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Research Article

Identification of a restriction point at the M/G1 transition in CHO cells

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Abstract. The regulation of cell cycle progression in normal mammalian cells is dependent on the presence of growth factors. In their absence, non-transformed cells will stop dividing and enter the quiescent state (G0). We show here that in Chinese hamster ovary cells, at least two serum-dependent points exist during G1 that lead to different cellular responses. The first point is located immediately after mitosis and is suggested to link with apoptosis. The second point is located late in G1, and probably corresponds with the 'classic' restriction point R. Cells depleted of serum after the first restriction point will not stop randomly in G1 but continue G1 progression until they reach the late restriction point, as marked by translocation of $p42^{MAPkinase}$ (ERK2) to the nucleus.

Key words. Cell cycle; restriction point; signal transduction; M/G1 transition; apoptosis.

The regulation of cell proliferation by growth factors of normal mammalian cells occurs during the transition from the quiescent G0 state to the G1 phase and during progression through the different sub-phases of G1. The G1 phase of the mammalian cell cycle of continuously cycling cells has been divided into two phases: G1-pm (G1-post-mitotis) and G1-ps (G1 pre-S phase) [1]. Non-transformed cells in the early phase of G1 (G1-pm) can enter the G0 phase, while those in the later phase (G1-ps) can not. The transition between these two phases is located at the end of G1 and is associated with the so-called restriction point (R), at which the cells acquire growth factor independence and become committed to DNA replication [2–4]. As long as growth factors are present, normal cells will proliferate, but when growth factors are withdrawn, these non-transformed cells will stop dividing and enter the quiescent state (G0). During G0, metabolic activity is low and protein expression is diminished. Cells will remain in this quiescent state until extracellular conditions change and cells are stimulated to reenter the cell cycle. Upon stimulation of quiescent cells by growth factors or other external signals, various signal transduction cascades are activated, leading to early gene expression and resumption of the cell cycle. Most research concerning cell cycle control makes use of this stimulation of quiescent cells as a way of cell synchronization, since cells that are stimulated to reenter the cell cycle are supposed to enter it at about the same point in G1 [5, 6].

One of the most important signal transduction pathways involved in growth factor stimulation of G0 cells is the mitogen-activated protein kinase (MAP kinase) pathway. MAP kinase [5, 7], also described as extracellular-regulated kinase (ERK), is rapidly activated by dual tyrosine and threonine phosphorylation in cells stimulated with various extracellular signals [5, 7–11]. Upon growth factor stimulation, MAP kinase translocates to the nucleus [12–17] where it can induce immediate early gene transcription by phosphorylating nuclear transcription factors like c-Myc, c-jun, Elk-1 and c-Ets [18–21]. MAP kinase

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activity is thought to be required for expression of cyclin D [10, 22–26] during G0/S and to facilitate assembly of cyclin D1 into catalytically active complexes [27]. In addition to the stimulation of quiescent cells, the MAP kinase signal transduction pathway has also been suggested to play an active role during G1 progression [5–7, 26, 28], for example by phosphorylating cdk2 [29] or by downregulating $p27^{kip1}$ [10, 24, 30, 31]. Furthermore, in continuously growing cells, MAP kinase has been shown to translocate to the nucleus at the end of mid-G1 [32]. Inhibition of this translocation by the use of MEK inhibitor, or inhibition of MAP kinase activation by a specific phosphatase, MKP-1, by the use of antisense constructs, by drug treatment or by overexpression of kinase-inactive mutants results in a block of DNA synthesis [32–35]. Besides the MAP kinase signal transduction pathway, other signal transduction cascades, such as the PI3 kinase signal transduction route [leading to activation of protein kinase B (PKB)/Akt], play important roles in the regulation of cell cycle progression and in the stimulation of quiescent cells by growth factors or other extracellular signals [36–40].

In contrast to non-transformed cells that move into the quiescent state upon removal of growth factors during G1-pm, most transformed cells appear to have lost the growth factor dependency. When growth factors are removed, transformed cells do not enter G0 but rather stop at some point in G1 or even pass the restriction point and continue cell cycle progression [41–43]. This lost ability to exit the cell cycle and become arrested in G0 is thought to represent one of the major differences between normal and transformed cells [1]. We show here that in Chinese hamster ovary (CHO) cells, a transformed cell line, a G0-like state exists which cells enter when growth factors are removed immediately after mitosis. Cells incubated in the absence of growth factors early in G1 (denominated $G0^-$ cells) show 'classic' G0 features, such as decreased expression of cyclin D, induction of $p27^{KIP1}$, dephosphorylation of proteins involved in signal transduction and transient activation of these proteins upon growth factor stimulation. However, unlike 'classic' G0 cells, these $G0^-$ cells were found to be unable to resume cell cycle progression after serum stimulation. Moreover, these cells showed a spherical phenotype and were not properly attached to the substratum after overnight starvation, indicating that $G0^-$ cells are different from G0 cells. Cells incubated in the absence of serum later in G1 appeared to continue cell cycle progression until the 'classic' restriction point (R), and were therefore denominated G_R cells. These cells did not show typical G0 characteristics upon overnight starvation, but seemed to stop at a point late in G1 that was marked by the translocation of MAP kinase to the nucleus. As opposed to $G0^-$ cells, G_R cells were readily able to reenter the cell cycle upon serum stimulation.

Materials and methods

Cell culture and synchronization of cells

CHO cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 7.5% fetal calf serum (FCS; Gibco) at 37 °C in a 5% CO_2 atmosphere. Three days prior to synchronization, cells were transferred in DMEM containing 7.5% FCS, 25 mM HEPES, and grown under low-CO_2 conditions. One day before mitotic shake-off, cells were plated at a density of 3×10^4 cells/cm^2. Mitotic cells were obtained by shaking an asynchronously growing cell population firmly for 1 min and pipetting off the medium as described previously [44]. The mitotic cells obtained after mitotic shake-off were centrifuged for 5 min at 1500 rpm and resuspended in DMEM, 25 mM HEPES, with or without serum. Cells were subsequently replated at a density of 1.5×10^4 cells/cm^2 on tissue culture dishes (Nalge Nunc). For serum stimulation, synchronized cells were incubated in the absence of serum for 24 h, washed twice with PBS, and medium containing 7.5% FCS was subsequently added for different periods of time. For induction of apoptosis, cells were incubated in the presence of 0.03 µg/ml doxorubicin.

Cell extraction and Western blotting

At the indicated times after replating, cells were washed twice in PBS and lysed in RIPA buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium desoxycholate, 0.1% SDS, 1 mM EDTA, 100 mM NaF, 1 mM benzamidine, 1 mM phenylmethanesulphonylfluoride (PMSF) and 1 mM Na_3VO_4]. The cell lysates were then centrifuged at 14,000 rpm for 5 min and the supernatants were subsequently used for Western blotting. For Western blotting, cell lysates of equal numbers of cells (3.0×10^4 cells/sample) were loaded on 10% acrylamide gels and electrophoretically transferred to a PVDF membrane (Boehringer Mannheim). The membranes were blocked with PBS containing 4% dried milk and 0.1% Tween 20 and then incubated with 1:40 diluted antibody against cyclin A (Calbiochem), 1:1000 diluted antibody against cyclin D ($p36^{cyclin\ D1}$ and $p34^{cyclin\ D2}$), PKBα (Transduction Labs) and $p42^{MAPK}$ (Upstate Biology) or phospho-specific antibodies for PKB (Ser473) and MAP kinase (New England Biolabs). After several washes in PBS containing 0.4% dried milk and 0.1% Tween 20, the membranes were incubated with 1:5000 diluted horseradish peroxidase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (1:5000; Jackson Immunoresearch). The membranes were washed twice in PBS containing 0.4% dried milk and 0.1% Tween 20 and twice in PBS alone, followed by detection of the immune signal using enhanced chemiluminescence (Dupont).

[^{3}H]thymidine incorporation

For thymidine incorporation, [^{3}H]thymidine, (specific activity 2 Ci/mmol; Amersham) was added to a concentration of 0.5 µCi/ml to medium containing mitotic 'shake-off' cells. Cells were grown in 24-well plates either in the presence or absence of 7.5% FCS, at 37°C. At the indicated times after replating, the cells were washed twice with PBS and 10% trichloroacetic acid and dissolved in 0.1 M NaOH. Radioactivity of the samples was measured using a liquid scintillation counter (Beckman LS 6000SE).

Immunofluorescence and apoptosis assay

Cells were grown on glass coverslips for the indicated times after replating and fixed for 30 min at room temperature with 3% paraformaldehyde (w/v) in PBS (pH 7.0). Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed and incubated with 50 mM glycine in PBS for 10 min. Incubation with primary antibody [αMAPK (ERK2) diluted 1:200 in 0.2% gelatine in PBS] was performed for 1 h at room temperature. Cells were washed four times in 0.2% PBS-gelatine for 5 min and incubated with fluorescein-conjugated secondary antibody goat-anti mouse IgG (GAM-FITC, 1:200; Jackson Immunoresearch). For detection of apoptosis, cells were treated with 10 µM of the CaspACE FITC-VAD-FMK in situ marker (Promega) for 30 min prior to fixation. Fluorescence was visualized with a Leitz Orthoplan microscope equipped with epi-illumination.

Results

The effect of serum withdrawal on asynchronously growing CHO cells

The proliferation of most non-transformed cells is dependent on the presence of growth factors and on cell attachment. When non-transformed cells are grown in the absence of growth factors, cell cycle progression is stopped in the G1 phase of the cell cycle and cells enter the quiescent state (G0) [45]. However, many cells lose the growth factor dependency upon transformation and do not enter G0 when serum is removed. These cells will often continue cell cycle progression without a growth factor-sensitive block at the G1/S transition or stop at some point in G1, depending on the nature of transformation [41–43].

Although the CHO cells used in our experiments are transformed cells, these cells do not pass the G1/S transition when serum is removed immediately after mitosis [46]. More remarkably, these cells appeared to enter the quiescent state (G0) upon serum removal during early G1. To determine whether (transformed) CHO cells indeed entered G0 upon serum removal, cell lysates were made of asynchronously growing cells, cultured in the presence or absence of serum, and used for Western blot experiments. In a typical quiescent state, signal transduction cascades, such as the MAP kinase pathway [8, 10] or the PI3 kinase pathway [39], are shut down and expression of cyclin D, which is thought to function as a growth factor sensor [47], is turned off. In asynchronously growing CHO cells, however, both MAP kinase and PKB/Akt, which is downstream of PI3 kinase [37], were found to be phosphorylated, both in cells incubated in the absence and in the presence of serum, as determined with a phospho-specific antibody (fig. 1 a). In addition, expression of cyclin D was detected both in cells grown in the presence of serum and in cells cultured in the absence of serum for 48 h. However, no expression of cyclin A in the serum-depleted cells was detected on a Western blot, indicating that these cells had not passed the G1/S transition. This suggestion was confirmed by cell count experiments; cells incubated in the presence of serum increased expo-

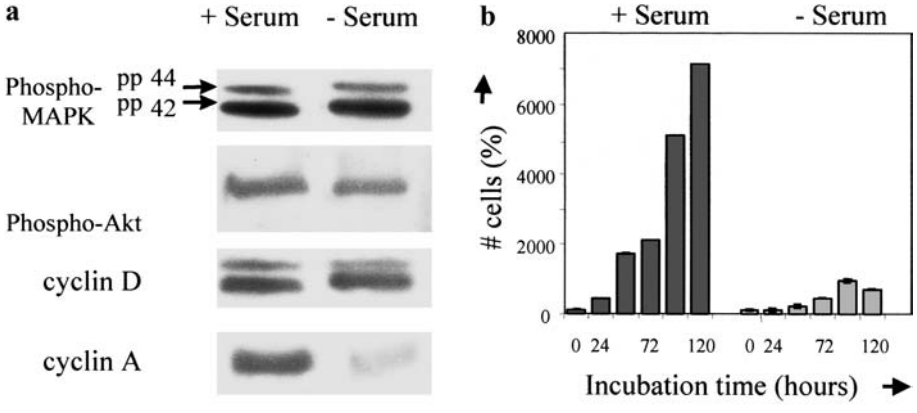

Figure 1. Effects of serum depletion on cell growth and the expression of cyclin A, cyclin D and the phosphorylation of MAP kinase in asynchronous CHO cells. (*a*) Western blot showing the phosphorylation of p44/p42^{MAPK} (ERK1/2) and Akt, and expression of cyclin D (p36$^{cyclin\ D1}$ and p34$^{cyclin\ D2}$) and cyclin A in asynchronous CHO cells incubated in the presence or absence of serum for 48 h. Cells were lysed in RIPA buffer and blotted as described in Materials and methods. (*b*) Quantitation of asynchronous CHO cells incubated in the presence or absence of serum, presented as means ± SD (n = 3).

nentially in cell number, whereas cells cultured in the absence of serum did not (fig. 1b). These data suggest that asynchronously growing CHO cells do not progress through G1/S and do not enter G0 but, rather, stop at some point in G1 when incubated in medium without serum.

Synchronized CHO cells react differently to serum starvation, depending on their position in the cell cycle

Although asynchronously growing CHO cells did not pass the G1/S transition when cultured in medium without serum, these cells did not appear to enter G0, but rather seemed to stop cell cycle progression in the G1 phase. Cells depleted of serum at the M/G1 transition, however, did seem to enter G0 as judged by the expression of cyclin D and the phosphorylation of MAP kinase [46]. Interestingly, the asynchronous cells that were stopped in G1 showed different characteristics from the cells arrested at the M/G1 transition (early in G1). Although these observations seem contradictory, they may be explained by assuming that CHO cells react differently to serum depletion at different phases in G1. Therefore, CHO cells were synchronized by mitotic shake-off and incubated in medium without serum either directly after mitosis (henceforth designated as $G0^-$ cells) or at 2 h after mitosis (now designated as G_R) and serum-starved for 24 h (fig. 2a). Cell lysates were subsequently made and analysed by Western blotting. As expected, cells that were depleted of serum immediately after mitosis ($G0^-$) did not exhibit phosphorylated signal transduction proteins, such as MAP kinase or PKB/Akt, although the non-phosphorylated form of these proteins was present. In addition, $G0^-$ cells did not show expression of cyclin D and had high levels of $p27^{KIP1}$, suggesting that they had entered the quiescent state [48]. Cells grown in the presence of serum for 2 h and then depleted of serum (G_R), however, did show phosphorylation of MAP kinase and PKB/Akt, expression of cyclin D and a low level of $p27^{KIP1}$ (fig. 2b), indicating that they had not entered G0. Neither of these cell types ($G0^-$ or G_R) was able to pass the G1/S transition, as determined by a [^{3}H]thymidine incorporation experiment. In cells incubated in the absence of serum either directly or at 2 h after mitosis, no significant thymidine incorporation was observed, indicating that no DNA had been synthesized. In contrast, control cells that were not depleted of serum did show [^{3}H]thymidine incorporation (fig. 3). In control cells, thymidine incorporation is low until 12 h after mitosis and subsequently increases rapidly, positioning G1/S at about 14 h after mitosis in this particular experiment. Thus, both $G0^-$ and G_R cells had stopped cell cycle progression, but reacted differently to serum depletion, as indicated by protein expression and phosphorylation patterns. Cells that were depleted of serum immediately after mitosis seemed to enter G0, while cells depleted of serum at 2 h after mitosis appeared to stop cell cycle progression at some point in G1.

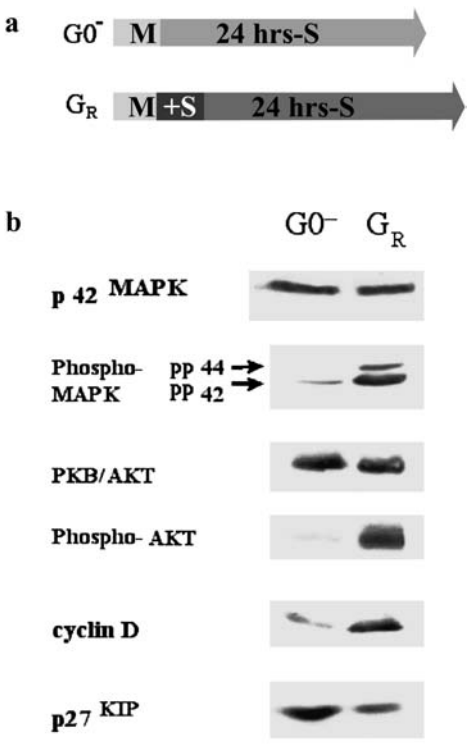

Figure 2. Effects of serum depletion at different points in G1 on the expression of cell cycle-related proteins and on the phosphorylation and expression of MAP kinase and PKB. (*a*) Schematic representation of $G0^-$ and G_R cells. CHO cells were synchronized by mitotic shake-off and depleted of serum either immediately after mitosis ($G0^-$) or at 2 h after mitosis (G_R) and serum-starved for 24 h. (*b*) Western blot showing the expression of cyclin D ($p36^{cyclin\ D1}$ and $p34^{cyclin\ D2}$), $p27^{KIP1}$, PKB/Akt and $p42^{MAPK}$ (ERK2) and phosphorylation of $p44/p42^{MAPK}$ (ERK1/2) and PKB (Ser473) in synchronized CHO cells incubated in the absence of serum at different times during G1. Cells were synchronized via mitotic shake-off, incubated in the absence of serum either immediately after mitosis ($G0^-$) or at 2 h after mitosis (G_R) and lysed in RIPA buffer as described in Materials and methods. Cell lysates of equal amounts of cells were put on the gel; a representative experiment is shown.

Stimulation of 'quiescent' cells

Although most research in the field of cell cycle control and signal transduction makes use of the stimulation of quiescent cells, no real definition of G0 cells exists. So far, the expression patterns and phosphorylation states of different proteins have suggested that cells incubated in the absence of serum immediately after mitosis had entered G0. When quiescent cells are stimulated to reenter the cell cycle, different signal transduction pathways are rapidly activated to resume protein expression and transcription of early genes. MAP kinase, for example, is known to be transiently phosphorylated upon growth factor stimulation in G0 cells, and to translocate to the nucleus, where it can activate transcription factors and in-

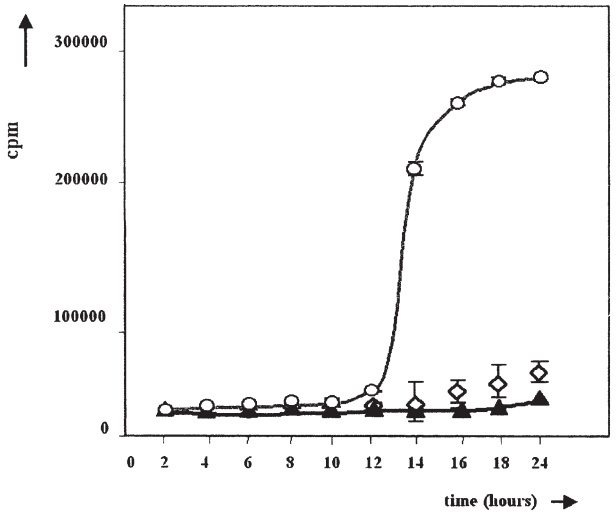

Figure 3. [^{3}H]thymidine incorporation in cells synchronized by mitotic shake-off and incubated in the presence or absence of serum. [^{3}H]thymidine was added at a concentration of 0.5 µCi/ml to medium containing mitotic CHO cells isolated by mitotic shake-off. Cells were replated in the presence of serum (circles) or in the absence of serum, either immediately (triangles) or at 2 h after mitosis (diamonds). At the indicated times after replating, the radioactivity of the samples was measured as described in Materials and methods. A representative experiment is shown; data are presented as means ± SD (n = 3).

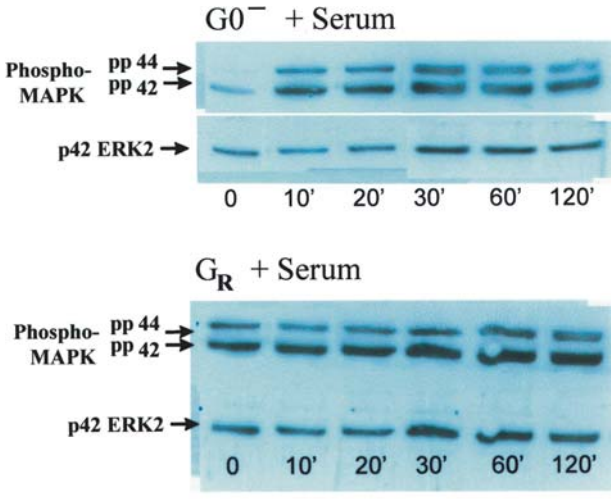

Figure 4. MAP kinase is transiently phosphorylated after serum stimulation in G0$^-$ cells but not in G$_R$ cells. Western blot showing the phosphorylation of p44/p42^{MAPK} (ERK1/2) in G0$^-$ and G$_R$ cells incubated in the absence of serum for 24 h and subsequently stimulated with 7.5% FCS for the indicated times. Cell lysates of equal amounts of cells were placed on the gel; a representative experiment is shown.

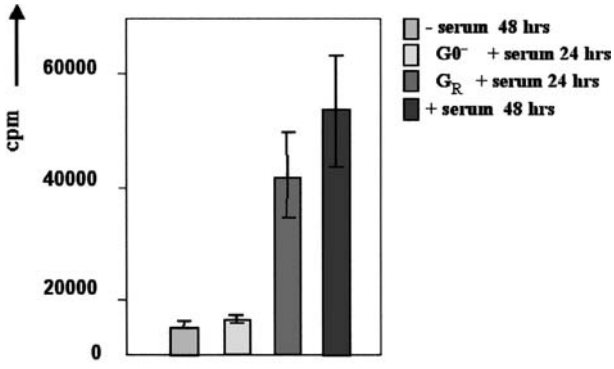

Figure 5. Effects of serum stimulation on cell cycle progression in G0$^-$ and G$_R$ cells. [^{3}H]thymidine-containing medium (0.5 µCi/ml) with 7.5% FCS was added to G0$^-$ cells and G$_R$ cells after overnight starvation, and radioactivity was measured after 24 h stimulation. Synchronized cells incubated for 48 h in medium with or without serum were used as a positive and negative control, respectively. A representative experiment is shown; data are presented as means ± SE (n = 3).

duce early gene expression [12–21]. To examine if G0$^-$ cells showed such a characteristic early response, cells were synchronized by mitotic shake-off, incubated in medium without serum immediately after mitosis or at 2 h after mitosis and serum-starved for 24 h. Then, cells were restimulated to enter G1 by addition of serum, and MAP kinase phosphorylation was determined by Western blotting using a phospho-specific antibody. As shown in figure 4, G0$^-$ cells did indeed show a transient phosphorylation of MAP kinase upon serum stimulation. In contrast, G$_R$ cells, which already had a high MAP kinase phosphorylation before stimulation (t = 0), did not show a transient response. However, when DNA synthesis was measured by [^{3}H]thymidine incorporation, G0$^-$ cells did not appear to resume their cell cycle upon stimulation (fig. 5). Even after 24 h of stimulation, the amount of incorporated thymidine was similar to that of cells depleted of serum immediately after shake-off and serum-starved for 48 h. In contrast, G$_R$ cells incorporated a significant amount of [^{3}H]thymidine, comparable to cells that had been incubated in the presence of serum for 48 h. Thus, G0$^-$ cells seemed to have entered an irreversible quiescent state, whereas G$_R$ cells did not appear to be affected by overnight starvation.

Features of G0$^-$ and G$_R$ cells

G0$^-$ cells did not continue cell cycle progression upon serum stimulation and therefore did not seem to be in a 'classic' G0 state. Besides this characteristic, G0$^-$ cells showed another remarkable feature that distinguished them from G0 cells, since changes in morphology occurred after overnight starvation. Cells incubated in the absence of serum immediately after mitosis initially appeared to attach properly to the substratum [46], but after longer starvation periods, adhesion decreased and cells became more spherical (fig. 6a). This spherical phenotype was observed only when serum was withdrawn within 10 min after mitosis. Cells incubated in the absence of serum at 10 min, 15 min or 4 h after mitosis did not show this characteristic phenotype, but remained

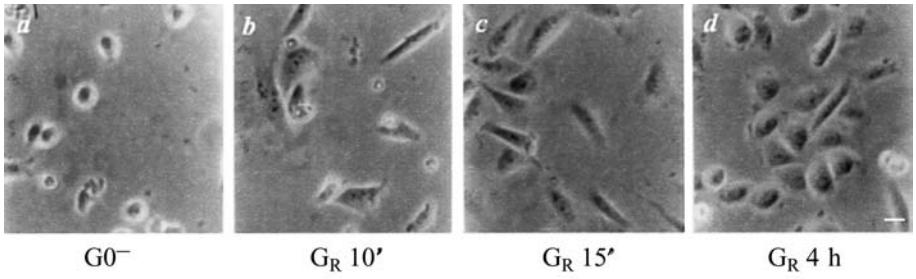

Figure 6. Morphology of CHO cells incubated in medium without serum at different times after mitosis. Cells were synchronized via mitotic shake-off and replated in the absence of serum, either immediately after mitosis ($G0^-$), at 10 min after mitosis (G_R 10′), at 15 min after mitosis (G_R 15′) or at 4 h after mitosis (G_R 4 h) and serum starved overnight.

firmly attached to the substratum (fig. 6b–d). Since $G0^-$ cells were found not to continue cell cycle progression upon serum stimulation and because mitotic cells that are incubated in suspension in the absence of growth factors die rapidly after replating [46], we tested the hypothesis that the $G0^-$ state was linked to apoptosis. Serum was withdrawn immediately after mitosis and cells were starved for 24, 48 and 72 h. At 30 min before cell fixation, the apoptosis marker CaspACE FITC-VAD-FMK was added to the cells to reveal activated caspase activity [49]. This marker is a cell-permeable FITC-conjugate of the pan-caspase inhibitor Z-VAD-FMK that binds irreversibly to activated caspases. As a control for apoptosis, asynchroneously growing CHO cells were incubated with doxorubicin for 24 or 48 h. As an additional control, non-synchronized CHO cells were incubated for 24, 48 or 72 h in the absence of serum. As expected, cells treated with doxorubicin showed apoptosis after 24 h as revealed by FITC staining (fig. 7). $G0^-$ cells only started to bind the apoptosis marker after 48 h of serum starvation and the FITC signal increased after 72 h. Interestingly, however, apoptosis was also observed in non-synchronous CHO cells, although to a much lesser extent. While approximately 35–40% of $G0^-$ cells incorporated the caspase inhibitor after 72 h of incubation in the absence of serum, only about 5–10% of the asynchronous CHO cells showed FITC staining (results not shown).

Whereas $G0^-$ cells entered the typical G0-like state, as described above, G_R cells attached normally to the substratum and appeared to stop somewhere in G1. Unlike $G0^-$ cells, G_R cells were able to reenter the cell cycle upon serum stimulation and even after overnight starvation these cells expressed cyclin D and activated signal transduction routes, such as the MAP kinase pathway. Phosphorylated MAP kinase is known to translocate to the nucleus both in response to growth factor stimulation of quiescent cells, and during the ongoing cell cycle in CHO cells [32]. This translocation during the ongoing cell cycle occurs at the end of mid-G1 and was shown to be necessary for cell cycle progression. Since G_R cells clearly contained phosphorylated MAP kinase, the localization of $p42^{MAPK}$ was determined in G_R cells at different times after mitosis to determine if the phosphorylated MAP kinase in G_R cells was also present in the nucleus. As shown in figure 8a, G_R cells indeed showed clear nuclear staining after 24 h of serum deprivation. Interestingly, the translocation of MAPK to the nucleus in these cells occurred at 8 h after mitosis (fig. 8b), at the same time as

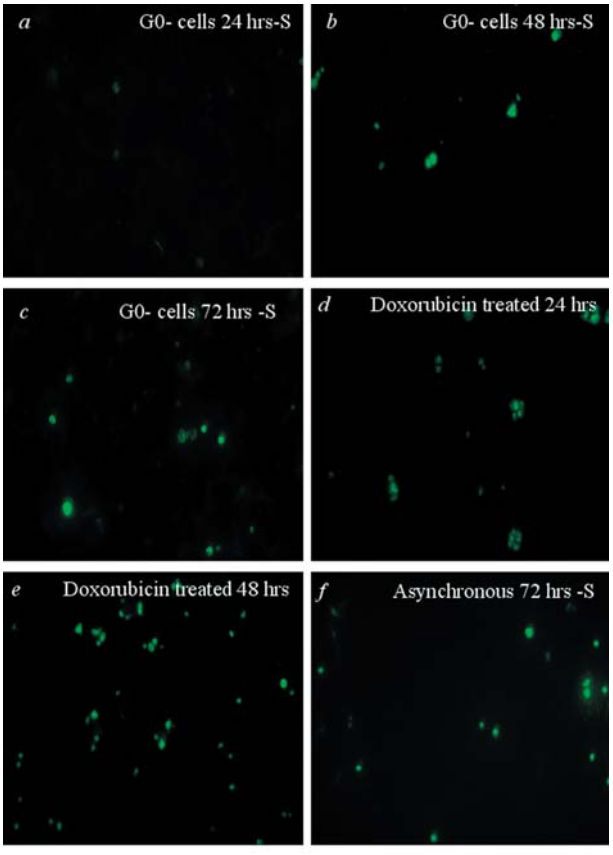

Figure 7. Apoptosis in CHO cells incubated in medium without serum at different times after mitosis. Cells synchronized via mitotic shake-off were replated in the absence of serum immediately after mitosis and serum-starved for the indicated times (*a–c*). At 30 min before fixation, the apoptotic marker CaspACE FITC-VAD-FMK was added as described in Materials and methods. Asynchronous CHO cells treated with doxorubicin for 24 or 48 h (*d, e*) or incubated in the absence of growth factors for 72 h (*f*) were used as controls.

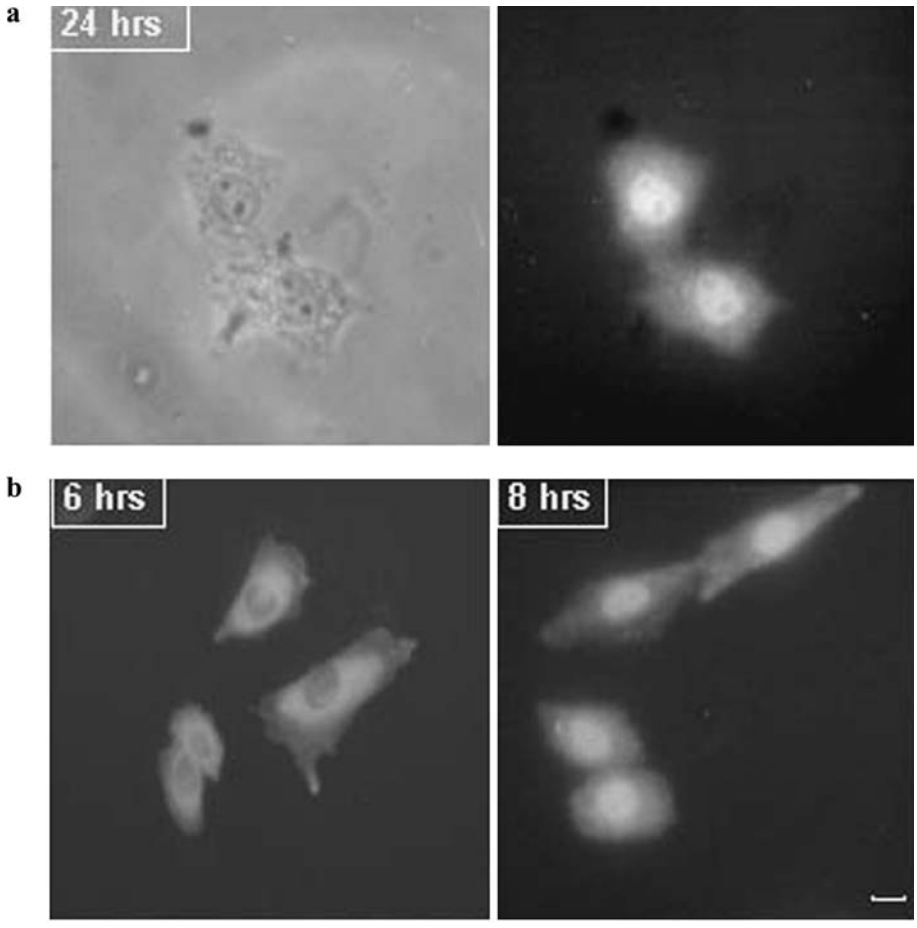

Figure 8. Localization of $p42^{MAPK}$ (ERK2) in G_R cells. Cells were synchronized via mitotic shake-off, replated and grown in the presence of serum for 2 h at 37°C. Serum was subsequently removed and at the indicated times after mitosis, cells were labelled with fluorescent probe directed against $p42^{MAPK}$, as described in Materials and methods. After overnight starvation, cells show a clear nuclear stain (*a*). Translocation occurs at 8 h after mitosis (*b*). A representative experiment is shown. Scale bar, 10 µm.

nuclear translocation of $p42^{MAPK}$ is observed in cells grown in the continuous presence of growth factors [32], suggesting that these cells progressed through G1 until a point late in G1. However, in G_R cells, this translocation appeared to occur independently of the presence of growth factors, although serum seemed to be required early after mitosis. Thus, the presence of growth factors during the first period of G1 is sufficient for nuclear translocation of $p42^{MAPK}$ and for cell cycle progression up to the restriction point at the end of mid-G1.

Discussion

We have shown here that CHO cells (a transformed cell line) contain at least two serum-dependent points during the G1 phase. One point is located very early in G1, immediately after mitosis, and another is located at the end of mid-G1. The early restriction point appears to lead to a G0-like state, whereas the second restriction point appears to correlate with the 'classic' restriction point as defined by Pardee [2]. The entry into the G0-like state is restricted to a limited period of time which appears to be overcome in asynchronously growing cells. Entry into this state can occur only during the first 2 h of G1 and this period can even be narrowed to 10–15 min after mitosis, as judged by the phosphorylation of MAP kinase (unpublished results). Interestingly, cells incubated in the absence of growth factors immediately after mitosis will initially attach normally to the substratum [46], but after overnight starvation, these cells will become more spherical and less well attached. In $G0^-$ cells, the expression of many proteins is decreased and signal transduction routes are switched off. Thus, proteins that are involved in integrin signalling, for example Grb2 and Sos, may no longer be present, causing the cells to become less well attached. Cells that are incubated in the absence of serum at 10 min after mitosis and serum-starved overnight, do not show this characteristic phenotype and exhibit MAP kinase phosphorylation, which suggests that the occurrence of

the spherical phenotype and the activation of signal transduction cascades are in some way correlated. In addition to the remarkable phenotype, cells incubated in the absence of serum immediately after mitosis ($G0^-$ cells) differ from 'classic' G0 cells in their inability to resume cell cycle progression upon serum stimulation. G0 cells reentered the cell cycle upon readdition of growth factors, but $G0^-$ cells were unable to do so. Even after 48 h of stimulation, $G0^-$ cells did not incorporate any [^{3}H]thymidine (unpublished results). This inability of $G0^-$ cells to resume cell cycle progression may be caused by the altered cell attachment, since integrin signalling is known to be required for expression of cyclin E [46]. Analogous to cells that are cultured in suspension, integrin signalling may be affected in $G0^-$ cells, resulting in cell cycle arrest at the restriction point R.

G_R cells stop cell cycle progression at a point late in G1

In contrast to non-transformed cells, most transformed cells will not enter G0 when growth factors are removed, but rather stop at the second restriction point later in G1, or even continue cell cycle progression through G1/S. Several transformed cell lines produce growth factors such as colony-stimulating factor (G-CSF) [50], fibroblast growth factor (FGF) [51, 52], PDGF-like growth factor [53–55] and insulin-like growth factor I and II (IGF I and IGF II) [53, 56, 57] via autocrine growth factor loops. These growth factors may be sufficient to drive the cells through different restriction points. Likewise, asynchronously grown cultures of CHO cells may produce a limited amount of growth factors, allowing the cells early in G1 to overcome the first restriction point, as described above. However, these growth factors appear to be insufficient to pass the second restriction point. Like cells that were incubated in medium without serum at 2 h after mitosis (G_R cells), asynchronous CHO cells incubated in the absence of serum seemed to continue G1 phase only until a point at the end of mid-G1. In G_R cells, this point at the end of mid-G1 is marked by the translocation of MAP kinase to the nucleus. Such a translocation also occurs in cells grown in the presence of growth factors and has been shown to be necessary for further cell cycle progression [32]. However, since G_R cells did not incorporate any thymidine, the translocation by itself apparently is not sufficient for progression through R. Since the translocation of MAP kinase observed in G_R cells occurs in the absence of growth factors, what triggers the transport to the nucleus is not clear. Apparently, processes that occur early in G1 can determine events later in G1.

Different points may lead to different responses

We have shown here that in CHO cells at least two serum-dependent points exist during the G1 phase, with different characteristics. Gurley and co-workers [58] identified similar points in CHO cells that respond to treatment with Br-cAMP. Br-cAMP treatment of CHO cells synchronized in G1 induces cell cycle arrest at a restriction point in mid-G1, whereas cells late in G1 or S phase continue cell cycle progression and undergo rapid apoptosis at the end of mitosis following cell division. Experiments using a fluorescent marker that binds to activated caspases indicate that $G0^-$ cells also enter an apoptotic state upon longer starvation periods. Interestingly, cell death was also observed in asynchronously growing CHO cells incubated in the absence of serum, although to a lesser extent. The apoptotic signal observed in those cells might derive from cells that were in the S or G2 phase at the time of starvation and had finished their cell cycle, thus representing $G0^-$ cells as well.

While the first restriction point is suggested to link with apoptosis, the second restriction point might link to cellular differentiation. cAMP is often used to induce differentiation [59–62] and preliminary results suggest that the cdk inhibitor (CKI) $p21^{CIP1/WAF1}$ is upregulated in cells depleted of serum later in G1. $p21^{CIP1/WAF1}$ has been found to be involved in cellular differentiation [63, 64] and expression of $p21^{CIP1/WAF1}$ has been shown to be induced by activation of the MAP kinase signal transduction pathway [65, 66]. In the budding yeast *Saccharomyces cerevisiae,* activation of the MAP kinase signal transduction pathway has been shown to induce a mating type response during G1 by upregulation and phosphorylation of the CKI FAR1 and induction of genes involved in cell fusion [10, 67, 68]. Differentiation of mammalian cells may follow a similar mechanism using $p21^{CIP1/WAF1}$ and differentiation-specific genes. A prolonged localization of MAP kinase in the nucleus as detected in G_R cells can thus result in high levels of $p21^{CIP/WAF1}$ and induce differentiation. Since CHO cells have lost the ability to differentiate, however, no differentiation is observed. Thus, entry into apoptosis and differentiation processes may occur at very distinct points in the cell cycle, which can be distinguished in transformed cells. In non-transformed cells, however, these points coincide at one single restriction point and are therefore presumed to occur at the same time.

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