Cyclin D1 production in cycling cells depends on Ras in a cell-cycle-specific manner

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Background: Cellular Ras and cyclin D1 are required at similar times of the cell cycle in quiescent NIH3T3 cells that have been induced to proliferate, but not in the case of cycling NIH3T3 cells. In asynchronous cultures, Ras activity has been found to be required only during G2 phase to promote passage through the entire upcoming cell cycle, whereas cyclin D1 is required through G1 phase until DNA synthesis begins. To explain these results in molecular terms, we propose a model whereby continuous cell cycle progression in NIH3T3 cells requires cellular Ras activity to promote the synthesis of cyclin D1 during G2 phase. Cyclin D1 expression then continues through G1 phase independently of Ras activity, and drives the G1–S phase transition.

Results: We found high levels of cyclin D1 expression during the G2, M and G1 phases of the cell cycle in cycling NIH3T3 cells, using quantitative fluorescent antibody measurements of individual cells. By microinjecting anti-Ras antibody, we found that the induction of cyclin D1 expression beginning in G2 phase was dependent on Ras activity. Consistent with our model, cyclin D1 expression during G1 phase was particularly stable following neutralization of cellular Ras. Finally, ectopic expression of cyclin D1 largely overcame the requirement for cellular Ras activity during the continuous proliferation of cycling NIH3T3 cells.

Conclusions: Ras-dependent induction of cyclin D1 expression beginning in G2 phase is critical for continuous cell cycle progression in NIH3T3 cells.

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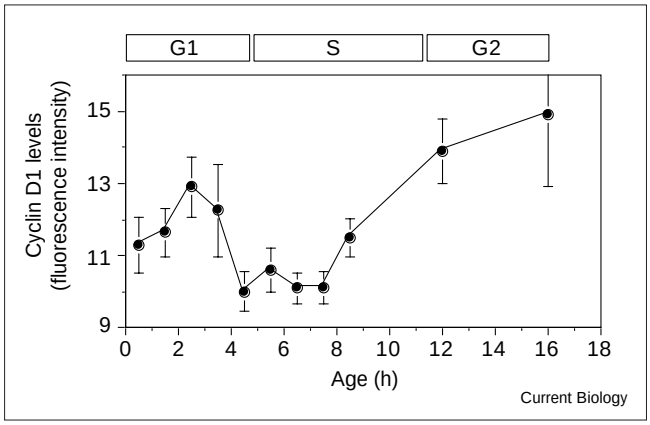
Background

The biological activities of cellular Ras and cyclin D1 are often closely connected [1–5]. Ras responds to activated growth factor receptors and is thus a growth-factor-sensitive molecular switch within the cell [6,7]. Its activity is transmitted through a cascade of kinases including Raf, MEK and the mitogen-activated protein (MAP) kinase Erk to induce the activity of transcription factors [8], some of which are directly involved in inducing cyclin D1 expression [2,9–11]. Thus, by virtue of its induction by Ras activity, cyclin D1 expression is directly responsive to the extracellular growth factor environment [12]. Both cellular Ras activity and cyclin D1 proteins are required for the proliferation of most normal cell types. Cyclin D1 expression is induced to high levels when quiescent NIH3T3 cells are stimulated to enter the cell cycle [13]. If induction of cyclin D1 expression is blocked, or its activity inhibited by anti-cyclin D1 antibodies, cell cycle progression is blocked [14,15], as is also observed when the cells are treated with anti-Ras antibodies [16]. In cells released from quiescence, Ras and cyclin D1 are both required near the restriction point [17,18], the time at which protein synthesis and growth factors cease to be required for entry into S phase [19]. The restriction point is located 4–5 hours before the beginning of DNA synthesis in NIH3T3 cells [18]. Most studies indicate that, upon entry into S phase, the cell is committed to complete mitosis [20], except under circumstances of unusual stress such as DNA damage.

The biochemical activity of cyclin D proteins is primarily related to their ability to combine with cyclin-dependent kinases (CDKs) 4 and 6, and to activate retinoblastoma protein (Rb) kinase activity [21]. This action releases the E2F transcription factor complex from inactivation by hypophosphorylated Rb, and promotes the synthesis of a variety of gene products directly involved in DNA synthesis. The critical role of cyclin D as a modifier of the Rb protein is demonstrated by the fact that it is dispensable in Rb-negative cells [22,23]. High levels of cyclin D protein shorten the length of G1 phase [1,15,24]. This might be due to its ability to activate CDK4/6, or to its ability to bind and thus inactivate CDK-inhibitory proteins, which would otherwise block the activity of other cyclin–CDK complexes [25]. Cyclin D1 expression has been reported during the G1 phase of quiescent cells stimulated to enter the cell cycle [26], and in the G2 phase of some tumor cells [27]. Of the three members of the cyclin D family, cyclin D1 is prominently expressed in NIH3T3 cells; other cyclins are also expressed in this and other cell types.

Figure 1



Time-lapse analysis of cyclin D1 expression in proliferating cells. A culture of cycling NIH3T3 cells was followed in time lapse for 20 h, fixed and stained with a fluorescent anti-cyclin D1 stain. The total amounts of fluorescence associated with the entire nuclear region of individual cells was determined quantitatively using a microscope equipped with a photomultiplier. The average fluorescence (cyclin D1 levels) of all cells of a given age is plotted (in arbitrary units ± SEM; the levels in quiescent cells being regarded as zero) against the average age of the cells. The approximate cell cycle position, on the basis of previous work [18], is indicated above the graph.

To determine the cell cycle requirement for Ras activity and cyclin D1 in cycling NIH3T3 cells, we have previously developed a time-lapse strategy to determine the effects of antibody microinjection on cell cycle progression. The results were unexpected as they were different to those previously described for quiescent cells [18]. Anti-cyclin D1 antibody was able to block entry into S phase even when introduced just before the beginning of DNA synthesis. Anti-Ras antibody, on the other hand, blocked entry into S phase only when introduced several hours before the beginning of DNA synthesis. Thus, in cycling NIH3T3 cells, Ras activity appeared to be largely unnecessary soon after cells had passed through mitosis [18]. This fact might indicate that Ras induces a factor(s) in G2 phase whose expression continues into G1 phase and is required for initiation of DNA synthesis. The fact that cyclin D1 is required through G1 phase, and typically is induced by cellular Ras activity makes it an attractive candidate for such a factor. Here, we have extended these observations. Experiments were designed to test the possibility that Ras activity during G2 phase induces the expression of cyclin D1, which then continues through G1 phase in a Ras-independent manner, and plays a critical role in promoting the G1–S phase transition. The validation of this hypothesis is important for understanding the signaling interactions required for cell cycle control in continuously proliferating cells.

Results

We postulated that, for cells to maintain active proliferation, growth factors must be present during G2 phase to induce cellular Ras activity, which then leads to the elevation of cyclin D1 levels. Cyclin D1 is required for the G1–S phase transition, so its expression must continue through mitosis and G1 phase, even though its expression becomes independent of Ras activity several hours before the G1–S phase boundary. This model makes four separate predictions which were tested here. First, cyclin D1 levels should be elevated before mitosis in rapidly cycling cells. Second, the expression of cyclin D1 should depend on Ras activity in all cell cycle phases. Third, once cycling cells enter G1 phase, cyclin D1 levels should remain high even in the absence of continued Ras activity. And fourth, cyclin D1 should be a primary proliferative target of Ras signaling, such that cyclin D1 plays a major role in promoting the cell cycle progression of cycling NIH3T3 cells even in the absence of continued cellular Ras activity.

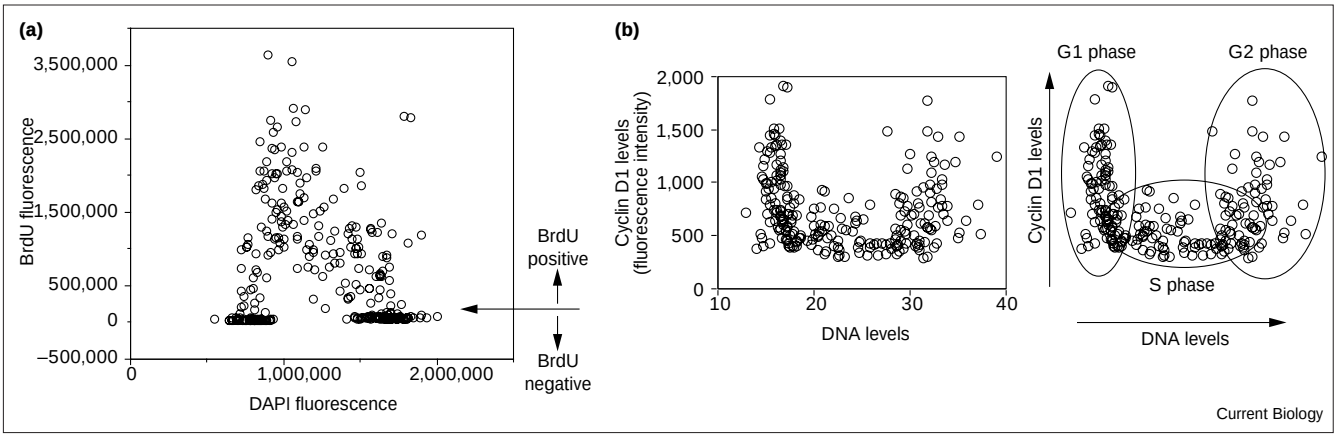
Time-lapse analysis of the expression of cyclin D1 during G2 phase

As a direct test of the first prediction, the levels of cyclin D1 in various cell cycle phases were determined using a time-lapse procedure to eliminate the need for cell synchronization [18]. An asynchronous culture was followed in time lapse for 20 hours, after which cells were fixed and stained for cyclin D1. The level of cyclin D1-associated fluorescence within the nuclear region was then quantitated on a cell-by-cell basis with a microscope equipped with a photomultiplier. From the time-lapse movie, it was possible to determine the approximate cell cycle position of an individual cell on the basis of its age (the time since it had passed through mitosis before fixation). Previous studies demonstrated that cells less than 4 hours old would be in G1 phase, cells between 5 and 12 hours old would most likely be in S phase, whereas cells greater than 12 hours old would begin entering G2 phase. The generation time of these cells is 16–18 hours [18]. For the purposes of presentation, cells of a given age were grouped together and the average fluorescence values were determined and plotted against the age of the group of cells (mean ± standard error). As expected, cyclin D1 levels were high in G1 phase and fell rapidly soon after the cells entered S phase. Significantly, after falling to low levels during early S phase, cyclin D1 levels began to increase as the cells progressed through S phase into G2 phase (Figure 1).

Double-staining strategy to determine cyclin D1 levels

To confirm the results from time-lapse analysis, a digital image analysis approach was used to determine cell cycle position from the DNA content of individual cells. The utility of this approach was first demonstrated by pulsing an asynchronous NIH3T3 culture with bromodeoxyuridine (BrdU) for 1 hour, followed by fixation and staining of DNA with 4,6-diamidino-2-phenylindole (DAPI) and the incorporated BrdU with a fluorescent antibody stain. Separate digital images of each fluorochrome were taken

Figure 2



Digital image analysis of cyclin D1 levels. (a) To test how reliably we could quantitate fluorescence levels in individual cells using the CCD camera, NIH3T3 cells were pulsed with BrdU for 1 h and fixed. DNA was stained with DAPI, and the incorporated BrdU was detected by indirect immunofluorescence. Separate fluorescence images of each fluorochrome were collected with the CCD camera and the fluorescence levels for individual cells determined by image analysis as described in the Materials and methods. The results (in arbitrary units) for each cell are plotted, with an indication of the position of BrdU-positive and BrdU-negative cells. (b) The above procedure was used to determine the levels of cyclin D1 throughout the cell cycle of cycling NIH3T3 cells. Cells in an asynchronous culture were fixed and stained with a fluorescent antibody stain against cyclin D1. The DNA was stained with DAPI. The nuclear intensities of cyclin D1 are plotted against the total DAPI staining for each cell analyzed. As an aid to interpretation, the same data are presented but with notations of the cells in different cell cycle stages (right). From these results, it is clear that the average cyclin D1 levels are high in cells in G1 and G2 phases, and low in S phase cells.

of identical areas of the coverslip with a charge-coupled device (CCD) camera. The DAPI staining identified nuclear regions in which the levels of both DAPI and anti-BrdU antibody fluorescence were quantitated. When DAPI fluorescence was plotted against BrdU-associated fluorescence for individual cells, cells in G1 and G2 phases were localized in well-separated groups without BrdU staining. S phase cells displayed intermediate DNA levels, and stained for BrdU, with the highest levels of staining found in cells in mid-S phase (Figure 2a). The DAPI fluorescence determined by this method was proportional to DNA content.

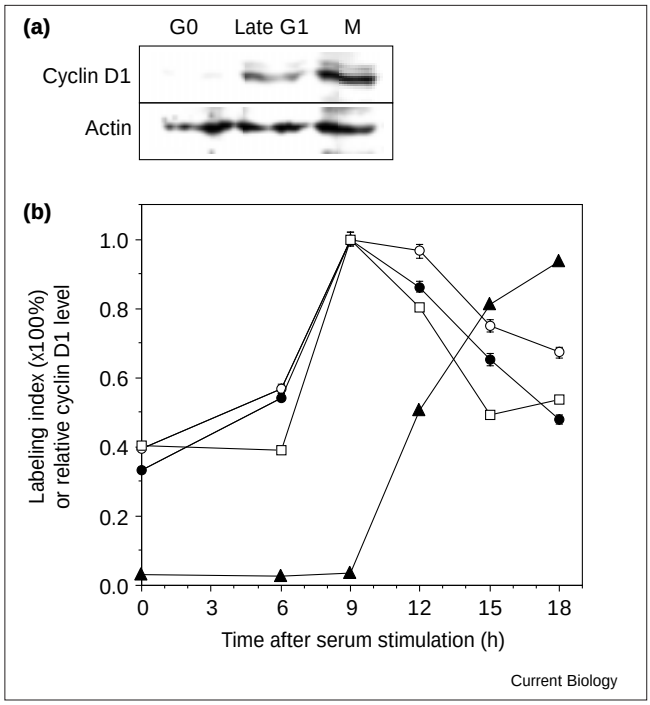
This quantitative immunofluorescence approach was used to determine cyclin D1 expression levels of individual cycling NIH3T3 cells. A rapidly proliferating culture was stained for cyclin D1 by indirect immunofluorescence and for DNA with DAPI. The nuclear fluorescence of individual cells was quantitated as above by digital image analysis. The results were completely consistent with those obtained by the time-lapse photomultiplier analysis. High levels of cyclin D1 were present in cells with G1 phase or G2 phase levels of DNA, but cyclin D1 levels were low in cells with S phase levels of DNA (Figure 2b). Together, these independent analyses confirm that cyclin D1 is indeed elevated during the G2 phase of cycling cells, as predicted by the model. In the time-lapse analysis, the absolute amount of cyclin D1-associated fluorescence was measured to obtain the total nuclear content of cyclin D1. In the double-staining experiments, on the other hand, the average fluorescence intensity was reported. In these cells, the fluorescence intensity measurement accurately corrects for the increase in size and DNA content of a cell as it progresses through the cell cycle, and indicates the relative cyclin D1 levels of cells in different cell cycle phases (see Supplementary material). The dramatic increase in cyclin D1 content as cells leave S phase and enter G2 phase was clearly evident from both measurements.

Confirmation of the antibody staining procedure

To demonstrate that the antibody staining procedure recognized only cyclin D1, analyses were performed on fibroblasts from a cyclin D1 knock-out mouse embryo and a wild-type littermate (generous gifts from Piotr Sicinski, [28]). *Cyclin D1*$^{+/+}$ mouse embryonic fibroblasts showed the same cell cycle-dependent cyclin D1 expression pattern as NIH3T3 cells. In *cyclin D1*$^{-/-}$ embryonic fibroblasts, on the other hand, only background levels of staining were observed regardless of the cell cycle phase (data not shown). Moreover, the pattern of cyclin D1 expression in NIH3T3 cells was also confirmed using a separate monoclonal antibody (DCS-6, a generous gift of Michele Pagano [14,18,29]).

Next, as levels of cyclin D1 were observed by antibody staining to be high in G2 and G1 phases, it followed that these levels would also be high in mitotic cells. To confirm this biochemically, M phase cells collected by mitotic detachment were immediately lysed and the cyclin D1 levels determined by western blot analysis.

Figure 3



Biochemical determination of cyclin D1 levels. (a) Mitotic cells (M) were collected from a cycling NIH3T3 culture by mechanical detachment and lysed. For comparison, lysates were prepared of quiescent cells (G0) and of quiescent cells 10 h after stimulation with serum. The amount of cyclin D1 is maximal around 9 h after serum stimulation of quiescent cells, as shown in (b). Equal amounts of protein were electrophoresed and subjected to western blotting with an anti-cyclin D1 antibody. As a control for loading, the amount of actin on the blot was also determined by western blotting. (b) Quiescent NIH3T3 cells were stimulated with serum and, after the indicated times, cells were harvested and analyzed by quantitative western blotting. Cyclin D1 levels were normalized to actin content (open squares), and the numbers reported as the percentage of the maximal signal at 9 h. A second set of parallel cultures were also fixed and stained for DNA and cyclin D1. The nuclear fluorescence associated with both stains was determined on a cell-by-cell basis by image analysis. The mean values (350–450 cells) of the fluorescence intensity of cyclin D1 (open circles), or the total cyclin D1 fluorescence normalized to DNA content (closed circles) are plotted. The error bars indicate standard errors. To monitor the G1–S transition, cells from a set of parallel cultures were labeled with tritiated thymidine soon after the serum addition. They were fixed at the indicated times, and the proportion of thymidine-labeled nuclei determined by autoradiography (triangles). It is clear from this analysis that the image analysis of stained cells accurately reflects the total amount of cyclin D1 in the culture as determined by western analysis.

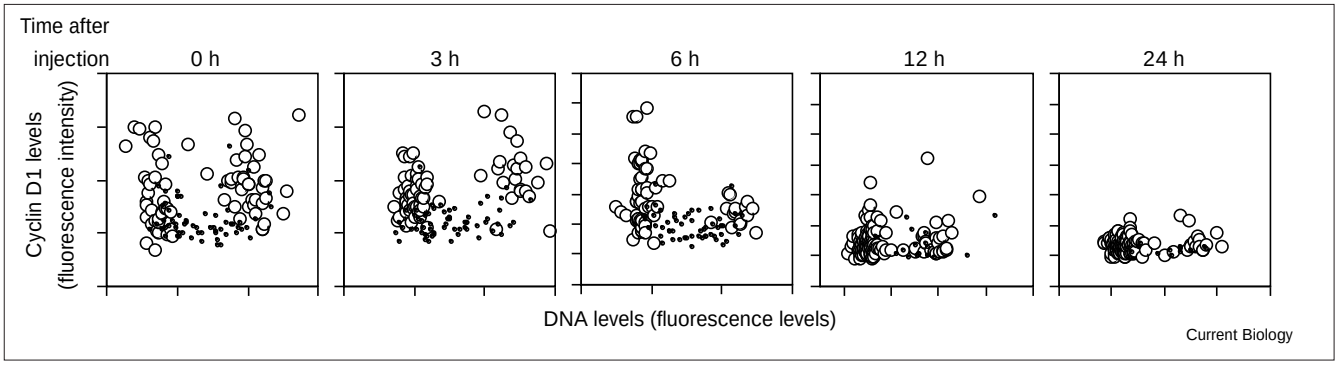
For comparison, the cyclin D1 levels were also determined in quiescent cells (in which cyclin D1 levels would be low), and in these cells stimulated for 10 hours with serum to induce high levels of cyclin D1 expression [18]. Mitotic cells contained even higher levels of cyclin D1 than stimulated NIH3T3 cells in late G1 phase (Figure 3a). The cyclin D1 protein levels in mitotic cells provide a direct biochemical confirmation of the staining results reported above.

Finally, it is possible that intermolecular associations might interfere with antibody binding to cyclin D1, resulting in underestimates of its level within the cell, even though the antibodies used for cyclin D1 staining (clone 72-13G) in this study can recognize cyclin D1 in an active protein complex [29–31]. To demonstrate directly that antibody staining intensity is proportional to cyclin D1 protein levels, both were determined in parallel cultures synchronized by serum deprivation (Figure 3b). At various times after serum re-stimulation, the levels of cyclin D1 determined in parallel cultures by quantitative western blotting or by digital analysis were compared. Cell cycle progression was monitored by thymidine labeling in a third set of parallel cultures. The fluorescence levels of cyclin D1 are presented as the simple fluorescent intensity as above, or as the total cyclin D1 fluorescence divided by the DNA content of each cell. The amount of cyclin D1 protein determined by western analysis was normalized to the levels of actin. Results at each time point are presented as a percentage of the maximum level (at 9 hours; Figure 3b). In each case, the levels of cyclin D1 were low for 6 hours following serum addition, increased to a maximum at 9 hours, and fell off as cells entered S phase beginning 12 hours after serum addition. This indicates that cyclin-D1-associated fluorescence intensity determined by the analysis of immunofluorescence faithfully represents the amount of cyclin D1 protein as determined by quantitative western blotting.

Dependence of cyclin D1 expression on endogenous Ras activity

Microinjection of neutralizing anti-Ras antibody followed by the double-staining strategy provided a means to test the second and third predictions of the model: that cyclin D1 expression is dependent on Ras, and that expression during G1 phase is relatively stable following removal of Ras activity. At various times after microinjection of anti-Ras antibody, cells were pulsed with tritiated thymidine for 1 hour and fixed. The cells were then stained for DNA with DAPI, and with separate antibodies against cyclin D1 and against the injected immunoglobulin (to identify injected cells). The cell cycle expression of cyclin D1 was determined using a microscopic photomultiplier by comparing its fluorescent intensity to total DNA-associated fluorescence; with thymidine-labeled and unlabeled cells noted from autoradiography. Although the method of determining fluorescence levels differed from that described above with the CCD camera, the overall approach was similar, and absolutely no differences were observed between results with the CCD camera (Figure 2b) and those obtained with the microscopic photomultiplier (Figure 4, 0 hours).

Figure 4



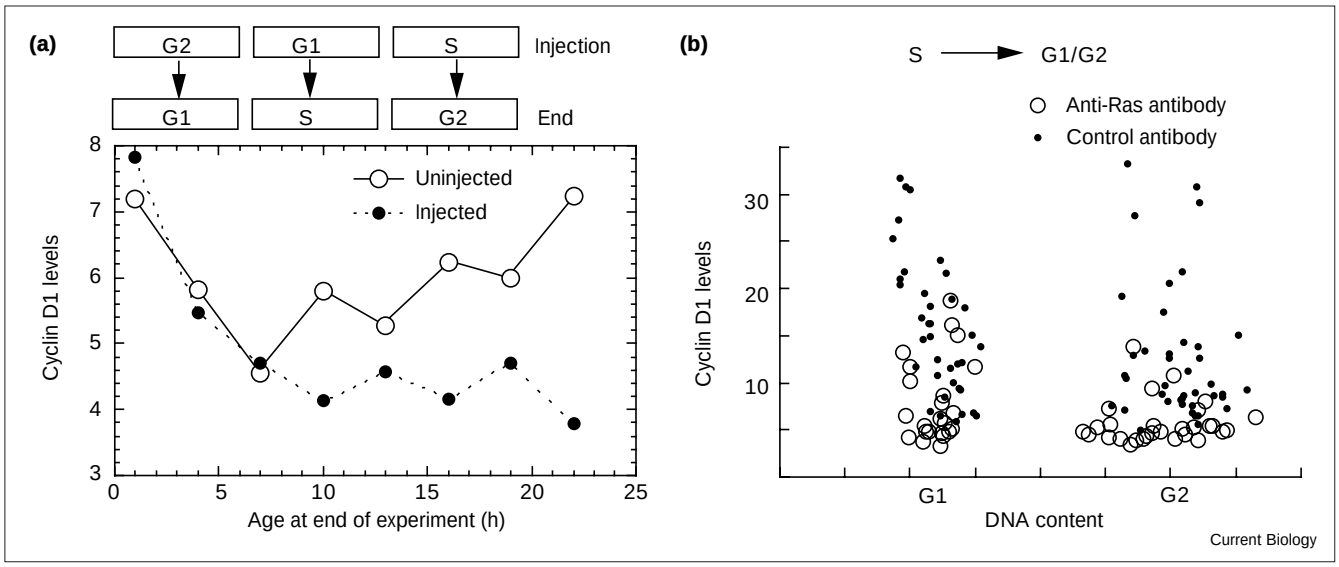
Ras dependence of cyclin D1 expression throughout the cell cycle. Anti-Ras antibody was injected into cells on several plates. At the indicated times after injection, the cells received a 1 h pulse of thymidine, were fixed and stained for cyclin D1, for injected immunoglobulin, and for DNA with DAPI. The fluorescence intensity of the cyclin D1-associated fluorochrome in the nuclear region of injected cells was determined on a cell-by-cell basis with a microscope equipped with a photomultiplier. The total DNA content was also determined in separate photomultiplier analyses. As in Figure 2, the fluorescence levels of cyclin D1 were plotted against the total amounts of DNA-associated fluorescence for each cell analyzed. The results for thymidine-labeled (closed circles) and unlabeled (open circles) cells are noted. The levels of cyclin D1 in G2 phase cells was dramatically reduced within 6 h following injection of anti-Ras antibody, whereas the levels in G1 phase cells required 12 h for efficient suppression. By 24 h after anti-Ras antibody injection, cyclin D1 levels had been reduced to the lowest levels observed. Only injected cells, as identified by staining for the injected immunoglobulin, were considered in this analysis.

As predicted, cyclin D1 levels in all cell cycle phases were strictly dependent on cellular Ras activity (Figure 4, 24 hours). Significantly, however, the rate at which the levels of cyclin D1 declined following anti-Ras antibody injection differed. At 6 hours after anti-Ras antibody injection, cyclin D1 levels in G1 phase cells remained high, but were reduced to low levels in most cells in G2 phase. By 12 hours after injection, cyclin D1 levels in G1 phase cells had also declined to low levels (Figure 4). After 24 hours, most cells were in G1 phase, but the few cells remaining in S and G2 phases expressed extremely low levels of cyclin D1 (Figure 4). A control injection of non-immune rat immunoglobulin G had no effect on cyclin D1 expression levels (data not shown). It is important to note that the cyclin D1 levels of all cells 24 hours after anti-Ras antibody injection were uniformly lower than even the S phase cells in the control (0 hours) culture. This means that, even in S phase cells, endogenous Ras activity results in the production of measurable levels of nuclear cyclin D1 protein.

It is possible that the injected anti-Ras antibody had functioned primarily by blocking the activation of cellular Ras during the transition between cell cycle phases, rather than by directly inhibiting the activity of Ras proteins that were already activated and therefore bound to effector molecules. To address this possibility, time-course analyses as above were performed in NIH3T3 cells transformed by a Ras mutant, Val12 Ras, which has a low intrinsic GTPase activity that is not enhanced by GTPase-activating proteins. A high proportion of these molecules would, therefore, be expected to be activated and thus bound to effector molecules throughout the cell cycle. In these transformed cells, however, the results were similar to those described above, with approximately 6 hours required to reduce the levels of cyclin D1 in G2 phase cells, and approximately 12 hours required to reduce levels in G1 phase cells (data not shown). The kinetics of suppression of cyclin D1 levels following anti-Ras antibody injection must, therefore, reflect the neutralization of all Ras protein within the cell, regardless of the state of activation.

The above time-course analyses support the predictions of the model and indicate that cyclin D1 expression is induced in a Ras-dependent manner as cells pass from S to G2 phase and that, once induced, cyclin D1 expression in G2 phase continues through G1 phase independently of Ras. It is critical, however, to perform separate experiments designed to specifically validate each of these important conclusions. A time-lapse analysis was first used to demonstrate directly that, when anti-Ras antibody is microinjected during G2 phase, the levels of cyclin D1 remain high into the next G1 phase. Cells were followed in time lapse for 18 hours before injection of anti-Ras antibody, and for an additional 6 hours thereafter. The cells were then fixed and stained for DNA, the injected immunoglobulin, and cyclin D1. This approach allows the direct identification of cells microinjected during G2 phase, and follows them into G1 phase. Cells that had passed through mitosis between anti-Ras antibody injection and the termination of the experiment would have been in G2 phase at the time of injection, but would generally be in G1 phase at the time of

Figure 5



The two approaches used to determine whether Ras activity is required for cyclin D1 expression during transitions between cell cycle phases. (a) Cells were followed in time lapse for 18 h before injection of anti-Ras antibody, and then for an additional 6 h after injection; the cells were then fixed and stained for cyclin D1 expression. The age of the cells at the end of the experiment is indicated; the age at the time of injection would be 6 h earlier. The approximate cell cycle positions at the time of injection and at the end of the experiment are indicated above the graph. Cells less than 6 h old at the end of the experiment must have divided between injection and fixation, and would therefore have passed from G2 to G1 phase during the analysis. Cells 6–14 h old would most probably have passed from G1 to S phase following antibody injection, while cells older than 14 h would probably have passed from S to G2 phase following injection. Cyclin D1 levels are expressed in arbitrary units, with background readings set at zero. In parallel experiments, no difference was observed between cells injected with a control immunoglobulin and uninjected cells. (b) The next analysis was designed to identify specifically cells passing from S to G2 phase following anti-Ras antibody injection. Cells were pulsed with BrdU for 1 h immediately after injection of anti-Ras antibody, and with tritiated thymidine for 1 h beginning 8 h after that injection. Following fixation, the cells were stained for DNA with DAPI, and for BrdU, injected immunoglobulin and cyclin D1 with antibodies linked to separate fluorochromes. Finally, the incorporation of thymidine was determined by autoradiography (see Materials and methods). As a control, parallel injections of a non-specific rat immunoglobulin were performed and analyzed identically (closed circles). For the purposes of this analysis, only injected cells that were BrdU-positive and thymidine-negative were considered. This would ensure that the cells had passed from S to G2 phase following injection. The cyclin D1 levels of such cells are plotted against their DNA content. It is clear that the injected anti-Ras antibody had efficiently blocked induction of cyclin D1 in these cells.

fixation. In such cells, the levels of cyclin D1 were indistinguishable from their uninjected neighboring cells in the same cell cycle position (Figure 5a). Clearly, anti-Ras antibody injection during G2 phase had not significantly reduced cyclin D1 expression in the subsequent G1 phase. On the other hand, the cyclin D1 levels of cells that did not pass through mitosis following injection of anti-Ras antibody was reduced in this experiment. These cells would have been in G1 or S phase at the time of injection and would have passed into S or G2 phase before fixation. In these cells, the anti-Ras antibody had apparently blocked the induction of cyclin D1 expression (Figure 5a).

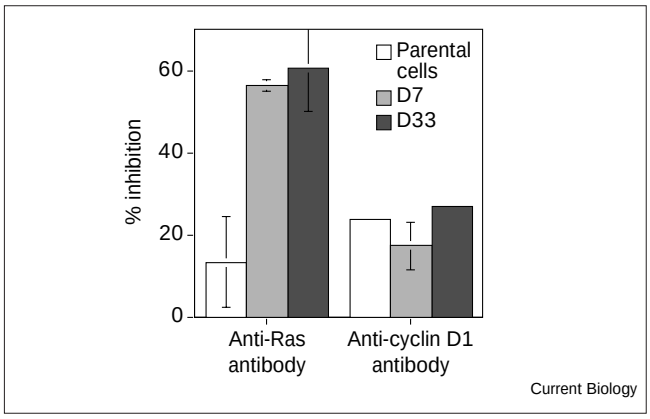
The second conclusion, that anti-Ras antibody had blocked the induction of cyclin D1 as cells passed from S to G2 phase could only be estimated from the above time-lapse analysis, as it is not easy to distinguish between cells in S and G2 phases in time-lapse analyses. Therefore, an independent labeling study was performed. NIH3T3 cells in an asynchronous culture were injected with anti-Ras antibody and then immediately pulsed with BrdU for 1 hour, then chased without BrdU for an additional 7 hours, and finally pulsed with tritiated thymidine for the final hour before fixation and staining as above. Parallel injections of non-specific rat immunoglobulin provided a control. We paid particular attention to injected cells that were labeled with BrdU but not with thymidine. These cells must have passed from S to G2 phase following injection. As predicted, the cyclin D1 levels in these cells were low following injection of anti-Ras antibody compared with such cells injected with control immunoglobulin (Figure 5b). This result directly demonstrates the requirement for Ras during the S–G2 phase transition period for efficient induction of cyclin D1 expression. Taken together, the above experiments provide strong support for the predictions of the model relating to cell cycle expression of cyclin D1.

Forced expression of cyclin D1 substitutes for Ras activity in cycling cells

The final prediction of the model suggests that Ras activity is unnecessary in the G1 phase of cycling cells because, by the time cells enter this cell cycle phase, the production of cyclin D1 has become Ras-independent. This suggests that, to a certain extent, the cell cycle function of Ras is accomplished upon the stable induction of cyclin D1. If this is true, forced expression of cyclin D1 would be expected to promote proliferation, at least in part, even in the absence of cellular Ras activity in cycling cells.

To test this possibility, NIH3T3 cells were stably transfected with a plasmid expressing the human cyclin D1 gene driven by the cytomegalovirus (CMV) promoter [32]. Two such clones were tested (D7 and D33). Careful analyses demonstrated that the production of human cyclin D1 in these cells was at nearly the same level as the production of cyclin D1 in human diploid fibroblast MRC5 cells. Anti-Ras antibody was injected into both clones of human cyclin-D1-expressing cells, as well as into parental NIH3T3 cells. The level of proliferation in injected cells was determined with a 1 hour pulse of thymidine at 24 hours after injection. Injected cells were positively identified by immunofluorescence staining for the injected immunoglobulin. Although the anti-Ras antibody suppressed thymidine labeling, and therefore proliferation in NIH3T3 cells by 90%, the proliferation of cells expressing the exogenous cyclin D1 was suppressed by only 40% (Figure 6). As a control, anti-cyclin D1 antibody was also injected into all three cell types and shown to reduce proliferation in all three similarly and efficiently (Figure 6). Finally, independent experiments demonstrated that injection of anti-Ras antibody into the two clones expressing exogenous cyclin D1 did not suppress expression of this transgene (data not shown).

Figure 6



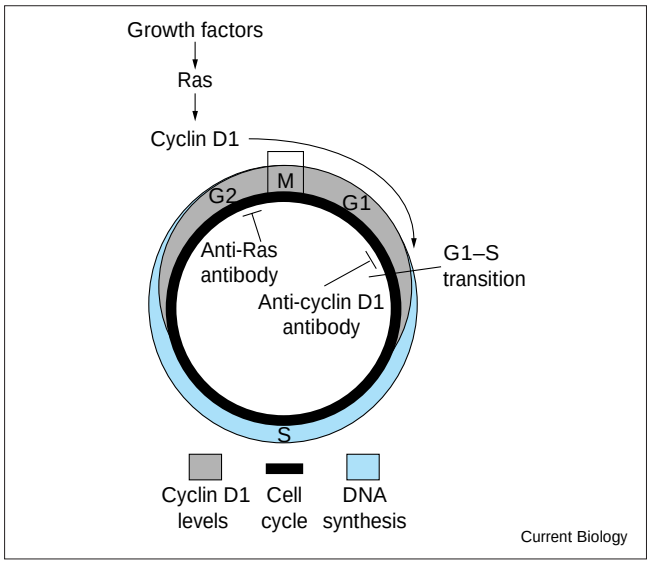
Exogenous expression of cyclin D1 reduces the requirement for cellular Ras. Two NIH3T3 cell lines (D7 and D33) expressing the human cyclin D1 gene controlled by the CMV promoter, together with parental NIH3T3 cells for comparison, received injections of anti-Ras and anti-cyclin D1 antibodies. The injected cells were cultured for 23 h, pulsed with labeled thymidine for 1 h, fixed and autoradiographed. The proportion of injected cells (as identified by staining with a fluorescent antibody against the injected immunoglobulin) that were labeled with thymidine (normalized against the labeling of surrounding uninjected cells) is plotted for each injection. Any cell in S phase 24 h following antibody injection would have escaped cell cycle inhibition. Whereas anti-cyclin D1 antibody efficiently inhibited cell cycle progression in all cells, the anti-Ras antibody was poorly inhibitory for the cyclin-D1-expressing cells. The results are from three separate experiments.

Discussion

Cyclin D1 plays a critical role in the control of cell proliferation. It is an important target of cellular Ras activity, is expressed at elevated levels in a number of tumor types [33], particularly in those tumors with activated *ras* genes [1,3,34], and constitutes an Rb kinase which is essential to cell cycle progression [14]. Cyclin D1 expression is low in quiescent cells, but is stimulated following serum addition to high levels at the time the cells pass the restriction point and become committed to enter S phase several hours later [14,19]. Little information, however, is known about the role of cyclin D1 in controlling the proliferation of continuously cycling cells. This question was addressed in a time-lapse analysis of asynchronous NIH3T3 cells injected with anti-cyclin D1 antibody. Cyclin D1 activity was found to be required throughout G1 phase until the beginning of DNA synthesis. On the other hand, cellular Ras activity was required to a minimal extent during G1 phase, but was necessary during the preceding G2 phase [18]. To explain these differences in the cell cycle requirements for Ras and cyclin D1, we propose that growth factors and other conditions conducive to continued proliferation must be available to rapidly cycling cells before mitosis, if the cell is to avoid quiescence and proceed through the next cell cycle. The growth factors stimulate Ras activity during G2 phase, resulting in the production of molecules required for cell cycle progression. The expression of these molecules becomes independent of Ras activity following passage through mitosis, and remains active until S phase begins. Because cyclin D1 is required through G1 phase, and because cyclin D1 is known to be a critical target of Ras activity in the control of proliferation, we predicted that cyclin D1 is one of the primary targets of cellular Ras activity induced in G2 phase and whose activity continues through G1 phase to promote entry into S phase (Figure 7). This possibility led to the four predictions tested in this study.

First, several independent experimental approaches were used to demonstrate that cyclin D1 is expressed to high levels in the nuclei of cells in G2 phase. Second, cellular Ras activity was necessary for the expression of cyclin D1 in all cell cycle phases. Third, upon neutralization of cellular Ras activity with injected anti-Ras antibody, the high level expression of cyclin D1 in G2 and G1 phases

Figure 7



Schematic illustration of the model supported by this work. The cell cycle is represented as a circle with mitosis (M) at the top and the approximate positions of other cell cycle phases indicated, together with G1–S phase transition and the points in the cell cycle at which anti-Ras and anti-cyclin D1 antibodies lose their abilities to block entry into S phase. The approximate cell cycle positions of nuclear cyclin D1 accumulation and DNA synthesis are indicated. As illustrated, growth factors stimulate Ras activity, which promotes cyclin D1 expression during G2 phase. Cyclin D1 is then continuously expressed through mitosis and G1 phase to promote passage through the G1–S transition.

continued for several hours. The expression of nuclear cyclin D1 in G1 phase was most stable, requiring more than 6 hours following injection of anti-Ras antibody for reduction to be observed. Separate analyses directly demonstrated that cyclin D1 levels are induced in a Ras-dependent manner as cells pass from S to G2 phase, and that, once induced in G2 phase, cyclin D1 levels remain high during G1 phase even in the absence of Ras activity. These results provide critical support for the model which predicts that Ras activity in G2 phase induces the stable expression of cyclin D1 until the beginning of the next S phase.

The means by which cellular Ras might stabilize cyclin D1 synthesis is not known, but several possibilities exist. Ras may regulate cyclin D1 stability by controlling the activity of signaling molecules able to phosphorylate a critical residue that controls the rate of proteasomal degradation [35]. In addition, the stability of cyclin D1 is regulated by direct degradation by calpain protease [36]. In any case, the half life of cyclin D1 is quite short, from 12–60 minutes depending on the signaling environment within the cell [35,37]. It would appear unlikely, therefore, that changes in protein stability alone could account for the above results [11,35]. Finally, it is generally believed that cyclin D1 must be localized in the nucleus to be active. We have focused throughout these studies on nuclear levels of this protein. It is also possible that the control over nuclear localization might also have a role in its cell cycle regulation [38], although preliminary studies revealed no evidence for the shuttling of cyclin D1 protein between nucleus and cytoplasm during the cell cycle of NIH3T3 cells (see Supplementary material).

Finally, the model makes a fourth prediction tested in this study, that a primary function of Ras activity in cycling cells is the induction of cyclin D1. This suggests that ectopic expression of cyclin D1 might overcome, to some extent, the requirement for cellular Ras during proliferation. To test this prediction, cells expressing human cyclin D1 from a viral promoter were analyzed. In these cycling cells, anti-Ras antibody was a poor inhibitor of proliferation, whereas anti-cyclin D1 antibody inhibited efficiently. Although this is the only observation in our studies that supports a role for cyclin D1 as a critical proliferative target of Ras activity, a number of other reports also support this idea [4,9,39]. Taken together, these results, along with a number of others that emphasize the role of cyclin D1 in control of proliferation, provide support for the idea that cyclin D1 has the ability to promote entry into S phase, independently of Ras activity, as predicted.

Taken together, these results strongly support all four predictions of the model, which postulates that the growth conditions necessary for continued cell cycle progression must be sensed during late stages of the cell cycle, leading to the induction of Ras activity together with the targets of this activity, including cyclin D1. Once induced during G2 phase in a Ras-dependent manner, cyclin D1 continues to be expressed through mitosis and G1 phase even in the absence of Ras activity. Cyclin D1 performs a vital function during the G1–S phase transition as evidenced by the fact that it can substitute for the proliferative requirement for Ras to some extent. Thus, cellular Ras activity is not required during the G1 phase in cycling cells, whereas cyclin D1 activity is required throughout G1 phase. These interactions between two molecules known to be vital for the control of proliferation apparently play a critical role in the control of continued cell cycle progression. It will be interesting in future studies to determine the role of other cell cycle control molecules, such as cyclin-inhibitory molecules, together with Ras and cyclin D1 in the control of cell cycle progression.

Materials and methods

Time-lapse video photography

Antibody preparation, cell culture, image capture and autoradiography were performed as described [18]. When cyclin D1 expression was assessed following time-lapse analysis and antibody injection, the cells were fixed and stained immediately following the final time-lapse frame. The staining characteristics of cells as determined by CCD or microscopic photomultiplier analysis were then combined with the analysis of cell age.

Measurement of fluorescent signal of each stained cell

For the quantitative fluorescence microscopy, the narrow band-pass filter cubes for DAPI, Cy2, Cy3 and Cy5 were used to minimize the cross-over signal reading among fluorochromes. The fluorescence intensity was determined by two separate devices, a photomultiplier (Leitz) or a cooled CCD camera (Princeton Instruments). Measurements with the photomultiplier were performed on a cell-by-cell basis. Digital images were captured using a cooled CCD camera controlled with Metamorph software (Universal Imaging). The exposure time was adjusted so that the brightest signal in the specimen gave less than 90% of the maximum linear exposure for the camera. The DAPI-stained image was thresholded to generate a binary mask which included each nucleus. With this mask, the intensity and total fluorescence of each individual nucleus in images of each fluorochrome were obtained. Shading corrections were used to adjust for uneven illumination. Careful analyses which failed to demonstrate translocation of cyclin D1 from the nucleus to the cytoplasm are described in Supplementary material. In addition, evidence that the size of the nucleus increases proportionally to DNA content is also presented in Supplementary material. This fact makes it possible to determine cyclin D1 content relative to cell cycle phase by measuring fluorescence intensity.

In Figure 5b, it was necessary to quantitate the fluorescence levels of four separate fluorochromes for each cell. Cells were first stained for cyclin D1 and injected IgG as described [18]. Then, after re-fixation with 70% ethanol in 50 mM glycine-HCl, pH 2.0 (–20°C), BrdU was stained with sheep anti-BrdU antibody following partial digestion of DNA (*Eco*RI and exonuclease III) [40], followed by incubation with Cy5-conjugated anti-sheep IgG. DNA was stained with DAPI.

Mitotic shake-off and western blotting

Cell culture plates of rapidly growing NIH3T3 cells were mechanically vibrated to detach mitotic cells. Only when greater than 85% of the detached cells displayed condensed chromatin were the cells used for determination of cyclin D1 content by western blotting [13]. To quantitate the immunoreactive bands, the blot was developed with chemifluorescence substrate (Amersham) after incubating with an alkaline-phosphatase-conjugated secondary antibody. The blot was scanned with Storm Imager 840 and the band intensity was determined by Image Quant software (Molecular Dynamics). Integrated cyclin D1 intensity was normalized to the corresponding actin band on the same blot.

Supplementary material

Additional methodological details are available at http://current-biology.com/supmat/supmatin.htm.

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Supplementary material

Cyclin D1 production in cycling cells depends on Ras in a cell-cycle-specific manner

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Supplementary materials and methods

Materials

Autoradiography emulsion type NTB2 was obtained from Eastman Kodak Company. DAPI was a product of Molecular Probes. Cy3-conjugated-anti-rat IgG, Cy2-conjugated-anti-rat IgG with minimal cross-reactivity against mouse IgG, Cy3-conjugated-anti-mouse IgG, Cy3-conjugated-anti-mouse IgG with minimal crossreactivity against rat IgG, and Cy5-conjugated-anti-sheep IgG were purchased from Jackson ImmunoResearch. Sheep polyclonal anti-BrdU antibody was a product of Fitzgerald Industries International. Anti-mouse-cyclin D1 (72-13G) was purchased from Santa Cruz Biotechnology. Anti-human-cyclin D1 (ZY-7D2) was a product of Zymed Laboratories Inc. Anti-actin mouse monoclonal antibody (clone C4), horseradish-peroxidase-conjugated and alkaline-phosphatase-conjugated anti-mouse IgG, and *Eco*RI were obtained from Boehringer Mannheim. Exonuclease III, [methyl-^{3}H]thymidine (80 Ci/mmol) and ECF (enzyme chemifluorescence) detection module were purchased from Amersham.

Time-lapse video photography

Antibody preparation, cell culture and autoradiography were performed as described [S1]. Digital images were obtained with a CCD camera (Sony) attached to a frame-capture board controlled by the NIH Image program [S1]. The area of the coverslip to be analyzed was marked with two contiguous circles of varying size using a diamond object marker (Leitz). This allowed realignment of the area of analysis and identification of individual cells following immunostaining, DAPI staining and/or autoradiography. In this approach, the cells were illuminated continuously with low-level green light.

To avoid possible complications with this constant illumination, several experiments were duplicated with a similar approach except that the light was shuttered. In this case, the cells were illuminated only during the time of exposure, less than 1 sec every 10 min. These images were collected with a highly sensitive, cooled CCD camera (Princeton Instrument) and controlled by the Metamorph software package (Universal Imaging). Because of the sensitivity of the cooled CCD camera, the cells were illuminated with extremely faint light. In these cases, there is little possibility that the illumination of cells would have any effects on the results obtained. In no case were the results with the two methods of illumination different.

When a second time-lapse analysis followed microinjection, care was taken to ensure that the same area of the coverslip and the same alignment were viewed in each movie. Antibodies to be studied were microinjected into all the cells, and only the cells, within the designated circular area. In this way, it was possible to follow the analysis from the beginning and determine which cells had arisen from originally injected cells. The injection was further confirmed by immunofluorescence staining for injected antibody. Immunofluorescence staining and detection of DNA synthesis were performed as described [S1]. When cyclin D1 expression was assessed following time-lapse analysis and antibody injection, the cells were fixed and stained immediately following the final time-lapse frame. The staining characteristics of cells, as determined by CCD analysis, were then combined with the analysis of cell age. It should be noted that, in such an analysis, the cell age is calculated from the end of the experiment (Figure 5 in the paper). As the analysis was terminated 6 h following anti-Ras antibody injection, the age at the time of injection can also be determined.

Measurement of fluorescent signal of each stained cell

A Leica fluorescent microscope DM 900 was used to quantitate fluorescence intensity. The Leica filter cubes, cube A, cube L4, cube N2.1, and a cube #41008 Cy5 from Chroma Technology were used to detect signal from DAPI, Cy2, Cy3, and Cy5, respectively. With these cubes, no significant crossover signal was detected except the faint crossover of Cy5 signal to the Cy3 cube (see below). We used two different devices for quantitation, a photomultiplier and a cooled CCD camera.

Photomultiplier (Leitz) readings were controlled by the software program, Scope 5 (Kinetek Co.), with settings gain = 1, voltage = 500 and expose = 0.5 sec. The area of measurement was defined so that it was large enough to include the entire nucleus (for total nuclear measurement), or small enough to fit inside the nucleus (for nuclear density measurement). Before photomultiplier measurement, a phase-contrasted photo of the field of interest was taken. As we measure the fluorescence intensity, each cell was given an identification number on this photo. In this way the fluorescence measurements could be related back to individual cells in the time-lapse movie, or in the autoradiogram. Thus, all the parameters for a given cell could be defined (the time of mitosis determined by the movie, the photomultiplier value for cyclin D1-associated fluorescence and the labeling status obtained after autoradiography).

Digital images were captured using a cooled (–25°C) CCD camera (Princeton Instrument) controlled with Metamorph software. The exposure time was adjusted so that the brightest signal in the specimen gave less than 90% of the maximum linear exposure for the camera (a gray scale of 0–4096, 12 bit gray scale). Integration of the fluorescence signal from each nucleus was obtained by processing the image as described below using Metamorph analysis features. Each captured image was corrected by subtracting the background image followed by shading correction (to correct for the uneven signal collection across the field). Both of the correction images were taken with the same exposure time as the image to be corrected. The background image was taken without a fluorescent specimen while the shading images were collected from a specimen with uniform fluorescence. This was achieved for Cy2 and Cy3 with a blank cover slip mounted with autofluorescent nail polish (Ultra Hard Strengthened, Avon) taken with cube L4 and cube N2.1, respectively. The shading image for DAPI was the image of a blank coverslip mounted with the mixture of anti-fade mounting media, Slowfade component A (Molecular Probes) and DAPI. This mixture gave moderate fluorescence detectable with cube A. The shaded image of the DNA fluorescence (taken with DAPI) was used to make a mask with which to measure nuclear fluorescence with each fluorochrome. This image was thresholded to generate a binary mask which included each nucleus. With this mask the intensity and total fluorescence of each individual nucleus in images of each fluorochrome were obtained. Thus, the fluorescence properties with each fluorochrome for each nucleus was obtained.

In Figure 5b in the paper, it was necessary to quantitate the fluorescence levels of four separate fluorochromes for each cell. The injected immunoglobulin (Y13-259, a rat monoclonal antibody, or non-immune rat IgG from Sigma) was stained with a Cy2-conjugated anti-rat IgG antibody as a marker of injection; cyclin D1 was stained with a mouse monoclonal followed by Cy3-conjugated anti-mouse IgG antibody as described [S1]. Then, after re-fixation with 70% ethanol in 50 mM glycine-HCl, pH 2.0 (–20°C), incorporated BrdU was stained with

sheep anti-BrdU antibody with partial digestion of DNA (*Eco*RI and exonuclease III), followed by Cy5-conjugated anti-sheep IgG antibody [S2]. DNA was stained with DAPI. Fluorescence cubes specific for each fluorochrome as described above were used. Very slight fluorescence crossover was detected only from the Cy5 fluorochrome to the Cy3 filter. Therefore, the staining of BrdU with the Cy5 fluorochrome was purposely rendered extremely faint. This still allowed identification of BrdU-positive cells, but did not effect the strong staining of the cyclin D1 with the Cy3-conjugated antibody.

Analytical considerations

As the cell progresses through the cell cycle, the nucleus increases in DNA content and in size. An increase in content of a nuclear protein through the cell cycle, therefore, might simply reflect the increase in the DNA content. This increase, however, would only result in at most a twofold increase in the content of a nuclear protein, and could not account for the dramatic increases in cyclin D1 protein seen here as cells progress from S- to G2-phases (Figure 2). Nevertheless, measurements were performed to determine the average intensity of fluorescence over the nucleus, rather than the total nuclear fluorescence, to correct for the increase in DNA content through the cell cycle. With this measurement, the cyclin D1 levels of S-phase cells were low compared with cells in G1 or G2 phases, but the levels of fluorescence of G1-phase cells compared with G2-phase cells was similar (Figure 3). To determine how well this measurement corrected for the increase in DNA content of the cell, the average volume of nuclei was compared with the average DNA content. For these cells, this was a strictly linear relationship, except for cells just before, during, or just following mitosis. Therefore, the fluorescence-intensity measurements for interphase cells accurately corrects for the increase in DNA content of the cell and reveals differences in the relative cyclin D1 content. To ensure that this was the correct conclusion, however, average fluorescence-intensity measurements were performed with both the photomultiplier (Figure 4) and with the CCD-captured image through software calculations (Figure 2). In this case, the window of the photomultiplier was extremely small, and measurements were performed at high magnification (40 ×) to ensure that the entire area being measured was localized within the nucleus. In these cells, measurement of the DNA content required a separate measurement with a window large enough to include the entire nucleus as it was necessary to measure the total DNA content of the nucleus for cell-cycle localization. In both types of measurements, the nuclear intensity as determined by completely different procedures yielded highly similar results, which together confirm the elevation of cyclin D1 in G2 phase even when the increase in size and DNA content were taken into consideration (Figure 2).

In other studies, the nuclear levels of cyclin D1 were found to be critical for the biological activity of cyclin D1 [S3]. It is possible that the appearance of cyclin D1 in the nucleus might reflect a redistribution of pre-existing cytoplasmic cyclin D1, rather than the *de novo* synthesis of the protein [S4]. To ensure that only active cyclin D1 was measured, only nuclear measurements are reported in this study. The possibility that an increase in nuclear cyclin D1 might result from cellular redistribution, however, does reflect directly on our biochemical measurements of total cellular cyclin D1 levels in mitotic cells (Figure 4). If significant cellular redistribution of cyclin D1 takes place, the high content of this protein in mitotic cells might not be informative. Should subcellular redistribution contribute to the observed changes in nuclear cyclin D1 levels, the cytoplasmic staining level should be high during S phase and low in G1 and G2 phases. Cells were stained with anti-cyclin D1 antibody and with DAPI. From CCD images, the nuclear fluorescence intensity and total fluorescence levels were determined for both fluorochromes, together with measurements of the cyclin-D1-associated fluorescent intensity of typical cytoplasmic regions of each cell. When the cytoplasmic cyclin D1 intensity was plotted against DNA content of the cells, the cytoplasmic cyclin D1 levels stayed constant through the cell cycle, or might have actually increased as cells entered G2 phase. Only in mitotic cells, where cyclin D1 seemed to be excluded from condensed chromosomes, was the cytoplasmic signal high. Soon after mitosis, the protein returned to the newly formed nuclei. It is concluded that, within the limits of these analyses, the loss of nuclear cyclin D1 in these NIH3T3 cells results primarily from the disappearance of cyclin D1 from the cell. We, therefore, conclude that the levels of cyclin D1 we observed to be present in mitotic shakeoff cells required the production of cyclin D1 during G2 phase. Moreover, the levels of cyclin D1 in mitotic cells was higher than observed at any time following serum stimulation of quiescent cells, further substantiating the *de novo* production of cyclin D1 during G2 phase.

Mitotic shakeoff and western blotting

NIH3T3 cells in a 15 cm plates were mechanically vibrated in a reproducible manner every 90 min. The medium from the plate was collected and the detached cells were pelleted by centrifugation at low speed. Pre-warmed medium was immediately added to each plate. The pelleted cells were suspended in 1 ml medium and a small number spread on a slide together with ethanol:acetic acid (3:1). The plate was dried and the chromatin stained with crystal violet. Only when greater than 85% of the detached cells displayed condensed chromatin were the cells used for determination of cyclin D1 content by western blotting [S5]. To quantitate the immunoreactive bands, the blot was developed with Chemifluorescence substrate after incubating with an alkaline-phosphatase-conjugated secondary antibody. The blot was scanned with Storm 840 and the band intensity was determined by a software, Image Quant (Molecular Dynamics). Integrated cyclin D1 intensity was normalized to the corresponding actin band on the same blot.

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S4. Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G: Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev* 1993, 7:812-821

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