responsible for phosphorylation of Thr286. Recall from the above studies that GSK3 had been proposed to be this kinase. If this were the case, we would anticipate that the activity of GSK3 would have to vary through the cell cycle to account for the increase in phosphorylation during S phase. Since GSK3 is controlled by the phosphatidylinositol-3 kinase/AKT pathway, we also tested the possibility that the activity of these kinases might also vary through the cell cycle. As described earlier, the fact that oncogenic Ras was able to induce cyclin D1 levels only during G2 phase supported the possibility that the activity of signaling molecules might vary in different cell cycle phases. Our first analysis focused upon the activity of phosphatidylinositol-3 kinase in individual cells of an asynchronous culture. This kinase produces a phosphorylated lipid located at the plasma membrane. To assess the activity of this enzyme, therefore, we microinjected a plasmid expressing the PH domain, able to bind to the lipid product of phosphatidylinositol-3 kinase at the plasma membrane. The PH domain was linked to the GFP protein, such that movement of GFP to the plasma membrane resulted from strong activation of phosphatidylinositol-3 kinase (21). We found no evidence for an increase in phosphatidylinositol-3 kinase activity during any cell cycle phase. Rather, this protein seemed to be activated upon the addition of serum to any culture following its removal (22). This fact emphasizes the potential problems in studying quiescent cultures following serum readdition.

Further biochemical studies with cells synchronized in S phase failed to reveal any alterations in the activity of AKT or GSK3 during this cell cycle period. Next, GSK3 activity was inhibited with LiCl, other chemical inhibitors, and microinjected siRNA. While the phosphorylation of β-catenin, a known substrate of GSK3, was inhibited, the phosphorylation of cyclin D1 was not affected (22). Finally, constitutively active GSK3 expressed from a microinjected plasmid failed to alter cyclin D1 levels within the cell (22). We concluded that this is

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**Figure 4.** Staining of multiple fluorochromes shows an increase in S phase phosphorylation. NIH3T3 cells were pulsed with BrdU, fixed and stained for BrdU, DNA, total cyclin D1, and phospho-cyclin D1 with different fluorescent antibody stains. Images of each fluorochrome were analyzed and the results are presented. The profiles of total cyclin D1 (A), phospho-cyclin D1 (B), and BrdU (D) are presented, with the DNA level of each cell plotted against the appropriate marker. In addition, to indicate the rate of phosphorylation of Thr286, the ratio of phospho-cyclin D1 divided by the total cyclin D1 content is also plotted against DNA content (C), and shows the expected increase during S phase. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
not the kinase responsible for regulation of cyclin D1 through the cell cycle of any of the cells we analyzed. Moreover, recall that the removal of serum or the microinjection of anti-Ras antibody inhibited the elevation of cyclin D1 during G2 phase, but had no effect upon the decline during S phase. We concluded, therefore, that the phosphorylation of Thr286 is not regulated by proliferative signaling or any kinase controlled by this signaling. Rather, we hypothesize that the kinase responsible for Thr286 phosphorylation is regulated by the cell cycle itself. Thus, no matter what the signaling environment of the cell, the decline in cyclin D1 levels during S phase will take place. In biological terms, this makes sense: if cyclin D1 must decline for DNA synthesis to take place efficiently, then this decline must be a nearly automatic event.

Our efforts, therefore, focused upon the identification of a kinase that might be controlled by DNA synthesis directly, and would be independent of any other signaling event. We were lead by the observation by Bartek and coworkers indicating that the checkpoint kinase ATR is activated to a limited extent during normal DNA synthesis, and that this activation is essential for the overall regulation of the rate of passage through S phase (23). The possibility that this kinase might direct phosphorylation of cyclin D1 on Thr286 was first tested by inducing the activity of ATR by UV irradiation, which is believed to specifically induce ATR and not other checkpoint kinases. The UV-irradiated cells clearly demonstrated an increase in overall phosphorylation of cyclin D1 Thr286. As evidence, this was the result of ATR activation, microinjection of a plasmid expressing TopBP-1, the natural activator of ATR, also induced Thr286 phosphorylation. Finally, these two phosphorylation events were blocked by microinjected siRNA against ATR in the case of UV irradiation, and by caffeine in the case of TopBP-1 expression (Hitomi and Stacey, in preparation). We conclude that ATR does indeed have the capacity to promote the phosphorylation of cyclin D1 on Thr286. It is also critical to point out that UV irradiation also reduced overall cyclin D1 levels, consistent with its ability to promote degradation through Thr286 phosphorylation.

Our next goal was to determine if ATR directs Thr286 phosphorylation during normal cell cycle progression (although it is possible that ATR regulates the Thr286 kinase, rather than directly phosphorylating cyclin D1 itself). For this purpose, siRNA against ATR was microinjected into normally cycling cells. There was a consistent if limited suppression of cyclin D1 Thr286 phosphorylation following siRNA treatment; and this was localized in S phase cells. Interestingly, ATM, the companion checkpoint kinase to ATR, was also found to promote cyclin D1 phosphorylation on Thr286 following double stranded DNA breakage (Hitomi and Stacey, in preparation). While it is possible that factors in addition to ATR are involved in promoting Thr286 phosphorylation and cyclin D1 decline during S phase, we conclude that ATR at least is involved in this suppression.

SUMMARY OF CYCLIN D1 STUDIES

The molecular biology of cyclin D1 has been clearly described in the literature. Important aspects of cyclin D1 activity, however, were missed in those studies because of the difficulty in studying actively cycling cells with traditional biochemical techniques. Amazingly, the simple fact that cyclin D1 levels fall during S phase was generally overlooked, even though this fact is supported by studies of synchronized cells (24–28), FACS analysis (29), and staining studies (30). It was not until quantitative image analysis of monolayer cells was conducted that the profound extent of this decline became clearly appreciated (12). Critically, the overall role of cyclin D1 in cell cycle control cannot be appreciated without an understanding of this decline, and the subsequent increase in cyclin D1 levels during G2 phase. As a direct confirmation of the overall approach, it should be remembered that these studies suggested the possibility that ATR/ATM might direct Thr286 phosphorylation. The fact that our work has thereby lead to the first identification of a kinase able to control Thr286 phosphorylation directly supports the validity of our technical approach.

SUMMARY OF p27 STUDIES

The techniques described herein have also been utilized in an extensive study of p27, the other protein that together with cyclin D1 is able to link proliferative signaling to cell cycle control. For the purposes of this review, however, the results with p27 only will be summarized. p27 blocks the activation of CDK2 bound to cyclin E or cyclin A, and thereby, passage through S phase. Its levels are suppressed late in the cell cycle following ubiquitination by the Skp2-SCF complex. We found p27 levels to increase beginning early in G1 phase and reach maximal levels later in G1 phase. In actively cycling cells, p27 and cyclin D1 levels are closely related. So long as the levels of cyclin D1 and p27 remain proportional, the cell is able to continue proliferation. If p27 levels increase above cyclin D1 levels, G1 phase is at first lengthened and finally blocked (31). The accumulation of p27 during G1 phase is specifically suppressed by a pathway involving both MEK and phosphatidylinositol-3 kinase (32). To understand how p27 retards or blocks passage through G1 phase, we found that in quiescent cells stimulated to re-enter the cell cycle p27 levels gradually decrease. When p27 reaches a low enough level the cell becomes committed to enter S phase. Thus, suppression of p27 accounts for passage through the traditional restriction point (33). The low levels of p27 promote passage into S phase by allowing subsequent phosphorylation of CDK2. Thus, one of the primary functions of p27 is to block the activating phosphorylation of CDK2 on Thr160. The ability of p27 to block phosphorylation of CDK2 in actively cycling cells, however, is regulated by cyclin D1. Thus, cyclin D1 serves to modulate the inhibitory activity of p27 allowing this profoundly inhibitory molecule to exert incremental effects upon the rate of passage through G1 phase. The interactions between cyclin D1 and p27 are summarized in the overall model of cell cycle control presented in the following.

MODEL OF CELL CYCLE CONTROL

We propose the following model to describe the control of cell cycle progression (Fig. 5). During S phase p27 is sup-
pressed by Skp-2 ubiquitination, while cyclin D1 is suppressed by DNA synthesis-induced Thr286 phosphorylation. Upon entry into G2 phase, cyclin D1 levels are able to increase as phosphorylation of Thr286 is reduced, but this takes place only if the proliferative signaling environment of the cell promotes stabilization of cyclin D1 mRNA. As the cell enters G1 phase, cyclin D1 levels remain generally at the level achieved during G2 phase, while p27 levels begin to increase since it is no longer ubiquitinated by Skp-2. Newly synthesized p27 at first binds cyclin D1/CDK4, which keeps it from blocking the activating phosphorylation of cyclin E/CDK2. In the absence of proliferative signaling, p27 is able to increase to molar excess over cyclin D1 and consequently to block CDK2 and entry into S phase. In the presence of proliferative signaling, however, any p27 formed in excess over cyclin D1 is degraded, and CDK2 becomes active and S phase begins. Thus, proliferative signaling during G2 phase promotes an increase in cyclin D1 levels that enables a cell to become competent to continue proliferation. During G1 phase a combination of cyclin D1 binding and p27 degradation allows proliferative signaling to regulate the rate of passage into S phase and thereby the overall rate of proliferation.

The Overall Strength of This Technical Approach

Our technical approach has allowed us to make observations not readily apparent with other techniques. For example, previous analyses suggested that cyclin D1 was localized in the nucleus except during S phase, when most fluorescence became cytoplasmic. Quantitative analysis, however, left little doubt that the cytoplasmic levels remained constant through the cell cycle, and that overall cyclin D1 levels declined during S phase. This fact is central to our overall model of cell cycle control. The technique of quantitative image analysis is most effective when combined with time-lapse and microinjection. For example, the injection of anti-Ras antibody resulted in all cells moving to G1 phase. Only time-lapse analysis, however, revealed that each of the cells passed through mitosis on the way to G1 phase, and therefore that the inhibition had taken place during G2 phase. This experiment relied upon the fact that microinjected antibody neutralized Ras protein within 15 min (11), making it possible to observe its biological effects within the same cell cycle period in which microinjection took place (see also Ref. 34). As an example of the value of the results obtained with our approach, they directly lead to the identification of check point kinases as regulators of cyclin D1 Thr286 phosphorylation. As indicated, the critical results with this cytometric approach have consistently been validated with the use of more conventional types of analyses. It is important to emphasize, however, that our initial observations would have been extremely difficult to obtain if we had been limited to more conventional analyses. Finally, while our procedures were designed for cell cycle analyses, they should also be highly valuable in a variety of other experimental systems, where cells in various stages of a biological process are present in the same culture.

Literature Cited


