Minireview

Ras-dependent cell cycle commitment during G2 phase

Masahiro Hitomi*, Dennis W. Stacey

Department of Molecular Biology, The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA

Abstract Synchronization used to study cell cycle progression may change the characteristics of rapidly proliferating cells. By combining time-lapse, quantitative fluorescent microscopy and microinjection, we have established a method to analyze the cell cycle progression of individual cells without synchronization. This new approach revealed that rapidly growing NIH3T3 cells make a Ras-dependent commitment for completion of the next cell cycle while they are in G2 phase of the preceding cell cycle. Thus, Ras activity during G2 phase induces cyclin D1 expression. This expression continues through the next G1 phase even in the absence of Ras activity, and drives cells into S phase. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Restriction point; Cell cycle; Ras; Cyclin D1; Single cell technique

1. Introduction

Multiple molecular events are required for transition from G1 to S phase [1–3]. Most of these were identified by analyzing quiescent cells stimulated to enter the cell cycle following mitogen stimulation. Results obtained during this cell cycle re-entry may apply in situations such as wound healing where many quiescent cells are required to re-enter the cell cycle and proliferate rapidly. However, there are many lines of evidence indicating a difference between this type of cell cycle re-initiation and continuous cell cycle progression. It is important to analyze the molecular events that control continuous cell cycle progression to understand the cell cycle control of continuously cycling cells from tissues such as skin, gut, bone marrow, and from cancer. Unfortunately, methods of cell cycle synchronization may not be ideal in the analysis of cell cycle progression because synchronization itself usually interrupts the cell cycle. In order to avoid such possible complications, by combining time-lapse, microinjection and quantitative fluorocytometry, we developed a cell cycle analysis method which does not require synchronization. In this review, we discuss the necessity of such methods, how they work and the results obtained. In rapidly cycling cells we observed a completely different sequence of molecular events from those observed in synchronous G0/S transition.

2. Growth phase transition from G0 to S phase

2.1. Restriction point

When cells are deprived of mitogens they exit the cell cycle and enter a quiescent state with G1 DNA content. This condition is often called G0 phase. When mitogens become available again to quiescent cells, they resume the cell cycle in a synchronous manner. During this re-entry process, mitogens are only required until a point located in mid-to-late G1 phase. Beyond this point, cells can complete one cell division cycle without growth factors. In other words, at this point the cells make a decision to complete the rest of the cell cycle and produce daughter cells. This critical commitment point is referred as the 'restriction point' [4]. The restriction point also coincides with the last point of the cell cycle in which essential amino acids or protein synthesis is required for the completion of cell division [5].

2.2. Molecular mechanism governing the restriction point

What is the molecular basis of the restriction point? Upon ligand binding, growth factor receptors dimerize and phosphorylate tyrosine residues in their own cytoplasmic domains. These phosphorylated tyrosine residues recruit signaling molecules to the membrane. Among the recruited molecules are nucleotide exchange factors for Ras protein, which promote binding to GTP [1]. The GTP-bound form of Ras actively signals to multiple downstream effectors, including the MAP kinase pathway which is vital in mediating the mitogenic activity of Ras protein [6,7]. This pathway, together with other pathways, phosphorylates and activates transcription factors to induce numerous genes [8]. Cyclin D1 is such a gene induced by mitogens through Ras activity.

Cyclin D1 was first identified as a delayed immediate gene induced by mitogenic stimulation [9]. Its promoter contains mitogen-responsive Ets and AP-1 binding sites [10]. The tandem occurrence of Ets and AP-1 sites has been shown to respond to Ras signaling [11]. In fact, Ras has been reported as a potent inducer of cyclin D1 expression [12–14]. Once induced, cyclin D1 protein forms complexes with cyclin-dependent kinase (CDK) 4 or 6 [15]. Cyclin association is required for CDK activity. Cyclin D1-associated kinases play an essential role in governing G1/S transition [16]. Cyclin D1 also titrates the inhibitors of other CDKs by physical association, resulting in their activation to further facilitate

---

*Corresponding author. Fax: (1)-216-444 0512.
E-mail: hitomi@ccf.org

G1/S transition [2]. Because of its responsiveness to mitogens and its requirement during cell cycle progression, cyclin D1 is a pivotal molecule connecting the mitogenic signaling system to the cell cycle control machinery [17].

Cyclin D1-associated CDKs play a vital role in phosphorylating the retinoblastoma protein (Rb). Rb is a well-established in vivo substrate of cyclin D1/CDK complexes [18,19]. Underphosphorylated forms of Rb bind the E2F/DP transcription factor complexes, and repress their transcriptional activity by recruiting repressor molecules, such as histone deacetylase and/or nucleosome remodeling complex to the proximity of the promoter regions containing the E2F/DP binding motif [20–22]. E2F/DP binding sites have been identified in promoters of many genes whose products are essential for G1/S transition (cyclin E or A) or DNA synthesis (dihydrofolate reductase, DNA polymerase α, cdc 6, etc.) [23]. Therefore, when Rb is underphosphorylated, the expression of these genes required for S phase is suppressed. Upon phosphorylation by cyclin D1/CDK complexes, E2F/DP complexes are released from Rb and activate target genes. Once E2F/DP target genes are expressed, growth factors are no longer required for entry into S phase. After the initiation of DNA synthesis, cells are committed to complete the cell cycle through mitosis without mitogens or de novo protein synthesis [3]. In this way, the signaling pathway from mitogens through Ras and cyclin D1 to Rb is believed to play an important role in controlling the restriction point observed during the growth phase transition from G0 to S phase. There are biological evidences to support this Ras–cyclin D1–Rb hierarchy. Forced expression of cyclin D1 can overcome growth suppression induced by dominant negative Ras [12]. Ras and cyclin D1 are no longer required for G1/S transition in Rb compromised cells [24–26].

2.3. Ras requirements during G0/S transition

During the G0/S transition, Ras has been reported to play critical roles at multiple points. Biochemical analyses detected two peaks of Ras activity as cells progress from G0 to S phase. The first peak is detected soon after serum stimulation, and the second one at mid-to-late G1 phase [27,28]. Biological analyses also indicate the requirement of the Ras activity multiple times during the re-entry into the cell cycle from quiescence [29].

3. The necessity for a new method to analyze progression through cell cycle without synchronization

The signaling interactions described above were identified in synchronous cultures of mitogen restimulated quiescent cells. There are, however, evidences to suggest that cell cycle control in continuously proliferating cells is quite different than in quiescent cells stimulated to re-enter the cell cycle. For example, (1) c-Fos, a target gene of the Ras signaling pathway, is rapidly induced following mitogen stimulation and is essential for cell cycle re-entry from quiescence. This gene, however, is not expressed at any time in actively cycling cells. Furthermore, neutralizing anti-c-Fos antibody (Ab) blocks re-entry into the cell cycle but has no effect in cycling cells [30]. (2) In Rb −/− cells, Ras is dispensable for continuous cell cycle progression, but is essential for transition from quiescence to S phase [26]. (3) The expression of proteins involved in the replication complex such as cdc6, orc1 and mcm, are ex-

pressed at low levels in quiescent cells, but are detected in all continuously cycling cells regardless of cell cycle position [31–35]. (4) C-myc expression peaks in G1 phase during the re-entry into cell cycle, but its expression in cycling cells is constant [36–38].

All these examples indicate that the mechanism of cell cycle control in continuous cycle may be very different from that observed in the G0/S transition. The above facts emphasize the need to study the control of cell cycle progression in continuously cycling cells. Unfortunately, such studies are complicated by the fact that continuously cycling cells have to be synchronized before analysis. Synchronization usually requires reversible growth arrest which might alter signaling patterns in the treated cells. For instance, reagents such as transforming growth factor β or nocodazole induce growth-arresting molecules, which are normally maintained at low levels during continuous cell cycle progression. Synchronization by means of selecting cells in specific cell cycle phases includes mitotic shake off and centrifugal elutriation. However, in cells collected by mitotic shake off, the G1 phase period doubles in length [39]. The G1 population collected from rapidly growing culture by centrifugal elutriation has been reported to have some properties of G0 cells [40]. Both methods require the detachment of the cells from a plate, which will disrupt signaling events essential for cell cycle progression [41]. Clearly, it would be best to avoid synchronization in the analysis of the cell cycle progression of continuously growing cells. Zetterberg and Larsson used time-lapse movies to circumvent the need for synchronization, to study the restriction point in rapidly cycling cells [42]. The cell cycle phase of individual cells was predicted by determining the time since each cell had passed through mitosis. We have combined this time-lapse technique with microinjection and quantitative fluorocytometry to determine the requirement point for Ras in rapidly proliferating cells.

4. Methods to analyze cell cycle without synchronization

We will begin with a description of the technical approach utilized in the study of proliferative signaling in continuously cycling cells. The cell cycle position of individual cells was determined by time-lapse and quantitative fluorocytometry. Microinjection in combination with time-lapse allowed us to manipulate signaling molecules at specified cell cycle phases. We could determine the expression levels of proteins of interest using quantitative immunofluorescence. Below are brief descriptions of each technique. Detailed information is described elsewhere [39,43–45].

Time-lapse movies were made of cells in an environmental chamber containing 5% CO_2 at 37°C on a microscope stage. Time-lapse images were captured with a CCD camera controlled by the NIH Image program [44,45]. By determining the time passed since mitosis (age of the cell), we could predict the cell cycle phase of each cell [39,42,44,45]. Time-lapse movies were also used to analyze cell migration.

In order to manipulate the activity of specific molecules of interest, we microinjected neutralizing antibodies, or constitutively active molecules [46–49]. Microinjection is ideal in studies of cell cycle progression, because a specific signaling event can be targeted in a short period of time. For example, neutralizing anti-Ras Ab can inhibit Erk activation within 20–30 min [44]; its inhibitory effect on Ras-dependent cell migration
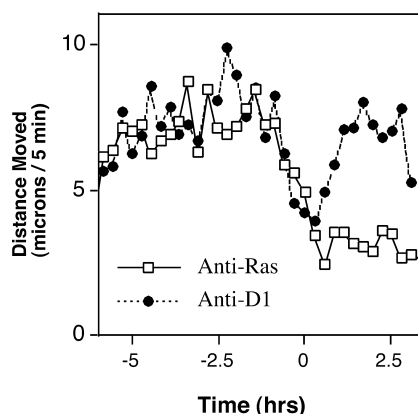
Fig. 1. Migration of Ras-transformed cells following injection of anti-Ras or anti-cyclin D1 antibodies. Neutralizing anti-Ras Ab immediately blocks cell migration. Ras-transformed cells have high cell motility due to constitutive Ras activity [50]. Motility of Ras-transformed NIH3T3 cells was monitored by time-lapse for several hours, at which time cells were injected with either anti-Ras (open square) or anti-cyclin D1 Ab (closed circle). Soon after the injection (0 h), these cells were observed with a second time-lapse analysis to determine post-injection migration characteristics. Approximately 50 cells were analyzed at each time point and mean values are plotted. The motility of the anti-Ras Ab-injected cells was immediately suppressed and remained at a low level, whereas control injection resulted in only a brief suppression of migration. This indicates that anti-Ras Ab injection blocks Ras activity in a short period even in the cells expressing constitutively active Ras.

[50] is detected within half an hour (Fig. 1). Injected oncogenic Ras induces a Ras-responsive reporter gene within 3 h [51]. Because of such rapid action, the effects of these manipulations can be assessed within a single cell cycle period. For example, in rapidly growing NIH3T3 cells with doubling times of 16–18 h, if the injection was done within a couple of hours after mitosis the cell was most likely in G1 phase; cells which received injections between 5 and 12 h after mitosis were in S phase at the time of injection; after 12 h following mitosis, most cells were in G2 phase [44,45]. This rapid action of microinjected molecules is advantageous over the other methods such as transfection, or viral transduction which require many hours to be effective.

Because time-lapse analyses are time consuming and involve limited numbers of cells, we also utilized quantitative fluorocytometry and digital image analysis to determine the DNA content of individual cells. 4′,6-Diamidino-2-phenylindole, dihydrochloride (DAPI) fluorescence values were proportional to DNA content [43], allowing us to determine the final cell cycle phase of individual cells in an asynchronous culture. These techniques were also able to determine the expression levels of proteins in individual cells stained by indirect immunofluorescence. The relative protein expression levels determined by these methods were in good agreement with those obtained by quantitative Western blot analyses [39,43].

5. The Ras requirement during continuous cell cycle

5.1. Ras is required during the G2 phase for the next cell cycle

In order to analyze the Ras requirement in rapidly growing cells, we injected anti-Ras into rapidly cycling NIH3T3 cells, which express functional Rb. Before injection, a time-lapse movie was made to determine the cell cycle phase of individual cells at the time of injection. The fate of the injected cells was monitored in a second time-lapse movie of the injected area. Studies of G0/S transition predict that anti-Ras injection during G1 phase would block cell cycle progression. In asynchronous culture, however, we found that the majority of the cells went through mitosis once following anti-Ras Ab injection regardless of their cell cycle position at the time of injection. Cells continued to progress through one cell cycle and completed mitosis once, even if anti-Ras Ab was introduced during G1 phase. The fact that most of the cells accomplish one cell cycle without significant delay indicates that there was no requirement for Ras during any cell cycle phase in continuously cycling cells [44]. Although cells without Ras activity divided once, almost none of them divided a second time. At the end of the experiment, most of the injected cells were arrested in G1 phase as judged by the DNA content [43,44]. These data indicate that, unlike re-entry into the cell cycle from quiescence where Ras is required during G1, in asynchronous cells Ras activity is required prior to mitosis to complete the next entire cell cycle. The progression from G1 to S phase, however, is still under the control of Rb as cyclin D1 was required just prior to the beginning of S phase [44]. The cyclin D1 result was obtained by microinjecting neutralizing anti-cyclin D1 Ab. In this case, cells injected during G1 phase were effectively blocked from entering into S phase.

Analogous determinations were performed by removing serum rather than injecting neutralizing antibodies. With this approach the serum-dependent commitment was observed during early G1 phase [42,44,52]. This indicates that mitogens are required after Ras activity in cycling cells. The order of Ras requirement in G2 phase and serum requirement in early G1 phase seems to be opposite to what is predicted by the fact that Ras functions downstream of mitogens. However, in addition to Ras, many signaling pathways have been identified to be activated by mitogens [53,54]. The signaling from growth factor receptor to myc through Src family tyrosine kinases constitutes an essential mitogenic signaling pathway independent of the Ras pathway [55]. The major mitogenic activity in serum is most likely to be platelet-derived lysophosphatidic acid, which signals through trimeric G proteins [56]. Trimeric G protein-mediated mitogenic signaling also stimulates multiple downstream molecules including Src and Ras, which are common among those activated by peptide growth factors [57]. Therefore, the serum requirement during early G1 phase of the continuous cell cycle suggests the presence of such mitogen-dependent, Ras-independent, essential pathways playing important roles in G1/S transition.

5.2. Our hypothesis

These observations suggest that in rapidly proliferating cells, Ras activity induces something essential for the next G1/S transition, but it does so prior to mitosis, during G2 phase. Moreover, this Ras-induced factor must remain active through mitosis and G1 phase to induce S phase entry even in the continued absence of Ras activity. Once cells start DNA synthesis, they will complete a cell cycle unless they are exposed to genotoxic reagents, which induce checkpoint-dependent arrests [3]. We hypothesized that this Ras-induced factor could be cyclin D1 for the following reasons: (1) cyclin D1 is induced by Ras [10,13,14]. (2) Cyclin D1 is required to abrogate Rb growth suppressive functions for G1/S transition

[16,24,58]. (3) Cyclin D1 can substitute, at least in part, for the Ras requirement during cell proliferation [12,26]. We therefore predicted that during continuous cell cycle progression, a cell senses favorable growth conditions during G2 phase and responds by activating Ras. The activated Ras then induces cyclin D1 during G2 phase. Once induced during G2 phase, the expression of cyclin D1 becomes independent of Ras through G1 phase allowing cells to transit from G1 to S phase in the absence of Ras activity.

5.3. Predictions from our hypothesis

Our hypothesis predicts the following in rapidly growing cultures:

1. Cyclin D1 should be expressed at high levels in G2 phase.
2. This G2 phase expression is Ras-dependent.
3. The subsequent expression of cyclin D1 during G1 is Ras-independent.
4. Forced expression of cyclin D1 can substitute for Ras activity in cycling cells.

5.4. Confirmation of our hypothesis

To test our hypothesis, we utilized quantitative immunofluorescence techniques to determine cyclin D1 expression levels together with the cell cycle position of individual cells in an asynchronous culture. As our hypothesis predicted, cyclin D1 was expressed at high levels not only in G1 phase but also in G2 phase (Fig. 2D). The expression levels during S phase are low, probably due to the repressive activity of free E2F [59] or inactivation of the growth inhibitory activity of Rb in S phase [60]. This pattern of cyclin D1 expression is not restricted to rapidly growing, immortalized 3T3 cells. The same pattern was observed in asynchronous cultures of mouse embryonic fibroblast (MEF) as well as in human diploid fibroblasts (Fig. 2). A similar expression pattern through the cell cycle phases have been documented for cyclin D1 message [61]. Upon random injection of neutralizing Ab against Ras protein, the expression of cyclin D1 in G2 phase was first suppressed (6 h after the injection), while G1 phase expression persisted for a longer period of time [43]. To confirm the Ras dependency more clearly, the S phase cells of an asynchronous NIH3T3 culture were tagged by a bromodeoxyuridine
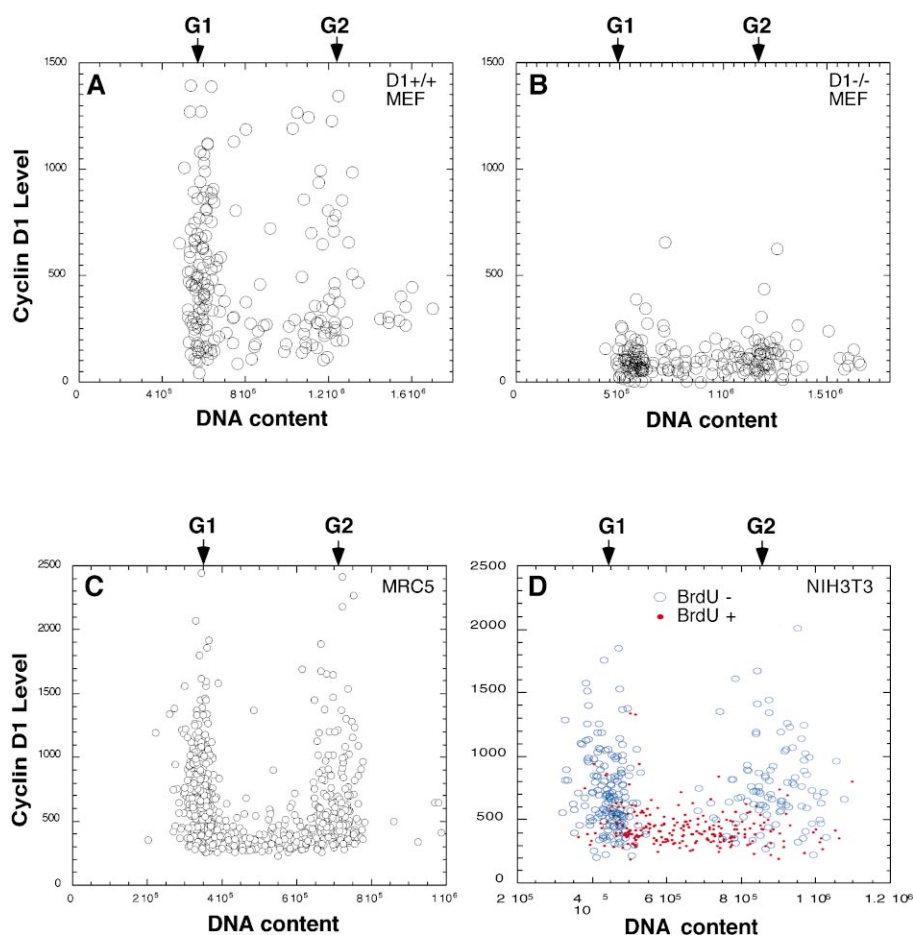

Fig. 2. Cyclin D1 is expressed at high levels in G2 phase. Rapidly growing cultures of the indicated cells were fixed and stained for cyclin D1 with indirect immunofluorescence using monoclonal anti-cyclin D1 antibodies, the clone 72-13G for mouse cells and the clone DCS-6 for MRC 5 cells. DNA was stained with DAPI and BrdU incorporation was detected with indirect immunofluorescence. DNA content and the expression levels of cyclin D1 of individual cells were determined using digital image analysis as described [43]. A: Cyclin D1 +/+ MEF. B: Cyclin D1 −/− MEF. C: MRC 5 cells, human diploid fibroblasts. D: NIH3T3 cells. Just prior to fixing, NIH3T3 cells were pulsed with BrdU for 30 min to identify S phase cells. S phase cells are indicated by closed circles, and open circles indicate BrdU negative cells (D). Notice that S phase cells express low levels of cyclin D1. A few D1 −/− cells with relatively high levels of cyclin D1 were in fact round mitotic cells displaying non-specific staining.

(BrdU) pulse soon after the injection, and cyclin D1 expression in those cells was followed thereafter. Anti-Ras Ab injection during S phase completely blocked the high expression of cyclin D1 which would have occurred in the next G2 phase, confirming the Ras requirement for cyclin D1 elevation during G2 phase. A similar analysis combined with time-lapse analyses to determine the cell cycle timing of the anti-Ras injection revealed that once induced during G2 phase in a Ras-dependent manner, cyclin D1 expression continued through G1 phase without Ras activity [43]. Finally, as we predicted, forced expression of cyclin D1 partially overcame the Ras requirement for cell cycle continuation, indicating that one of the primary functions of Ras activity in cyclin cells is to induce cyclin D1 expression to drive G1/S transition [43]. In each case, the predictions of our model were confirmed.

The continued expression of cyclin D1 during G1 phase in the absence of Ras activity raises interesting questions. This might result from the fact that each signaling step from activated Ras to cyclin D1 expression has a given half-life. The overall half-life of cyclin D1 expression following Ras neutralization is an integration of the half-lives of individual signaling steps, which together serve as a reservoir for the total signaling activity. Thus, molecules of the first step must be inactivated before their deficiency initiates decay of the activities in the second signaling step and so forth. In this way the expression of cyclin D1 can persist even after Ras neutralization, despite the fact that induction of cyclin D1 expression is dependent upon Ras. Alternatively, cell cycle specific altera-

tions in Ras signaling might reduce the Ras dependency of cyclin D1 expression during G1 phase. JunB, which represses cyclin D1 transcription by antagonizing c-Jun, is phosphorylated by cdc2 during mitosis. It is suggested that this phosphorylation triggers the degradation of JunB resulting in low expression levels during G1 phase [62]. With low levels of this repressor, Ras activity might be required to a lesser extent for cyclin D1 expression during G1 phase. The third possibility is that Ras is required only to activate cyclin D1 expression, while Ras-independent mechanisms promote its continued expression. In support of this idea, many signaling systems have been reported to activate the cyclin D1 promoter [63–70]. Finally, the stability of cyclin D1 protein might play a role in this continued expression in G1 phase. Cyclin D1 is actively degraded through ubiquitination [71]. Phosphorylation by glycogen synthase kinase 3β (GSK3β) triggers degradation of cyclin D1. During G1 phase, GSK3β is mainly localized in the cytoplasm whereas cyclin D1 is concentrated in the nucleus [72]. This subcellular separation of GSK3β from cyclin D1 would favor the stabilization of cyclin D1, contributing to its Ras-independent expression during G1 phase.

6. The biological significance of cyclin D1 expression in G2 phase

Cyclin D1 is known as a G1 cyclin because it plays an important role in the cell cycle transition from G1 to S phase. The high levels of cyclin D1 expression during G2 phase,
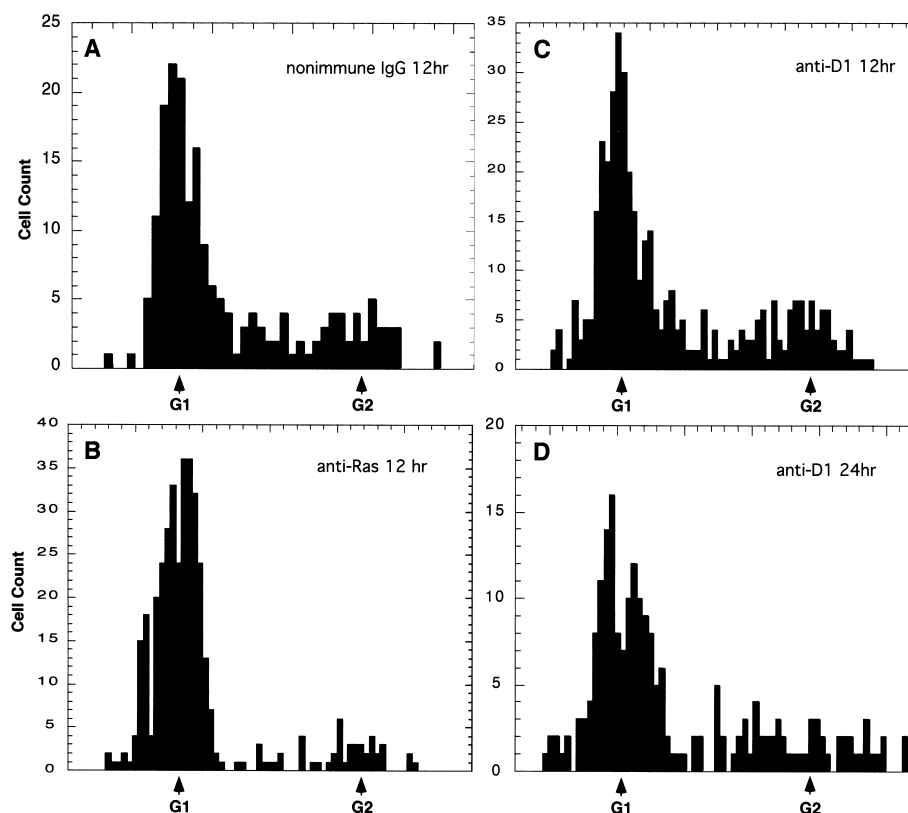

Fig. 3. G2 arrest induced by anti-cyclin D1 Ab injection. Rapidly growing NIH3T3 cells were injected with anti-cyclin D1 Ab, anti-Ras Ab or non-immune chicken IgG. After the indicated times, cells were fixed and DNA was stained with DAPI. To detect injected cells, injected IgG was stained. Non-immune IgG injection did not alter the DNA histogram pattern (A). When cells received anti-Ras Ab, the majority of the cells were arrested at G1 phase by 12 h (B). Anti-cyclin D1 Ab, on the other hand, induced G2 arrest at 12 h after injection (C). This arrest subsided by 24 h (D).

however, prompted us to consider the possibility that cyclin D1 has a biological role during G2 phase. From our previous studies, it was clear that anti-cyclin D1 Ab injection did not induce a prolonged G2 phase arrest [44]. It is possible, however, that a slight delay of cell cycle progression might be seen following anti-cyclin D1 Ab injection. Therefore, in order to carefully determine if cyclin D1 plays some role in progression through G2 phase, neutralizing Ab against cyclin D1 was injected into rapidly growing cells and the DNA content of the injected cells was determined as a function of time after the injection. For comparison, anti-Ras Ab or non-immune chicken IgG was injected into parallel cultures. Non-immune IgG injection did not change the cell cycle distribution at any time (Fig. 3A). G1 arrest became obvious 12 h after anti-Ras injection (Fig. 3B). In contrast, the G2 population increased at 12 h after anti-cyclin D1 injection (Fig. 3C). This increase was not very large, but similar results were obtained in multiple experiments. This delay in G2 phase progression, however, is temporary, because the G2 population decreased with time (Fig. 3D).

Since cyclin D1 protein is unstable, it is possible that its continued synthesis following injection had begun to saturate the neutralizing capacity of the injected Ab. This might result in a gradual release of cell cycle blockage in G1 phase, and allow for the appearance of G2 phase cells 15 h following injection of anti-cyclin D1 seen above. To avoid this potential complication we performed a time-lapse experiment. Cells were followed for 15 h prior to and following injection with anti-cyclin D1 Ab, and pulsed with ^{3}H-thymidine at the end of the analysis to determine cells in S phase. The DNA content of each cell was determined by image analysis following DAPI staining and the time-lapse movies were analyzed to determine the age of each cell at the end of the analysis. This analysis allowed us to determine approximate cell cycle position at the time of injection, and the ability of cells to pass through the cell cycle thereafter (Fig. 4).

The results confirm the conclusions from previous experiments and indicate that injected anti-cyclin D1 does interfere with passage through G2 phase. Many cells in G2 and S phases at the time of injection were found in G2 phase at the end of the experiment without ever passing through mitosis. These cells had, therefore, been retained in G2 phase by the injected Ab for 20–30 h (Fig. 4A). Cells in G1 phase at the time of injection had either been retained in G1 phase, or had recently been released from the G1 block and had entered S phase. This observation confirms the transient nature of the anti-cyclin D1 blockage. Cells that divided following injection are listed with negative ages. Most of these cells had G1 DNA content as a result of anti-cyclin D1 inhibition (Fig. 4A). As a control, anti-Ras Ab did not inhibit progression through G2 phase. Most of these injected cells divided following injection and were retained with a G1 DNA content (Fig. 4B). While cyclin D1 production is dependent upon Ras activity, it is apparent that anti-Ras was not able to decrease cyclin D1 levels enough to inhibit G2 transit within the time frame of this experiment. Taken together, these experiments indicate that when cyclin D1 levels are eliminated by anti-cyclin D1 Ab injection, G2 phase is increased in length by up to 10 h. Recently, abrogation of cyclin D1 or inhibition of cyclin D-associated kinase has been shown to cause G2 arrest [73,74] and over-expression of cyclin D1 is reported to shorten the G2 arrest induced by γ-irradiation [75]. It is therefore possible
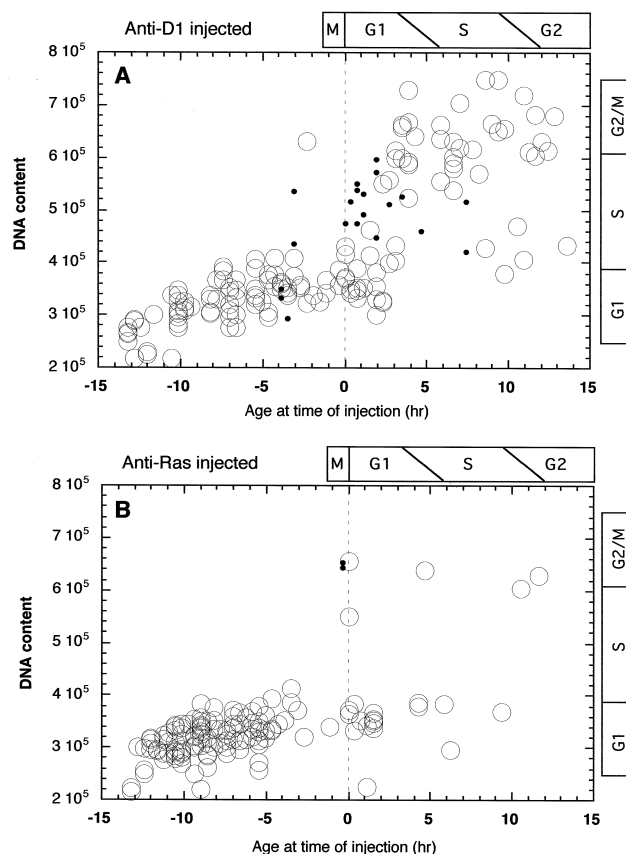

Fig. 4. Neutralization of cyclin D1 slows down the progression through G2 phase. A: An asynchronous culture of NIH3T3 cells was monitored in time-lapse for 15 h to determine the cell cycle timing of anti-cyclin D1 injection. After the injection, a second 15 h time-lapse movie was taken, with a ^{3}H-thymidine pulse during the final hour. Cells were fixed and stained with DAPI for determination of DNA content. Age of the cells at the time of injection is plotted against DNA content. Thymidine-labeled cells are indicated by closed circles, and non-labeled cells with open circles. The cells with negative age divided following the injection. For example, a cell with the age of minus 6 h divided 6 h after the injection. The cells with positive age divided at the indicated times prior to the injection and never divided thereafter until the end of the experiment. The cell cycle bar at the top indicates the cell cycle position at the time of injection, estimated from the age of the cell. The cell cycle bar on the right indicates the cell cycle phase at the end of the experiment, determined by the intensity of DAPI staining. A significant number of anti-cyclin D1-injected cells had G2 content of DNA at the end of the experiment. The majority of such cells received injection while they were in S or G2 phase. This indicates that these cells stayed in S or G2 for at least 15 h after the injection. B: Under the same conditions, anti-Ras Ab injection did not show any significant G2 population at the end of the experiment, indicating no G2-arresting activity of this Ab.

that high levels of cyclin D1 expression during G2 phase might facilitate progression through G2 phase.

7. Synchronization alters the nature of cell cycle control

In order to perform the studies described above, it was necessary to analyze individual cells in asynchronous culture. It would be desirable to confirm these conclusions with biochemical analyses in synchronous cultures. Our goal was to confirm our observation with biochemical means, such as Western or Northern blotting using synchronous cultures.
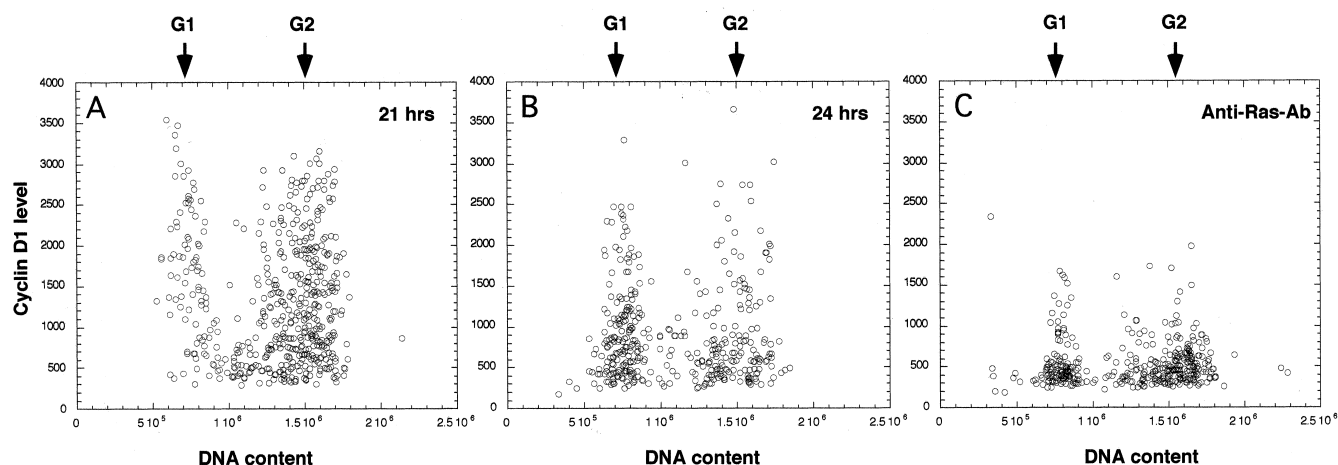
Fig. 5. Synchronization alters Ras dependency of cyclin D1 expression. NIH3T3 cells were made quiescent by serum starvation (0.5% serum) for 2 days. Cells were stimulated with 10% serum. At indicated time after serum addition, cells were fixed and stained for cyclin D1 and DNA with immunofluorescence and DAPI. The intensity of cyclin D1-associated fluorescence was plotted against DNA content. Each circle represents a single cell. After 21 h, cells progress into G2 phase with good synchrony and cyclin D1 levels are high (A). At this time point, anti-Ras Ab was injected and further cultured for 3 h, during which half the cells completed mitosis. Anti-Ras Ab suppressed the cyclin D1 expression levels both in G1 and G2 phase (C) in comparison to non-injected surrounding cells (B). This is quite different from rapidly cycling cells, where anti-Ras Ab injection failed to suppress the already induced cyclin D1 in G1 phase [43].

Although synchronization may alter the nature of cell cycle, the second cell cycle phases after the release from serum starvation may be similar to those in rapid cell cycle. For example, the expression of c-myc in the second G1 phase following release from quiescence is similar to the constitutive expression observed in G1 phase of rapidly growing cultures [36,37]. We tested the cyclin D1 expression from G2 through the second G1 phase following serum stimulation of quiescent NIH3T3 cells. Using the methods described above, we analyzed cell cycle synchrony, cyclin D1 expression pattern and the Ras dependency of cyclin D1 expression to determine if the regulation of cyclin D1 was similar to an asynchronous culture. Judging by DNA content, the cell cycle synchrony was maintained well even at 21 h after serum stimulation, when the majority of cells had progressed into G2 phase (Fig. 5A). These synchronous G2 cells expressed high levels of cyclin D1, and the high levels of expression were maintained through the next G1 phase (the second G1), 24 h after serum addition (Fig. 5B). This expression pattern of cyclin D1 was the same as in rapidly growing asynchronous cultures. Next we examined the Ras dependency of the cyclin D1 expression during G2 and the second G1 phases. Anti-Ras Ab was injected at 21 h after the serum stimulation, the time point when the majority of cells had progressed into G2 phase and expressed high levels of cyclin D1. When the cells were fixed 3 h after the injection, i.e. 24 h after the serum addition, anti-Ras injection suppressed the level of cyclin D1 expression in both G1 and G2 populations (Fig. 5C). In a rapidly growing culture, on the other hand, cyclin D1 expression continued through G1 at least for 6 h after anti-Ras injection, once it has been induced during the preceding G2 phase in a Ras-dependent manner [44]. This indicates that serum starvation altered the Ras dependency of cyclin D1 expression in the second G1 phase.

We also tested the synchronized cultures released from S phase arrest induced by double thymidine block or aphidicholin–thymidine block. One previous report observed that cyclin

D1 is induced as the cells progressed into G2 phase following release from S phase arrest [61]. Our image analysis detected the same increase in cyclin D1 protein expression as the cells synchronously progress into G2 phase as was observed in asynchronous cultures. Under these conditions, however, the cyclin D1 expression in the next G1 phase again became dependent upon Ras activity (data not shown). In mitotic shake off, we could not achieve enough synchrony to perform biochemical analysis after the cells passed through the S phase. Therefore, no synchronization protocol tested faithfully reproduced the delicate control patterns of cyclin D1 through the cell cycle as seen in continuous cell cycle progression. These results emphasize the importance of our methods to analyze rapidly proliferating cells without synchronization.

8. Conclusion

Based upon these results, the following model is proposed (Fig. 6). During continuous cell cycle progression, cells sense the presence of mitogens leading to Ras activation during G2 phase or earlier. Activated Ras induces cyclin D1 expression during G2 phase. Once induced by Ras action, cyclin D1 expression continues until the beginning of the next S phase in a Ras-independent fashion. This Ras-independent continued expression of cyclin D1 drives, in part, cell cycle progression from G1 to S phase even when the activity of Ras is abrogated during G1 phase. In this way, anti-Ras injection during G1 phase failed to arrest rapidly cycling NIH3T3 cells in the current G1. In other words, the Ras-dependent decision to pass through the G1/S transition is already made during the preceding G2 phase.

It seems that rapidly proliferating cells start to prepare for the next G1/S transition while they are still in G2 phase, so long as the conditions remain favorable for proliferation. Such early preparation may allow for a shortened G1 phase. In support of this model, we recently observed that the elevation of cyclin D1 expression levels took place only in G2

Reentry into Cell Cycle
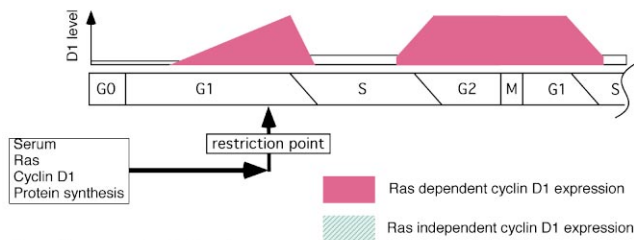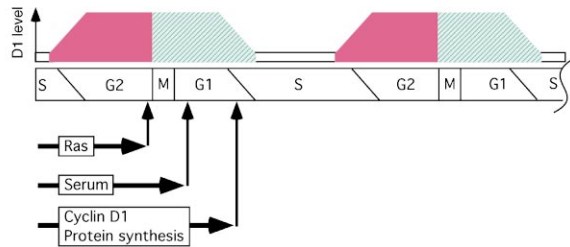

Continuous Cell Cycle

Fig. 6. Schematic presentation of our model. During re-entry into the cell cycle from quiescence, serum, Ras, cyclin D1 and protein synthesis are each required until the same point in G1 phase, several hours before the start of DNA synthesis. The expression of cyclin D1 depends upon Ras during the entire process, even during the second G1 phase (see Fig. 5). On the other hand, rapidly growing cells sense growth conditions during S/G2 phase, leading to Ras activation. In turn, Ras induces cyclin D1 expression to high levels during G2 phase, which continues through the next G1 phase. Cyclin D1 induced during G2 phase promotes the progression through G2 phase. The expression of cyclin D1 becomes Ras-independent following mitosis and plays a major role in driving the cell cycle progression into S phase. Although Ras becomes dispensable after mitosis, serum, protein synthesis and cyclin D1 are still required in G1 phase for G1/S transition.

phase when oncogenic Ras was injected [51]. The expression of cyclin D1 during G2 also promotes rapid passage through G2 phase contributing to a shortened cell cycle. Although Ras becomes dispensable after mitosis in asynchronous cultures, the cyclin D1-dependent and protein synthesis-dependent restriction point exists at the end of the G1 phase, and serum is still required during early G1 phase for the completion of the next cell cycle [44]. These requirements may serve as G1 check points to monitor the concerted activities of molecules required for G1/S transition, preventing the premature or undesirable entry into S phase during the rapid cell cycle progression.

This new concept concerning the control of continuous cell cycle progression was obtained because the utilized analytical methods, which were designed to analyze continuously proliferating cells, avoided the alterations in cell cycle signaling caused by synchronization. By applying the methods described here, it is possible that we may learn new aspects of cell cycle control in cancer cells. Furthermore, we may even identify differences in proliferative signaling between cancer cells and rapidly proliferating normal tissues such as epidermis, gut epithelium and bone marrow, which are often inadvertently targeted by cancer therapeutics. The information obtained from such studies might suggest cancer therapeutic strategies which are less toxic for these continuously proliferating normal tissues, without compromising the toxicity against cancer cells.

References

[1] Schlessinger, J. (2000) Cell 103, 211–225.
[2] Sherr, C.J. and Roberts, J.M. (1995) Genes Dev. 9, 1149–1163.
[3] Weinberg, R.A. (1995) Cell 81, 323–330.
[4] Pardee, A.B. (1974) Proc. Natl. Acad. Sci. USA 71, 1286–1290.
[5] Rossow, P.W., Riddle, V.G.H. and Pardee, A.B. (1979) Proc. Natl. Acad. Sci. USA 76, 4446–4450.
[6] McCormick, F. (1999) Trends Biochem. Sci. 9, M53–M56.
[7] Campbell, S.L., Khosravi-Far, R., Rossman, K.L., Clark, G.J. and Der, C.J. (1998) Oncogene 17, 1395–1413.
[8] Hill, C.S. and Treisman, R. (1999) Science's STKE, www.stke.org/cgi/content/full/OC_sigtrans;1999/3/pe1.
[9] Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.Y., Hanks, S.K., Roussel, M.F. and Sherr, C.J. (1992) Cell 71, 323–334.
[10] Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A. and Pestell, R.G. (1995) J. Biol. Chem. 270, 23589–23597.
[11] Galang, C.K., Der, C.J. and Hauser, C.A. (1994) Oncogene 9, 2913–2921.
[12] Aktas, H., Cai, H. and Cooper, G.M. (1997) Mol. Cell. Biol. 17, 3850–3857.
[13] Filmus, J., Robles, A.I., Shi, W., Wong, M.J., Colombo, L.L. and Conti, C.J. (1994) Oncogene 9, 3627–3633.
[14] Liu, J.-J., Chao, J.-R., Jiang, M.-C., Ng, S.-Y., Yen, J.-J. and Yang-Yen, H.-F. (1995) Mol. Cell. Biol. 15, 3654–3663.
[15] Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J. and Kato, J.Y. (1994) Mol. Cell. Biol. 14, 2066–2076.
[16] Baldin, V., Lukas, J., Marcote, M.J., Pagano, M. and Draetta, G. (1993) Genes Dev. 7, 812–821.
[17] Sherr, C.J. (1994) Cell 79, 551–555.
[18] Connell-Crowley, L., Harper, J.W. and Goodrich, D.W. (1997) Mol. Biol. Cell 8, 287–301.
[19] Zarkowska, T. and Mittnacht, S. (1997) J. Biol. Chem. 272, 12738–12746.
[20] Magnaghi-Jaulin, L. et al. (1998) Nature 391, 601–605.
[21] Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J. and Kouzarides, T. (1998) Nature 391, 597–601.
[22] Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X., Harbour, J.W. and Dean, D.C. (2000) Cell 101, 79–89.
[23] DeGregori, J., Kowalik, T. and Nevins, J.R. (1995) Mol. Cell. Biol. 15, 4215–4224.
[24] Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M. and Pagano, M. (1995) Mol. Cell. Biol. 15, 2612–2624.
[25] Lukas, J., Bartkova, J., Rohde, M., Strauss, M. and Bartek, J. (1995) Mol. Cell. Biol. 15, 2600–2611.
[26] Peeper, D.S., Upton, T.M., Ladha, M.H., Neuman, E., Zalvide, J., Bernard, R., DeCaprio, J.A. and Ewen, M.E. (1997) Nature 386, 177–181.
[27] Satoh, T., Endo, M., Nakafuku, M., Nakamura, S. and Kaziro, Y. (1990) Proc. Natl. Acad. Sci. USA 87, 5993–5997.
[28] Taylor, S.J. and Shalloway, D. (1996) Curr. Biol. 6, 1621–1627.
[29] Dobrowolski, S., Harter, M. and Stacey, D.W. (1994) Mol. Cell. Biol. 14, 5441–5449.
[30] Kovary, K. and Bravo, R. (1992) Mol. Cell. Biol. 12, 5015–5023.
[31] Stoeber, K., Mills, A.D., Kubota, Y., Krude, T., Romanowski, P., Marheineke, K., Laskey, R.A. and Williams, G.H. (1998) EMBO J. 17, 7219–7229.
[32] Williams, G.H. et al. (1998) Proc. Natl. Acad. Sci. USA 95, 14932–14937.
[33] Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stiliman, B., Nevins, J.R. and Williams, R.S. (1998) Proc. Natl. Acad. Sci. USA 95, 3603–3608.
[34] Ohtani, K., DeGregori, J., Leone, G., Herendeen, D.R., Kelly, T.J. and Nevins, J.R. (1996) Mol. Cell. Biol. 16, 6977–6984.
[35] Freeman, A., Morris, L.S., Mills, A.D., Stoeber, K., Laskey, R.A., Williams, G.H. and Coleman, N. (1999) Clin. Cancer Res. 5, 2121–2132.
[36] Persson, H., Gray, H.E. and Godeau, F. (1985) Mol. Cell. Biol. 5, 2903–2912.

[37] Thompson, C.B., Challoner, P.B., Neiman, P.E. and Groundine, M. (1985) Nature 314, 363–366.

[38] Campan, M., Desgranges, C., Gadeau, A.P., Millet, D. and Belloc, F. (1992) J. Cell Physiol. 150, 493–500.

[39] Stacey, D.W., Hitomi, M. and Chen, G. (2001) Mol. Biol. Cell, in press.

[40] Zickert, P., Wejde, J., Skog, S., Zetterberg, A. and Larsson, O. (1993) Exp. Cell Res. 207, 115–121.

[41] Assoian, R.K. (1997) J. Cell Biol. 136, 1–4.

[42] Zetterberg, A. and Larsson, O. (1985) Proc. Natl. Acad. Sci. USA 82, 5365–5369.

[43] Hitomi, M. and Stacey, D.W. (1999) Curr. Biol. 9, 1075–1084.

[44] Hitomi, M. and Stacey, D.W. (1999) Mol. Cell. Biol. 19, 4623–4632.

[45] Stacey, D.W., Hitomi, M., Kanovsky, M., Gan, L. and Johnson, E.M. (1999) Oncogene 18, 4254–4261.

[46] Stacey, D.W. (1981) Methods Enzymol. 79, 76–88.

[47] Stacey, D.W. and Kung, H.F. (1984) Nature 310, 508–511.

[48] Mulcahy, L.S., Smith, M.R. and Stacey, D.W. (1985) Nature 313, 241–243.

[49] Smith, M.R., DeGudicibus, S.J. and Stacey, D.W. (1986) Nature 320, 540–543.

[50] Fox, P.L., Sa, G., Dobrowolski, S.F. and Stacey, D.W. (1994) Oncogene 9, 3519–3526.

[51] Sa, G., Hitomi, M., Stacey, A., Chen, G. and Stacey, D.W. (2001), in preparation.

[52] Yen, A. and Pardee, A.B. (1978) Exp. Cell Res. 116, 103–113.

[53] Roche, S., Koegl, M., Barone, M.V., Roussel, M.F. and Courtneidge, S.A. (1995) Mol. Cell. Biol. 15, 1102–1109.

[54] Valius, M. and Kazlauskas, A. (1993) Cell 73, 321–334.

[55] Barone, M.V. and Courtneidge, S.A. (1995) Nature 378, 509–512.

[56] Moolenaar, W.H. (1995) J. Biol. Chem. 270, 12949–12952.

[57] Silvio, G. (2000) Science's STKE, www.stke.org/cgi/content/full/OC_sigtrans;2000/40/re1.

[58] Lukas, J., Jadayel, D., Bartkova, J., Nacheva, E., Dyer, M.J.S., Strauss, M. and Bartek, J. (1994) Oncogene 9, 2159–2167.

[59] Watanabe, G., Albanese, C., Lee, R.J., Reutens, A., Vairo, G., Henglein, B. and Pestell, R.G. (1998) Mol. Cell. Biol. 18, 3212–3222.

[60] Yageta, M., Tsunoda, H., Yamanaka, T., Nakajima, T., Tomooka, Y., Tsuchida, N. and Oda, K. (1999) Oncogene 18, 4767–4776.

[61] Motokura, T., Bloom, T., Kim, H.G., Juppner, H., Ruderman, J.V., Kronenberg, H.M. and Arnold, A. (1991) Nature 350, 512–515.

[62] Bakiri, L., Lallemand, D., Bossy-Wetzel, E. and Yaniv, M. (2000) EMBO J. 19, 2056–2068.

[63] Guttridge, D.C., Albanese, C., Reuther, J.Y., Pestell, R.G. and Baldwin Jr., A.S. (1999) Mol. Cell. Biol. 19, 5785–5799.

[64] Hinz, M., Krappmann, D., Eichten, A., Heder, A., Scheidereit, C. and Strauss, M. (1999) Mol. Cell. Biol. 19, 2690–2698.

[65] Page, K. et al. (1999) J. Biol. Chem. 274, 22065–22071.

[66] Westwick, J.K., Lambert, Q.T., Clark, G.J., Symons, M., Van Aelst, L., Pestell, R.G. and Der, C.J. (1997) Mol. Cell. Biol. 17, 1324–1335.

[67] Joyce, D. et al. (1999) J. Biol. Chem. 274, 25245–25249.

[68] Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999) Proc. Natl. Acad. Sci. USA 96, 5522–5527.

[69] Tetsu, O. and McCormick, F. (1999) Nature 398, 422–426.

[70] Matsumura, I. et al. (1999) EMBO J. 18, 1367–1377.

[71] Diehl, J.A., Zindy, F. and Sherr, C.J. (1997) Genes Dev. 11, 957–972.

[72] Diehl, J.A., Cheng, M., Roussel, M.F. and Sherr, C.J. (1998) Genes Dev. 12, 3499–3511.

[73] Gabrielli, B.G., Sarcevic, B., Sinnamon, J., Walker, G., Castellano, M., Wang, X.Q. and Ellem, K.A. (1999) J. Biol. Chem. 274, 13961–13969.

[74] Lahti, J.M., Li, H. and Kidd, V.J. (1997) J. Biol. Chem. 272, 10859–10869.

[75] Coco Martin, J.M., Balkenende, A., Verschoor, T., Lallemand, F. and Michalides, R. (1999) Cancer Res. 59, 1134–1140.