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Progression Through the G_1-Phase of the On-Going Cell Cycle

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Abstract Cell cycle progression is dependent upon the action of cyclins and their partners the cyclin dependent kinases (CDKs). Each cell cycle phase has its own characteristic cyclin-CDK combination, cyclin D-CDK4,6 and cyclin E-CDK2 being responsible for progression through G_1-phase into S-phase. Progression through G_1-phase is regulated by signal transduction cascades activated by polypeptide growth factors and by extracellular matrix (ECM) components. Studies aiming to unravel the molecular mechanism by which these extracellular components activate the cyclin-CDK complexes in the G_1-phase, are usually performed using serum-starved cells (G_0 cells). These cells are activated by addition of growth factors, or the cells are detached from the substratum by trypsinization and subsequently allowed to re-attach. An alternative approach, however, is to study the effects of growth factors and attachment in the ongoing cell cycle by synchronization of the cells by the mitotic shake-off method. These cells are not serum starved and not actively detached from the substratum. In this contribution it is shown that both methods yield significant different results. These observations demonstrate that data obtained with model systems should be interpreted with care, especially if the findings are used to explain cell cycle progression in cells in an intact organism. J. Cell. Biochem. 90: 244–252, 2003.

Key words: cell cycle; G_1-phase; growth factors; attachment; G_1 cyclins; signal transduction

The most fundamental property of living organisms is their ability to reproduce themselves. This property is based upon the feature that cells are able to duplicate by a process known as the cell cycle. Research on the regulation of cell cycle progression has gained enormous attention during the last decades, mainly because this knowledge is of utmost importance in fighting cancer as well as many other diseases. Furthermore knowledge on cell cycle regulation is essential in understanding embryonal development, and in applied sciences, such as production of heterologous proteins. However, studying cell cycle regulation in cells in intact organisms is extremely complicated, and therefore such studies are usually performed in model systems, as tissue culture conditions using stabile cell lines and applying state-of-the-art molecular biological and cell biological methods. Extrapolation of the results obtained from these model systems to the cell in the intact organism may face, however, serious problems, as the model system itself may induce responses of cells, which are at least artificial of nature. In this contribution, I will describe studies aiming to solve the molecular mechanisms that regulate progression through the G_1-phase of the cell cycle of mammalian cells and demonstrate that the model systems strongly affect the results.

In virtually all cells, the cell cycle is composed of four discrete phases, being the DNA synthesis phase (S phase), the cell division phase (M phase) and the gap phases between these two: the G_1-phase between M and S phases and the G_2-phase between S and M phases. During the last decades, a wealth of knowledge has become available that gives insight in the molecular mechanisms that control cell cycle regulation. Cyclins and their partners the cyclin dependent kinases (CDKs) constitute the basis of these molecular mechanisms. A number of excellent reviews has appeared that describe

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the mechanisms by which cyclin-CDK activity is regulated in different cell cycle phases and how these activities result in controlled cell cycle progression, cell cycle arrest, cell differentiation, and even cell death (apoptosis) [amongst many others: Norbury and Nurse, 1992; Nigg, 1995; Pines, 1995; Schafer, 1998; Ekholm and Reed, 2000; Bird, 2003].

Whether cells progress through the cell cycle or not, depends to a large extent on extracellular signals. Cell cycle progression of free-living unicellular organisms, such as yeasts, is dependent upon the availability of nutrients in their environment, pheromones, which induce a sexual reproduction, or stress conditions, for example high temperatures [Herrero et al., 2003; Verrips, 2003]. In multicellular organisms, cell cycle progression is regulated by growth factors, the extracellular matrix (ECM), cell–cell contacts as well as stress conditions [Agami and Bernards, 2002; Hulleman and van Rossum, 2003; Juliano, 2003; Martínez Muñoz and Post, 2003]. Upon deprivation of the cells of an essential component (nutrients or growth factors) the cells become quiescent, the so-called G_0-phase. Most non-proliferating cells in an organism have a G_1-phase amount of DNA, indicating that progression through the cell cycle stops in the G_1-phase. Therefore, studies aimed to understand the regulation of cell cycle progression are focused usually on the G_1-phase and the molecular mechanisms underlying progression through this phase. In this contribution, I will shortly summarize the current knowledge on the molecular mechanisms that control G_1-phase progression followed by a discussion on the role of the used model systems in the understanding of regulation of G_1-phase progression.

MOLECULAR MECHANISMS REGULATING CELL CYCLE PROGRESSION

Cyclins and their partners the cyclin dependent kinases (CDKs) are responsible for progression through the cell cycle. Cyclins are activating subunits that interact with specific CDKs to regulate their activity and substrate specificity. CDKs are serine/threonine protein kinases that require binding of a cyclin in order to be ready to become activated. Mammalian cells contain multiple CDKs that are activated by multiple cyclins [reviewed amongst others in Reed et al., 1994; Pines, 1995; Roussel, 1998; Schafer, 1998]. CDK activity is regulated by several processes, including phosphorylation on threonine and tyrosine residues; some of these phosphorylation steps being stimulatory, others inhibitory [Obaya and Sedivy, 2002; Bird, 2003]. In addition, a large family of inhibitor proteins have been discovered, which may inhibit CDK activity by either binding to CDK alone or to the complex of CDK with its cyclin [Tyner and Gartel, 2003]. The most important mammalian cyclin-CDK complexes known so far are the mitotic cyclins A and B in association with CDK1, and the G_1-cyclins D and E in complex with CDK 4/6 and CDK2 [Nigg, 1995; Sherr, 1995; Arellano and Moreno, 1997; Reed, 1997] (Fig. 1). The first cyclin-CDK complex, to be activated during the G_1-phase, is composed of a D-type cyclin in association with CDK4 or CDK6 depending on the cell type [Sherr, 1995]. As cells progress through the G_1-phase, cyclin E is synthesized with a peak late in G_1. Cyclin E associates with CDK2 and is essential for entry into S phase [Ohtsubo et al., 1995]. Once cells enter S phase, cyclin E is degraded and CDK2 then associates with cyclin A [Fotedar and Fotedar, 1995]. Finally, cyclin A and the B-type cyclins associate with CDK1 to promote entry into mitosis. Cyclin A binds to CDK1 with a peak of activity in G_2-phase and is then suddenly degraded, whereas entry into mitosis is triggered by cyclin B–CDK1. For exit from mitosis, cyclin B destruction is required [Murray, 1995].

One of the most important G_1-phase cyclin/CDK substrates in mammalian cells is the product of the retinoblastoma tumor suppressor gene (*pRB*) [Yee and Wang, 2003]. pRB is phosphorylated in a cell-cycle-dependent manner and binds in the hypophosphorylated state to transcription factors, particularly members of the E2F family. E2F consists of at least five different isoforms that form heterodimers with a second group of proteins known as DP-1 [Yee and Wang, 2003]. pRB is present in this hypophosphorylated form during early G_1–and becomes phosphorylated on several residues during mid to late G_1. This phosphorylation causes the release and activation of the E2F transcription factors, allowing transcription of genes that mediate progression through S phase [DeGregori et al., 1995]. Initial activation of pRB is thought to occur in the G_1-phase by phosphorylation by cyclin D/CDK complexes. D-type cyclins can bind directly to pRB in the

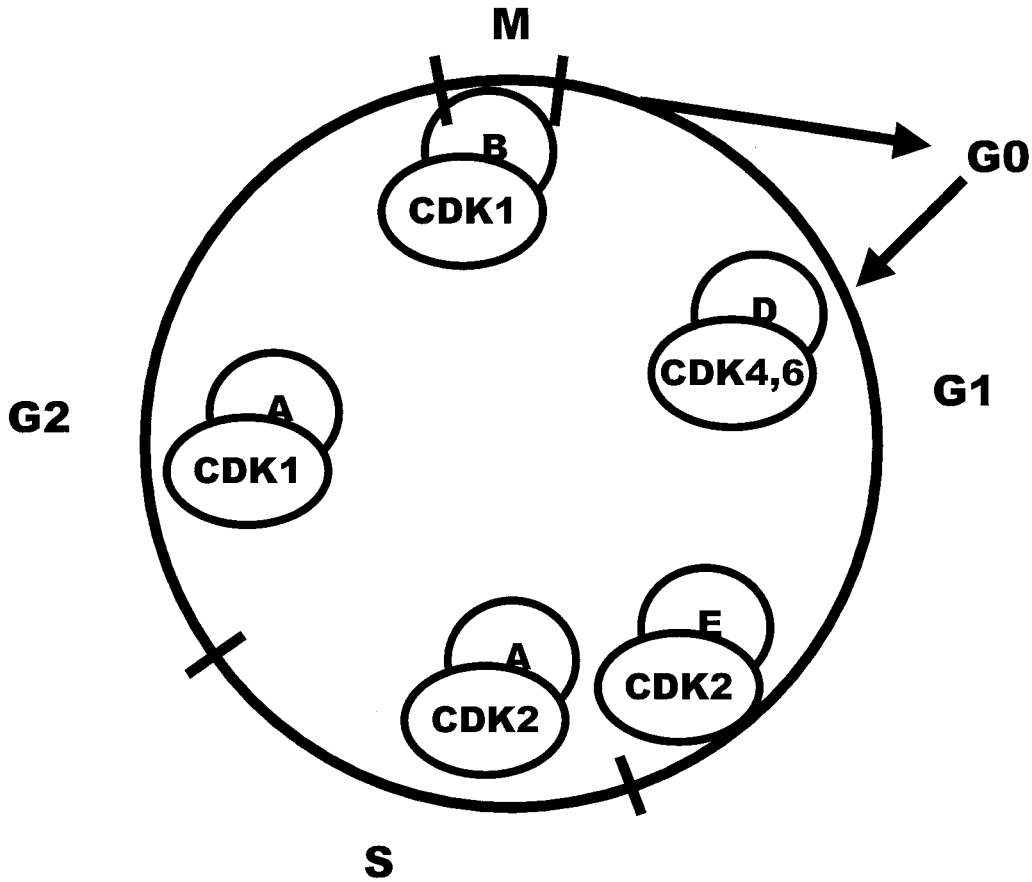

Fig. 1. Overview of the cell cycle in mammalian cells. The mammalian cell cycle basically consists of four phases: first gap phase (G_1), DNA synthesis (S), second gap phase (G_2), and mitosis (M). The transition between the different phases is regulated by cyclin/cdk complexes. Different cyclins (**A**, **B**, **D**, and **E**) are present during different cell cycle phases and interact with different CDKs. As long as growth factors are present, adherent cells will continue to proliferate. In the absence of growth factors, cells will stop dividing and enter the quiescent state (G_0).

absence of a kinase, and thus might target the pRB to CDK4/CDK6 kinases. After the initial phosphorylation by cyclin D/CDK, cyclin E/CDK2 complexes are thought to subsequently phosphorylate pRB late in G_1, thereby triggering the onset of S phase [DeGregori et al., 1995].

As external factors regulate G_1-phase progression, understanding of the regulation of cell cycle progression has to be focused on the mechanisms by which these external factors regulate the G_1-cyclin-CDK activities.

EFFECT OF GROWTH FACTORS ON G_1-PHASE PROGRESSION

The G_0 to G_1-Transition

A widely used model system to investigate cell proliferation, and thus G_1-phase progression, is stimulation of serum-starved cells with growth factors. Polypeptide growth factors as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), or fibroblast growth factor (FGF) exert their effects in the target cells by binding to specific plasma membrane bound receptors belonging to the class of receptor tyrosine kinases. Upon binding of growth factors, the receptors dimerize which leads to activation and autophosphorylation of the receptor on tyrosine residues in the intracellular domain. This phosphorylation triggers the recruitment of a number of target proteins to the receptor, like for example phosphoinositide-specific phospholipase Cγ (PLCγ), the p85 kDa subunit of phosphatidylinositol 3-kinase (PI3-kinase), GTPase-activating protein (GAP), growth factor receptor binding protein 2 (Grb2) and members of the Src family of cytoplasmic tyrosine kinases. In some instances, binding of the receptor to a target molecule may result in phosphorylation and direct activation of this target (e.g., PLCγ). In other cases, however, proteins without any enzymatic activity

are bound, such as Grb2 and p85. These proteins serve as adaptor proteins to couple the activated receptor to other intermediates. All protein interactions consequently lead to modification (e.g., phosphorylation or dephosphorylation) and activation of other target proteins, thus creating signal transduction cascades that form a signal transduction network, which finally results in activation of nuclear transcription factors and induction of gene expression [amongst many others: Bokemeyer et al., 1995; Malarkey et al., 1995; Hulleman and Boonstra, 2001]. One of the best-known signal transduction cascades activated by growth factors is the mitogen-activated protein kinase (MAPK) pathway. In general, the MAPK isoforms are activated by phosphorylation on regulatory threonine and tyrosine residues by dual specificity protein kinases, the MAP kinase kinases (MAPKK), which on their turn are activated by phosphorylation by MAP kinase kinase kinases (MAPKKK). One of the most important MAPK pathways activated by growth factor receptors is the signal transduction pathway that leads to phosphorylation of $p44^{MAPK}$ and $p42^{MAPK}$ (also called ERK1 and ERK2, respectively). When growth factor receptors are activated, the adaptor protein Grb2 is bound to the receptor, together with the guanine-nucleotide exchange factor Sos. Binding of Sos, leads to the activation of Ras, which subsequently recruits Raf-1 to the plasmamembrane. Subsequently, Raf-1 is activated and can, in turn, activate the MAP kinase kinase MEK (MAPK- or ERK Kinase), which finally phosphorylates $p44/p42^{MAPK}$ (ERK1/2). Upon activation, ERK can phosphorylate targets in the cytoplasm, such as $p90^{RSK}$, cytoskeletal elements, cytosolic phospholipase A_2 ($cPLA_2$) and others, or translocate to the nucleus where it may phosphorylate and activate several transcription factors, such as c-myc, c-jun, $p62^{TCF}$/Elk-1, c-Ets-1, and c-Ets-2 [amongst many others: Bokemeyer et al., 1996; Kolch, 2000; Hulleman and Boonstra, 2001; Hulleman and van Rossum, 2003; Jones and Kazlauskas, 2003].

In serum-starved fibroblasts, activation of the MAPK pathway by growth factors has been shown to induce expression of cyclin D [Brunet et al., 1999]. Furthermore, it was demonstrated that the MAPK cascade is involved in the assembly of cyclin D with its partner CDK4 [Cheng et al., 1998]. In most quiescent cells, the MAPK phosphorylation and activation by growth factors is transient, with a maximum around 30 min after addition. At the same time, a translocation was observed from the cytoplasm to the nucleus. Furthermore, growth factors induce usually a phosphorylation of all MAPK molecules in the cells.

However, it is known for a long time that growth factors have to be present in the culture medium for at least hours in order to induce DNA synthesis in quiescent cells. So the initial transient MAPK activation and nuclear translocation appears not sufficient for completing the G_1-phase, and it seems tempting to suggest that the transient MAPK activation is required for G_0 to G_1-transition, and that later signaling is required for passage of the G_1-phase. Thus a biphasic activation of Ras was observed in serum-stimulated NIH 3T3 fibroblasts [Gille and Downward, 1999]. The first activation within 20 min was followed by a second one, several hours later. Similar observations were done in HEPG2 cells activated with PDGF [Jones et al., 1999; Balciunaite et al., 2000]. Recently, it was demonstrated that the prolonged exposure of cells to growth factors required for entry into S phase, could be substituted by two short pulses of growth factor, the first at the onset of the experiment and the second 8 h later [Jones and Kazlauskas, 2001]. It was suggested that the first burst of signaling is required to commit the cells to engage the cell cycle program, or the G_0 to G_1-transition, while the second burst in fact is responsible for G_1-phase progression [Simm et al., 1998; Jones and Kazlauskas, 2003].

The On-Going Cell Cycle

As described briefly above, cell cycle studies and in particular those related to progression through the G_1-phase, are usually performed using serum-starved cells. The interpretation of the results is always complicated because in addition to G_1-phase progression, the cells are also involved in G_0 to G_1-transition, while in addition all sorts of recovery processes occur due to the long period of starvation. Furthermore, the G_0-phase is a rather special condition to the cells, as in an organism cells are usually not growth arrested due to the absence of serum. Therefore, we have decided to study the role of the MAPK pathway in cell cycle progression by using a relatively old method to synchronize the cells. This method, the so-called mitotic shake-off method, was used about 25 years ago to study

cell cycle related processes [de Laat et al., 1975; Boonstra et al., 1981; de Laat and van der Saag, 1982]. The method is based upon the property that mitotic cells are less well attached to the substratum than interphase cells. Thus by shaking an a-synchronously growing cell population, the mitotic cells can be easily detached from the substratum. Replating this mitotic cell suspension yields a cell population that synchronously progresses through the G_1-phase. The advantage of this method as compared to serum starvation is, that progression through the cell cycle is not disturbed by the synchronization method. Furthermore, the cells need not to recover from a starvation period. We have studied the role of MAPK signaling in the ongoing cell cycle of Chinese hamster ovary (CHO) cells, synchronized by mitotic shake-off [Hulleman et al., 1999a; Hulleman and Boonstra, 2001, van Rossum et al., 2001]. The expression of $p42^{MAPK}$ increased in mid G_1-phase. Of particular interest appeared the observation that MAPK is phosphorylated already within 15 min after mitosis, far before the G_1/S phase transition, which is apparent after 10 h after mitosis in these cells as deduced from thymidine incorporation studies. The MAPK phosphorylation is entirely due to the presence of serum growth factors and not to cell attachment, since incubation of mitotic cells in suspension for up to 6 h in the presence of serum resulted in MAPK phosphorylation as in control cells [Hulleman et al., 1999b]. The phosphorylated state of MAPK persists during the entire G_1-phase, in the early S phase a de-phosphorylation is observed. Of interest is the observation that MAPK translocates to the nucleus about 8 h after mitosis [Hulleman et al., 1999a]. This nuclear translocation is transient and lasts for about 1 h. Furthermore, as deduced from gel-shift analysis, only approximately 10% of the total MAPK population is phosphorylated, the majority remaining in the un-phosphorylated form. Treatment of the cells with the MEK inhibitor PD 098059 in the early G_1-phase prevents the phosphorylation and nuclear translocation of MAPK and the entry into S phase. This behavior of MAPK during the G_1-phase was observed in CHO cells and in the non-related neuroblastoma N2A cell line [van Rossum, 2001]. These results demonstrate some interesting features so far not obtained from studies using serum-starved cells: (1) MAPK is phosphorylated throughout the G_1-phase and only dependent upon serum growth factors; (2) MAPK phosphorylation and nuclear translocation are clearly separate processes, the latter occurring only in a limited period during G_1-phase; (3) the phosphorylation of MAPK concerns only on a part on the total MAPK population, apparently only a small fraction of phosphorylated and activated MAPK is sufficient to induce G_1-phase progression. It is of course well possible that during progression through G_1-phase, the phosphorylated MAPK molecules are subject to continuous dephosphorylation, so that the population of activated MAPK is changing. But the observation that only a small fraction of activated MAPK is sufficient for progression through G_1-phase is of interest, as it has been demonstrated that the amount of activated signal transduction proteins may determine cell fate. Thus low levels of Raf activity were demonstrated to activate cell cycle progression, while high amounts of Raf caused cell cycle arrest [Woods et al., 1997].

Other interesting observations concerned the expression of the G_1-cyclins during the ongoing cell cycle [Hulleman et al., 1999a]. Cyclin D is present in mitotic cells and during the whole G_1-phase, in agreement with the notion that the cyclins D are considered as growth factor sensors. Cyclin E expression is known to occur in the second half of the G_1-phase, but our studies show that in the ongoing cell cycle, cyclin E is expressed as early as 2 h after mitosis, while the G_1-phase lasts for 10–12 h in CHO cells. Cyclin E expression apparently occurs in the early rather than in late G_1-phase [Hulleman et al., 1999a].

The studies described above indicate that the results obtained under laboratory conditions to unravel the molecular mechanisms underlying cell cycle regulation, should be interpreted with care with respect to extrapolation to the normal cell living in an organism. Cell starvation, protein overexpression or in contrast protein inhibition, may induce reactions in the cells that are far from the physiological response and consequently may suggest mechanisms, which do not occur under physiological conditions.

EFFECT OF ATTACHMENT ON G_1-PHASE PROGRESSION

The G_0 to G_1-Transition

Interactions between cells and the components of the ECM have profound effects on cell

survival, growth, and differentiation [Hanks and Polte, 1997; Danen and Yamada, 2001; Alahari et al., 2002; Reenstra et al., 2002; Juliano, 2003]. The ECM is composed of a network of proteins and proteoglycans that provides both structure and information to the cells. The protein network of the ECM is based upon many members of the collagen family, which are embedded in the proteoglycans. The interactions between cells and the ECM, are realized by linker proteins, as fibronectin, laminin, and vitronectin [Aumailley and Gayraud, 1998]. These linker proteins bind to the components of the ECM and in addition to specific transmembrane proteins of the plasma membrane, the integrins. Integrins form a large family of cell surface hetero-dimeric proteins. At the cytoplasmic side, integrins interact with the cytoskeleton and are involved as such in cell morphology and cell migration. The importance of integrins lies in their adhesive function as well as in their capacity to induce signal transduction that affects gene expression [Hanks and Polte, 1997; Danen and Yamada, 2001]. The interaction between integrins and the ECM triggers tyrosine phoshorylation due to activation of focal adhesion kinase (FAK). FAK associates with the cytoplasmic tail of integrins upon integrin stimulation and is subsequently autophosphorylated on tyrosine residues. Activated FAK subsequently associates with c-Src, which further phosphorylates FAK on additional tyrosine residues, leading to full activation of FAK. This phosphorylation results in the binding of the Grb2/Sos complex and the adaptor protein Shc, thereby linking to the MAPK pathway [for reviews see: Gutkind, 1998; Danen and Yamada, 2001; Hulleman and Boonstra, 2001; Howe et al., 2002; Juliano, 2003]. In addition, FAK is able to bind to a number of other signaling and structural proteins, including PI3 kinase, a Rho-GAP, paxillin, talin, and $p130^{CAS}$ [Juliano, 2003]. Some of these interactions depend on the phosphorylation status of FAK. Tyrosine phosphorylation and activation of FAK is dependent upon integrin-mediated adhesion of the cells, de-phosphorylation occurring when the cells are detached [Schaller, 1996]. Thus, integrin signaling may induce the same signal transduction cascades as growth factors, and consequently may have comparable effects on cell cycle progression.

Adhesion of cells allows an efficient activation of cyclin D/CDK4,6 and cyclin E/CDK2 and a suppression of CDK inhibitor proteins [Assoian and Schwartz, 2001]. In contrast, in non-adherent cells, a poor induction of cyclin D1 and high levels of CDK inhibitors result in G_1-arrest. Consistent with this idea is the observation that ectopic expression of cyclin D1 induces anchorage-independent RB phosphorylation and cyclin A expression [Zhu et al., 1996]. In addition to cyclin D1, also other processes occurring later in G_1-phase appear to be dependent upon adhesion of the cells, for example cyclin A expression is anchorage-dependent in NRK fibroblasts [Guadagno and Ohtsubo, 1993; Zhu et al., 1996].

The On-Going Cell Cycle

It should be realized, however, that most of the studies related to the effects of cell adhesion on cell cycle progression have been performed using serum-starved cells, which are detached from the substratum by trypsinization. These cells may pose comparable problems in the interpretation of the results as described above. The only cell cycle phase during which cell adhesion to the ECM is minimal, is the M phase. From this point of view, it seems more appropriate to study the effects of cell adhesion on cell cycle progression during the M to G_1-phase transition of cells exposed to serum growth factors. During this transition, the new daughter cells will attach to the ECM, followed by a drastic morphological change from a round cell to a flattened G_1-phase cell. Therefore, we have studied these properties using cells synchronized by the mitotic shake-off method. These cells are not serum starved and need not to be trypsinized for detachment.

CHO and N2A neuroblastoma cells, synchronized by mitotic shake-off, exhibit cyclin D expression, but no phosphorylated MAPK or phosphorylated FAK, and cyclin E is not detectable [Hulleman et al., 1999b]. The expression of cyclin D in mitotic cells was dependent on the presence of serum, because incubation of the cells in serum free medium results in a rapid disappearance of cyclin D within 2 h. Incubation of the mitotic cells on petri dishes coated with poly-L-lysine in the presence of serum does prevent integrin activation, but leads to a sustained cyclin D expression, but no cyclin E expression is detected up to 6 h after mitosis. Under these conditions also MAPK appears to be phosphorylated. In contrast, incubation of mitotic cells on petri dishes coated

with fibronectin results in sustained cyclin D expression, MAPK phosphorylation and cyclin E expression within 2 h after mitosis. These results demonstrate clearly that during the M to G_1-phase transition, integrin-induced signal transduction is required for cyclin E expression during the G_1-phase, but not for cyclin D expression and MAPK phosphorylation. These latter two are solely dependent upon the presence of serum growth factors [Hulleman et al., 1999b]. In addition, no differences are observed in the level of expression of the CDK inhibitor proteins p21 and p27 [Hulleman et al., 1999b], suggesting that the level of expression of these inhibitor proteins is not changed significantly during the ongoing cell cycle. These observations clearly demonstrate that the role of integrin-induced signaling is different during the M to G_1-transition as compared to adhesion of serum-starved trypsinized cells with respect to G_1-phase progression.

STUDIES ON REGULATION OF G_1-PHASE PROGRESSION

It has been well documented that progression through the G_1-phase by mammalian cells requires growth factor- and ECM component-induced signal transduction. Both are able to activate the MAPK cascade and this cascade has been demonstrated to be involved in cyclin D expression and S phase entry. However, as described above, the model system that is used to study these phenomena has a significant effect on the results and these results may be even conflicting with each other. Therefore, it is important to judge the validity of the results with respect to the questions to be answered. In this respect, two different types of questions can be addressed: (1) what are the features of the proteins under study, are they able to interact with each other, do they respond to particular conditions and (2) what are the physiological roles of the proteins under study. With respect to the first type of questions, a study protocol using serum-starvation and/or detachment of the cells is an attractive protocol, because answers may be obtained about the potential role of proteins in G_1-phase progression. However, this approach may not provide an answer to the in vivo role of the proteins under study. In an organism, growth factor deprivation is not the usual way to induce cell cycle arrest, and cells are only detached from the ECM during mitosis, and not in any other phase of the cell cycle. Using the mitotic shake-off method for synchronization, cells are not starved from growth factors and are not mechanically or enzymatically detached from the substratum. But also here it should be realized that also under these conditions a tissue culture approach is used, which on its own is far away from the physiological situation.

Similar considerations concern the use of molecular biological approaches. Over-expression or knock-out of a specific gene may provide information on the potential role of this particular protein, but since it has been demonstrated that the amount of active signal transduction molecules and their localization may have a profound effect on the ultimate biological effect, over-expression, and knock-out studies will not provide the solution to the role of this specific protein under physiological conditions. Analysis of the molecular mechanisms underlying cell cycle progression requires a very accurate inventory of the expression, activity, interaction, and localization of the proteins of interest during the cell cycle of cells under optimal physiological conditions, i.e., in the intact organism. This knowledge should be combined with the knowledge from molecular biological studies and studies using starvation, inhibitors, activators, to deduce the real molecular regulatory circuit.

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