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Review

Regulation of G1 phase progression by growth factors and the extracellular matrix

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Abstract. Cell cycle progression is regulated by both intracellular and extracellular control mechanisms. Intracellular controls ensure that cell cycle progression is stopped in response to irregularities such as DNA damage or faulty spindle assembly, whereas extracellular factors may determine cell fate such as differentiation, proliferation or programmed cell death (apoptosis). When extracellular factors bind to receptors at the outside of the cell, signal transduction cascades are activated inside the cell that eventually lead to cellular responses. We have shown previously that MAP kinase (MAPK), one of the proteins involved in several signal transduction processes, is phosphorylated early after mitosis and translocates to the nucleus around the restriction point. The activation of MAPK is independent of cell attachment, but does require the presence of growth factors. Moreover, it appears that in Chinese hamster ovary cells, a transformed cell line, growth factors must be present early in the G1 phase for a nuclear translocation of MAPK and subsequent DNA replication to occur. When growth factors are withdrawn from the medium immediately after mitosis, MAPK is not phosphorylated, cell cycle progression is stopped and cells appear to enter a quiescent state, which may lead to apoptosis. Furthermore, in addition to this growth-factor-regulated decision point in early G1 phase, another growth-factor-sensitive period can be distinguished at the end of the G1 phase. This period is suggested to correlate with the classical restriction point (R) and may be related to cell differentiation.

Key words. G1 phase; signal transduction; cell cycle; growth factors; integrins; MAP kinase; restriction point.

Cyclins and CDKs

The cell cycle consists of four phases: the S phase, in which DNA replication occurs; M phase or mitosis when the DNA is segregated and the cell actually divides; and two gap phases: G1 (before DNA synthesis) and G2 (before mitosis). Progression through the cell cycle depends on both cell adhesion and the presence of growth factors. As long as growth factors are present, most adherent cells will continue to proliferate. In the absence of growth factors, however, cells will stop dividing and enter the quiescent state (G0). The regulation of cell proliferation by growth factors of normal mammalian cells occurs at two levels in the cell cycle: during transition from the quiescent G0 state to the G1 phase, and during progression through the different subphases of G1. At a point in G1 called the restriction point (R) the cells acquire growth-factor independence and become committed to enter the S phase [1].

Proper cell cycle progression is regulated accurately by a family of protein kinases termed cyclin-dependent kinases (CDKs). CDKs are serine/threonine protein ki-

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nases which control the transition between successive phases of the cell cycle and which require binding of regulatory subunits, named cyclins, as an initial step in their activation process. Whereas in yeast one single CDK regulates cell cycle progression (*cdc2* in *Schizosaccharomyces pombe* and CDC28 in *Saccharomyces cerevisiae* [2–4]), in mammalian cells at least nine CDKs (CDK1–CDK9) have been described [5, 6]. Their positive regulatory subunits, the cyclins, also constitute a large family. So far, 12 different cyclins have been described in vertebrates and at least nine distinct cyclins are involved in cell cycle regulation in the yeast *S. cerevisiae* [5]. Although cyclins were originally denominated on basis of a characteristic cell-cycle-dependent pattern of synthesis and degradation, some of the cyclins identified more recently are expressed at fairly constant levels, and their inclusion into the cyclin family is based on structural and functional criteria. Cyclins can form catalytically active complexes with different types of CDKs in mammalian cells, and different cyclin/CDK complexes are assembled and activated at specific points in the cell cycle (fig. 1).

The first cyclin/CDK holoenzyme which is activated during the G1 phase in mammalian cells is composed of a D-type cyclin (cyclin D1, D2 or D3) in association with CDK4 or CDK6, depending on the cell type (reviewed in [7]). D-type cyclins are localized almost exclusively in the cell nucleus [8] and are absent in serum-deprived cells. In contrast to other cyclins, their expression depends on stimulation by growth factors, and only a modest peak of accumulation is observed late in G1. As cells progress through the G1 phase, cyclin E is synthesized with a peak late in G1. Cyclin E associates with CDK2 and has been shown to be essential for entry into S phase [9]. Cyclin E is localized in the nucleus during mid-G1 to early S-phase, and once cells have entered S phase, cyclin E is degraded. CDK2 then associates with cyclin A, whose synthesis is initiated during late G1. Activity of the cyclin A/CDK2 complex is essential for progression through S phase and appears to be required for phosphorylation of transcription factors and some of the proteins required for DNA replication [10]. Finally, both cyclin A and the B-type cyclins associate with cdc2 (= cdk1) to promote entry into mitosis; cyclin A binds to cdc2 with a peak of activity in G2 and is then suddenly degraded, whereas entry into mitosis is triggered by cyclin B/cdc2. For exit from mitosis cyclin B destruction is required (fig. 2).

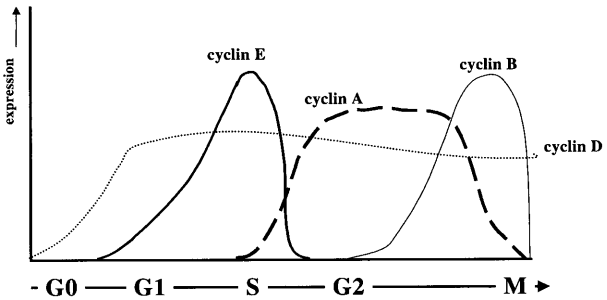

Figure 2. Expression of cyclins during the mammalian cell cycle. Upon stimulation of quiescent cells (G0), different cyclins are expressed in an orderly way. Cyclin D is absent in quiescent cells but rapidly accumulates after growth factor stimulation. Cyclin D is subsequently expressed constitutively throughout subsequent cell cycles, whereas expression of cyclins E, A and B is related to a specific cell cycle phase.

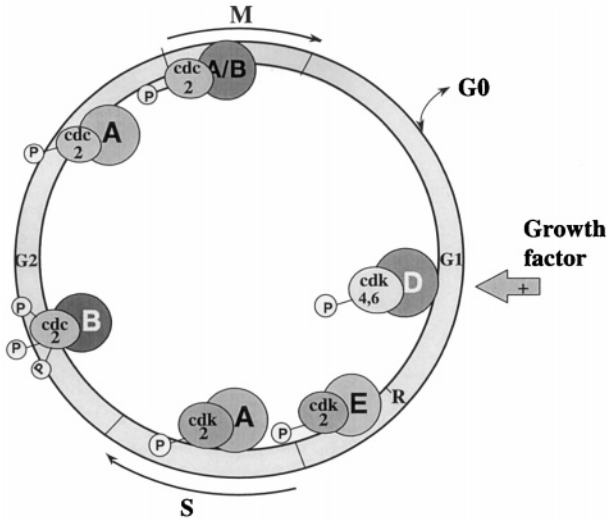

Figure 1. Overview of the cell cycle in mammalian cells. The mammalian cell cycle basically consists of four phases: first gap phase (G1), DNA synthesis (S), second gap phase (G2) and mitosis (M). The transition between the different phases is regulated by cyclin/cdk complexes. Different cyclins (A, 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 (G0).

Activation of cyclin/CDK complexes

Passage from one stage of the cell cycle to another is regulated not only by the presence of cyclins but also by modifications of the kinase subunits. Multiple phosphorylation and dephosphorylation events occur on both the cyclins and the CDKs. Phosphorylation controls the activity of CDKs both positively and negatively, depending on the phosphorylation sites and may also serve in the stabilization of the cyclin/CDK complex [5,

11, 12]. Besides phosphorylation, the presence of cyclin-dependent kinase inhibitors (CKIs) may regulate CDK activity. CKIs inhibit the activity of CDKs by binding in vivo with the CDK subunit, the cyclin or the cyclin/CDK complex. In mammalian cells, two families of CKIs have been described, based on protein sequence similarity: the Cip/Kip family, composed of $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$, and the INK family (inhibitors of cdk4), including $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$ (fig. 3) [12, 13]. $p21^{CIP1}$ (also known as WAF1, CAP20 and SDI1) binds to multiple cyclin/CDK complexes, with the greatest affinity for G1 complexes [14] and has been suggested to function either as an assembly factor or as an inhibitor [15], depending on the fraction of the kinase that is in complex with $p21^{CIP1}$ [16]. Expression of $p21^{CIP1}$ can be induced by the tumor suppressor p53 in response to DNA damage. This upregulation of $p21^{CIP1}$ can induce cell cycle arrest in response to DNA damage not only by inhibiting cyclin/CDK complexes but also by binding to the DNA polymerase-δ subunit PCNA, which has roles in DNA replication and repair [17]. However, expression of $p21^{CIP1}$ can also be induced independent of p53. In several cell types $p21^{CIP1}$ expression correlates with terminal differentiation, and $p21^{CIP1}$ may therefore be involved in cell cycle exit by inducing G1 arrest.

Another CDK inhibitor which is structurally related to $p21^{CIP1}$, is $p27^{KIP1}$ [18]. $p27^{KIP1}$ is expressed both in proliferating and in differentiated cells [19, 20], and like $p21^{CIP1}$, $p27^{KIP1}$ binds to both D- and E-type cyclin/CDK complexes and inhibits several cyclin/CDK complexes in vitro. Like $p21^{CIP1}$, $p27^{KIP1}$ binds with higher affinity to the cyclin/CDK complex than to the CDK alone. Unlike $p21^{CIP1}$, $p27^{KIP1}$ does not bind PCNA and is not regulated by p53. In fact, the expression of $p27^{KIP1}$ does not appear to be regulated at the transcriptional level at all [21]. Protein levels seem to change during the cell cycle due to translational and posttranslational regulation. $p27^{KIP1}$ is phosphorylated at the end of the G1 phase by cyclin E/CDK2 [22] and subsequently degraded via the ubiquitin pathway at the G1/S transition [23]. $p27^{KIP1}$ is expressed at high levels in quiescent cells and is rapidly downregulated upon growth factor stimulation. $p27^{KIP1}$ is also present in proliferating cells, but at lower levels and apparently in a sequestered state. It has been proposed that when cells progress through G1, $p27^{KIP1}$ is sequestered by cyclin D/CDK4 or CDK6 complexes, which need to overcome a $p27^{KIP1}$ threshold to become active [13]. Since $p27^{KIP1}$ can interact with multiple cyclin/CDK complexes, its availability for inhibition of cyclin E/CDK2 also depends on the abundance of cyclin D/CDK4 [24].

A more recently identified member of the Cip/Kip family of CKIs is $p57^{KIP2}$ [25, 26]. This CKI, like $p21^{CIP1}$ and $p27^{KIP1}$, can interact and inhibit several cyclin/CDK complexes, and its overexpression blocks cells in G1. $p57^{KIP2}$ is expressed in terminally differentiated cells, suggesting an involvement of this CKI in the cell cycle exit of specific cell types.

$p15^{INK4B/MTS2}$, $p16^{INK4A/MTS1}$, $p18^{INK4C}$ and $p19^{INK4D}$ constitute a different family of CDK4/CDK6 specific inhibitors, structurally distinct from $p21^{CIP1}$ and $p27^{KIP1}$. INKs bind exclusively to CDK4 and CDK6 and prevent their binding to cyclins or block the kinase activity of pre assembled complexes [27–29]. The precise role of these CKIs is yet unknown; it has been suggested that $p16^{INKA}$ acts as a tumor suppressor in vivo, since the $p16^{INK}$ gene has been found mutated in the majority of tumor cell lines tested. Furthermore, $p16^{INK4A}$ has been shown to associate with the C-terminal domain (CTD) of RNA polII and the general transcription factor TFIIH [30], regulating transcription. Since CAK has also been shown to phosphorylate the CTD of RNA polII and to associate with TFIIH [31, 32], $p16^{INKA}$ may be involved in the regulation of CAK.

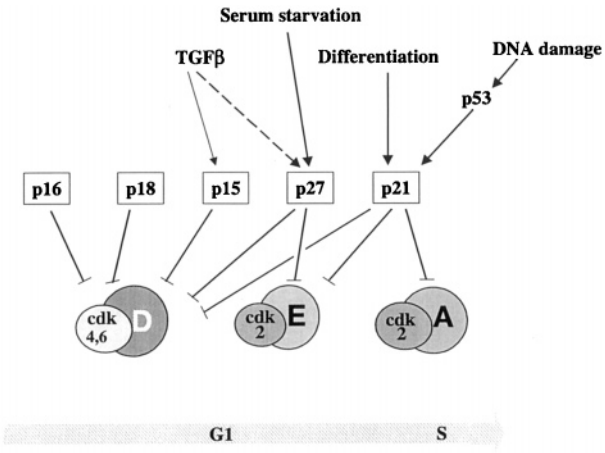

Figure 3. Function of CDK-inhibitors (CKIs) during G1/S. CDK inhibitors belonging to the INK family (p15, p16 and p18) are specific for interaction with the cyclin/CDK4,6 complexes, whereas p21 and p27 are able to inhibit all cyclin/CDK complexes. p27 is mainly involved in cell cycle arrest by serum starvation, whereas p21 plays an important role in cell cycle arrest due to DNA damage and in cell differentiation. Inhibition of cell growth by transforming growth factor β (TGFβ) is due to activation of p15 and partly to p27.

Substrates of cyclin/CDK complexes

Progression through the cell cycle has to be tightly regulated, and cell cycle progression has to be stopped in response to irregularities such as DNA damage or faulty spindle assembly. The presence of cyclin/CDK

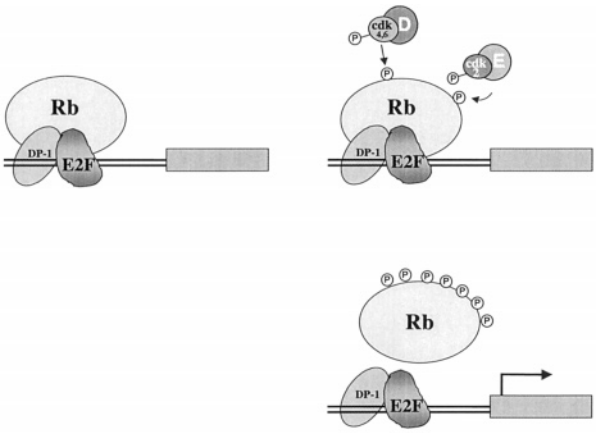

Figure 4. Phosphorylation of retinoblastoma (Rb) by cyclin D and cyclin E leads to gene expression. Nonphosphorylated retinoblastoma binds to the transcription factor complex E2F/DP-1, thereby inactivating transcriptional activity. Phosphorylation of Rb by cyclin D/CDK4,6 complexes during the G1 phase, followed by phosphorylation by cyclin E/CDK2 at the end of the G1 phase, results in release of the transcription complex from Rb, and consequently in the activation of the E2F complex and entrance into S phase.

complexes plays an important role in this regulation, and cyclin/CDK activity is especially required at the so-called checkpoint controls at the G1/S transition and at the transition from G2 to mitosis.

One of the most important cyclin/CDK substrates in mammalian cells during G1 is the product of the retinoblastoma tumor suppressor gene (pRb). Rb is phosphorylated in a cell-cycle-dependent manner and binds in the hypophosphorylated state to transcription factors, particularly members of the E2F family [33–36]. E2F consists of at least five different isoforms that form heterodimers with a second group of proteins known as DP-1 [35, 37, 38]. pRb is present in this hypophosphorylated form during early G1 and becomes phosphorylated on several residues during mid to late G1. This phosphorylation causes the release and activation of the E2F transcription factors, allowing transcription of genes that mediate progression through S phase [39] (fig. 4).

Initial activation of Rb is thought to occur in the the G1 phase by phosphorylation by cyclin D/CDK complexes. D-type cyclins have been shown to bind directly to Rb in the absence of a kinase and thus might target the CDK4/CDK6 kinase to Rb. After the initial phosphorylation by cyclin D/CDK, cyclin E/CDK2 complexes are thought to subsequently phosphorylate pRb late in G1, thereby triggering the actual onset of DNA replication [40]. Phosphorylation of the Rb protein and subsequent activation of E2F have been suggested to be essential for progression through the restriction point [34].

Signal transduction

Growth factor signaling

The restriction point (R) has been defined as the point in G1 after which cells no longer respond to withdrawal of growth factors [41]; once cells have passed the restriction point, they will continue cell cycle progression independent of the presence of growth factors. When growth factors are removed before the restriction point, however, cells will stop cell cycle progression and enter the quiescent state (G0). Cells will remain in the quiescent state until extracellular conditions change and cells are stimulated to reenter the cell cycle.

Extracellular signals induce not only reentry of the cell cycle and cell proliferation but also differentiation or programmed cell death (apoptosis), depending on the nature of the signal. This kind of signal transduction mostly occurs during the G1 phase of the cell cycle. The transmission of extracellular signals to intracellular targets, like the cell cycle machinery, is mediated by specific signal transduction pathways, which involve protein-protein interactions and phosphorylation and dephosphorylation of proteins. One of the most important signaling pathways in the complex network of signal transduction is the mitogen-activated protein kinase (MAPK) pathway [42, 43]. This pathway is evolutionarily conserved, as it is present in *Xenopus*, *Drosophila*, yeast, *C. elegans* and mammals. In mammalian cells different MAPK isoforms have been described, based on sequence homology and function, such as JNK/SAPK, p38 HOG1 kinase and extracellular regulated kinase (ERK). These MAP kinases (MAPKs) respond to distinct extracellular stimuli and have different intracellular substrates.

In general, the MAPK isoforms are activated by phosphorylation on regulatory threonine and tyrosine residues by dual specificity protein kinases, also referred to as MAP kinase kinase (MAPKK). The MAPKK themselves are phosphorylated and activated by serine/threonine kinases that function as MAPKK kinases (MAPKKKs). The best-studied MAPK pathway is the signal transduction pathway that leads to phosphorylation of $p44^{MAPK}$ and $p42^{MAPK}$ (also called ERK1 and ERK2, respectively) and their translocation to the nucleus. When growth factor receptors are activated, the Grb2 adaptor protein is bound to the receptor, together with the guanine-nucleotide exchange factor Sos. Upon binding of Sos, the *ras* GTPase is activated, leading to recruitment of Raf-1 to the plasma membrane. Raf-1 is subsequently activated and can, in turn, activate the MAPKK MEK (MAPK- or ERK kinase), which finally

phosphorylates p44/p42^{MAPK} (ERK1/2) on thr183 and tyr185 (TEY motif). Subsequently activated ERK may phosphorylate targets in the cytoplasm, such as cytoskeletal elements, phospholipase A_2 (PLA_2) and others [44–46], 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 [47–50] (fig. 5). Besides growth factor receptors, the MAPK pathway can also be regulated by G-protein-coupled receptors (GPCRs). G-protein-coupled receptors can be activated by different external stimuli such as growth factors, hormones and neurotransmitters (reviewed in [51]), and mostly consist of seven hydrophobic transmembrane helixes with a large hydrophobic tail at the C-terminus [52] which interacts with GTP-binding proteins (G proteins). G proteins are heterotrimers composed of an α subunit and a $\beta\gamma$ dimer, which can bind GTP and hydrolyze GTP to GDP (reviewed in [53]). Upon binding of GPCRs to hormones or other ligands, the G proteins are activated, leading to conversion of G_α from the inactive GDP bound state into the active GTP-bound state and dissociation of the $G_{\alpha\beta\gamma}$ complex. Thereupon, both the GTP-bound G_α as well as free $\beta\gamma$ subunits can activate several effector enzymes, such as phospholipase C (PLC) species or adenylyl cyclases. Activation of adenylyl cyclases leads to the generation of cyclic AMP (cAMP) from ATP which can subsequently induce activation of protein kinase A (PKA) [54]. However, most GPCRs activate MAPK via ras-dependent signaling pathways. $G_{\beta\gamma}$ subunits can activate either nonreceptor tyrosine kinases, such as Shc, or phosphorylation of RTKs, thus inducing the cascade described above [51, 55]. MAPK activation by GPCRs in a ras-independent manner, through PLC or PKC, has also been reported; G_α activates PKC, which can directly phosphorylate Raf-1 [56, 57].

Integrin signaling

The MAPK signal transduction pathway may be induced not only by growth factor receptors and GPCRs but also by integrins. Integrins are signal-transducing cell surface receptors that have an essential role in cell adhesion [58]. Integrin receptors bind to a variety of ligands, including extracellular matrix (ECM) components such as fibronectin, vitronectin, laminin and collagen. In addition, integrins interact with the cytoskeleton, and through this interaction integrins participate in cell migration, differentiation and cell growth. Integrins are heterodimers that are composed of an α and a β subunit, each with a large extracellular domain, a single membrane-spanning region and usu-

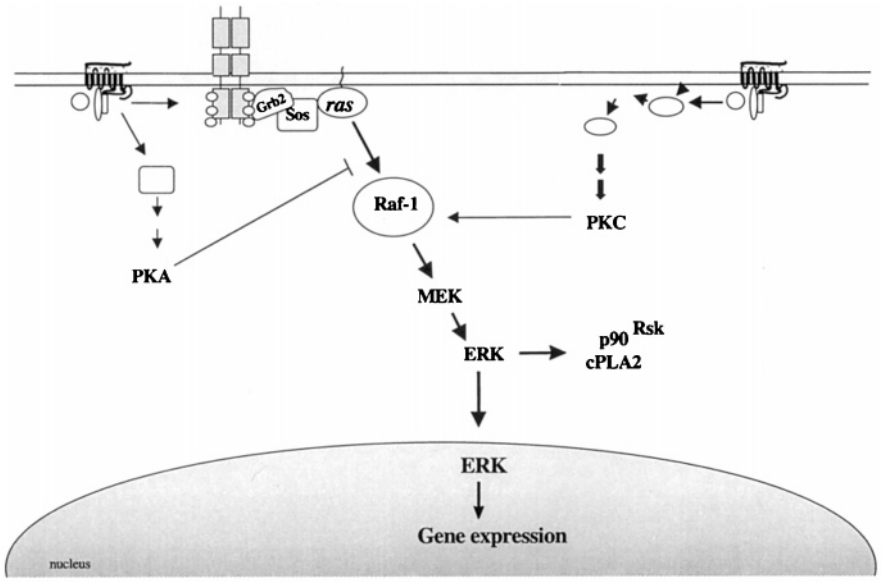

Figure 5. Overview of the MAPK signal transduction pathway. The MAP kinase signal transduction pathway consists of MAP kinase (ERK), the MAP kinase kinase (MEK) and MAP kinase kinase kinase (raf-1). Upon binding of growth factors to receptors located on the cell surface, the G-protein ras is activated, which subsequently activates raf. Ras can also be activated by G-protein-coupled receptors. In addition, an interaction between the MAPK signal transduction pathway and other signal transduction pathways [amongst them protein kinase C (PKC)] and protein kinase A (PKA) has been described. Upon activation, MAPK phosphorylates and activates proteins in the cytoplasm [amongst them p90^{Rsk} and phospholipase A_2 (cPLA2)] and transcription factors in the nucleus, leading to induction of gene expression. MAPK clearly acts as a shutle between the cytoplasm and the nucleus in signal transduction.

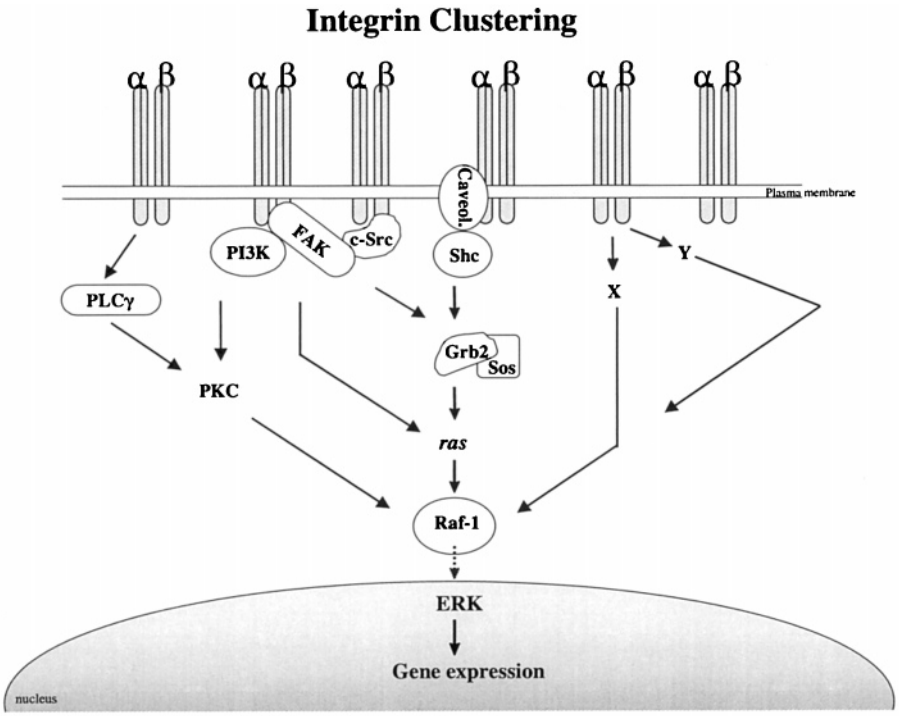

Figure 6. Different mechanisms of MAPK activation by integrin signaling. Activation of integrins (consisting of two different subunits, α and β) by binding to the ECM results in the activation of MAPK by various signal transduction cascades. Upon activation of integrins, various proteins can be activated, such as kinases [amongst them focal adhesion kinase (FAK), c-Src and phosphatidylinositol-3 kinase (PI3K)], adaptor proteins (amongst them Shc, Grb2/SoS) and others [amongst them phospholipase Cγ (PLCγ)].

ally a short cytoplasmic domain without kinase activity (reviewed in [59] and references therein). So far, at least 16 different α subunits and eight β subunits are known, which can form at least 22 distinct integrins. The α/β combinations determine the ligand binding specificities of the integrin heterodimer for various ECM proteins, and various combinations might transduce different signals from the ECM to the cell interior. Some integrins, such as the 'classic' fibronectin receptor $\alpha 5/\beta 1$, interact with only a single ECM protein [60]. More commonly an individual integrin will recognize several distinct ECM proteins. In general, integrins recognize specific amino acid sequences of the ligand, such as the Arg-Gly-Asp (RGD) motif.

The interaction between integrins and surrounding extracellular matrix molecules triggers tyrosine phosphorylation intracellularly [61]. In contrast to growth factor receptors, integrin receptors lack intrinsic kinase activity; but upon ligand binding, specific tyrosine kinases such as focal adhesion kinase ($p125^{FAK}$) can be activated [62, 63]. FAK associates with the cytoplasmic tails of β integrins upon integrin stimulation and is autophosphorylated on tyrosine. Activated FAK subsequently associates with c-Src, which further phosphorylates FAK on additional tyrosine residues, leading to full activation of FAK [64]. This phosphorylation results in the binding of the Grb2/Sos complex and the adaptor protein Shc, thereby linking to the MAPK pathway [65]. In addition, the adaptor protein Shc can be activated in a FAK-independent way via the membrane protein caveolin [66]. Recently it was demonstrated that FAK associates with activated platelet-derived growth factor and epidermal growth factor receptor signalling complexes [67]. Furthermore, a number of other signal transduction pathways have been suggested to activate MAPK upon integrin stimulation, which would act independent of ras, like activation of PKC or direct activation of raf (fig. 6). The exact mechanism of these pathways, however, is still unclear.

Integrins are not only involved in direct signaling after ligand binding as described above but can also participate in collaborative signaling. Integrins and growth factors frequently regulate the same pathways, and activation of both integrins and growth factor receptors is often required for activation of downstream events. For example, integrin-mediated adhesion itself does not result in mitogenesis, but integrin binding can increase the

activation of MAPK by growth factor receptors by 75–90% [68–70] and may thus contribute to cell proliferation. Apparently, integrins can regulate transmission of signals to MAPK by altering the activity of MEK or raf [65], but association between integrins and growth factor receptors has also been reported [71–74]. Thus, integrins might regulate the MAPK signal transduction pathway at several levels.

Although much is known about the separate processes that control cell cycle progression or induce signal transduction cascades upon stimulation by extracellular stimuli, it still is not entirely clear how single signal transduction cascades can have different cellular responses and how signal transduction pathways couple to the cell cycle machinery. In this respect, it is of interest to note that at mitosis the focal adhesions disassemble and the signal transduction from focal adhesions is inactivated [75]. In this review we describe the role of MAPK during the ongoing cell cycle and suggest a model that explains how integrins and growth factors cooperate to regulate progression through G1 phase, based on experiments in Chinese hamster ovary (CHO) cells.

MAPK activity during the cell cycle

Many studies regarding the activation of MAPK in relation to cell cycle progression have been performed in *Xenopus* oocytes and starfish oocytes (reviewed in [76, 77]). In *Xenopus*, MAPK activation has been shown to play a crucial role in meiotic cell cycles during oocyte maturation, early embryonic development and metaphase arrest of unfertilized eggs [78–80]. MAPK activity was found to induce oocyte maturation, probably by activation and stabilization of the M-phase-promoting factor (MPF = cyclin B/cdc2 complex) [81]. However, in fertilized eggs, activation of the MAPK pathway results in growth arrest, which may be caused by the phosphorylation of Wee1 by MAPK [82].

In starfish eggs MAPK was found not to be required for either MPF activation or subsequent oocyte maturation [83]. Instead, its major role seems to be the suppression of DNA synthesis in unfertilized, haploid eggs. Unfertilized starfish eggs, which are arrested at the G1 phase, have elevated levels of MAPK activity, and inactivation of MAPK is necessary and sufficient for triggering the G1/S phase transition at fertilization [84]. The mechanism by which MAPK regulates this G1 arrest in mature starfish eggs has been suggested to resemble the pheromone response pathway in budding yeast. The pheromone pathway is necessary for haploid strains of yeast, which are of mating type **a** or α, to mate with each other. Haploid cells produce pheromone, **a**-factor or α-factor, which acts on the receptor of its mating partner. Upon binding of the factor on the receptor, a MAPK pathway is activated, consisting of STE11 (MAPKKK), STE7 (MAPKK) and FUS3 and KSS1 (MAPKs) (reviewed in [85]). These activated MAPKs then induce the transcription of genes that are necessary for cell fusion, like *fus1* and *far1*. Furthermore, FUS3 phosphorylates the cdk inhibitor FAR1, thus inducing G1 arrest and concomitant cell fusion (fig. 7).

In addition to the functions described above, in *Xenopus*, MAPK was also found to act in the cell cycle checkpoint that detects defects in mitotic spindle assembly [86, 87]. Activation of MAPK at the G2/M transition results in stabilization of cyclin B and presumably also of proteins that are involved in the separation of sister chromatids. This protein stabilization causes a stop in mitosis which can be overcome by downregulation of MAPK activity, for example by treating cell extracts with the MAPK phosphatase MKP-1 [88]. During the specific stages of M phase, MAP kinase is present in the mitotic spindle, consistent with the possibility that MAPK regulates the spindle or monitors the status of the spindle [86]. Since MAPK has also been found to localize to spindle poles during M phase in NIH3T3 cells [89], it has been suggested that the MAPK signal transduction pathway may have a function during mitosis in mammalian cells as well.

MAP kinase during the cell cycle in mammalian cells

Most research done on the role of MAPK during the cell cycle in mammalian cells concerns the stimulation of quiescent cells to reenter the cell cycle (G0/G1). Upon activation, MAPK translocates to the nucleus, where it phosphorylates transcription factors and induces early gene transcription [90]. In fibroblasts, activation of the MAPK pathway at the G0/G1 transition has been shown to induce expression of cyclin D [91, 92]. D-type cyclins are synthesized as long as growth factors are present, and therefore, cyclin D has been suggested to act as a growth factor sensor [93]. As opposed to cyclin D, the CKI $p27^{KIP1}$ is upregulated in quiescent cells [94] and decreases upon growth factor stimulation [95]. MAPK has been shown to phosphorylate $p27^{KIP1}$ in vitro [96], and this may trigger degradation by the ubiquitin pathway [23]. Thus, activation of the MAPK signal transduction pathway regulates the exit from G0, both by downregulating the CKI $p27^{KIP1}$ and by inducing the expression of cyclin D [97]. Ever more data now indicate that the same regulation of cyclin D and $p27^{KIP1}$ might occur at the G1/S transition in mammalian cells [98–100]. Activation of $p42^{MAPK}$/$p44^{MAPK}$ is observed at the G1/S and G2/M transition in CHO cells [101], and a sustained activation of MAPK is required for fibroblasts to pass the restriction point. Recently, evidence was obtained that the sus-

tained MAPK activity was due to the combined activity of growth factor- and integrin-activated signal transduction cascades [102]. A nuclear translocation of $p42^{MAPK}$ at the end of mid-G1 has been described in continuously cycling CHO cells grown in the continuous presence of growth factors [103]. Inhibition of the $p42^{MAPK}/p44^{MAPK}$ cascade by antisense constructs, overexpression of kinase-inactive mutants and inactivation by MKP-1 or inhibition of the nuclear translocation by the use of MEK inhibitor PD098059 blocks DNA synthesis and cell proliferation [103–105].

Although sustained MAPK activity seems essential for cell proliferation in fibroblasts, activation of this same MAPK signal transduction pathway during G1 can also lead to cell cycle arrest. By using inducible forms of mammalian raf in fibroblasts, several groups showed that the level of activation of the raf/MAPK pathway controls cell cycle progression [106–108]. Low level activation of raf promotes cell proliferation, whereas high level activation of the raf/MAPK signal transduction pathway causes cell cycle arrest. This stop in cell cycle progression is correlated with the upregulation of $p21^{CIP1}$ and concomitant inhibition of cyclin/CDK activity. It is now firmly established that the expression of $p21^{CIP1}$ can be induced by the MAPK signaling pathway in a p53-independent manner [109, 110]. In addition, ever more data now indicate that the induction of $p21^{CIP1}$ in response to MAPK activation may also be involved in the differentiation of rat pheochromocytoma (PC12) cells.

The role of MAPK during differentiation in PC12 cells

Treatment of PC12 cells with nerve growth factor (NGF) leads to exit of the cell cycle and differentiation to a neuron like phenotype [111]. This neuronal differentiation is accompanied by a persistent activation and subsequent translocation of $p42^{MAPK}/p44^{MAPK}$. In contrast, receptors for other growth factors such as epidermal growth factor (EGF) that only transiently activate MAP kinase do not induce its nuclear translocation and do not result in neuronal differentiation (reviewed in [112]). Forced activation of the MAPK signal transduction pathway in PC12 cells, either by overexpression of EGF receptors or expression of constitutive active mu-

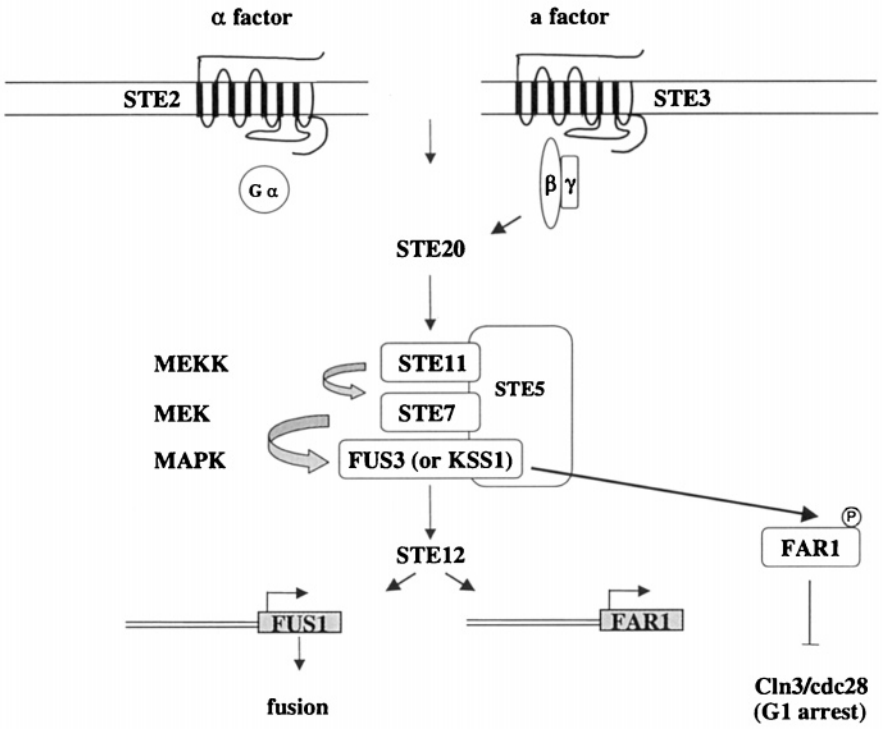

Figure 7. The MAPK pheromone pathway in yeast (adapted from [128]). The MAPK pheromone pathway in yeast consists of MAPK (FUS3), MAPKK (STE7) and MAPKKK (STE11). The MAPK cascade is activated upon stimulation of the α-factor receptor and **a**-factor receptor, G-protein-coupled receptors in the plasma membrane. The activated G proteins subsequently stimulate STE20, the activator of STE11. The members of the MAPK family in yeast are bound to a binding protein, STE5. Interestingly, MAPK in yeast causes the phosphorylation and activation of a CKI, FAR1.

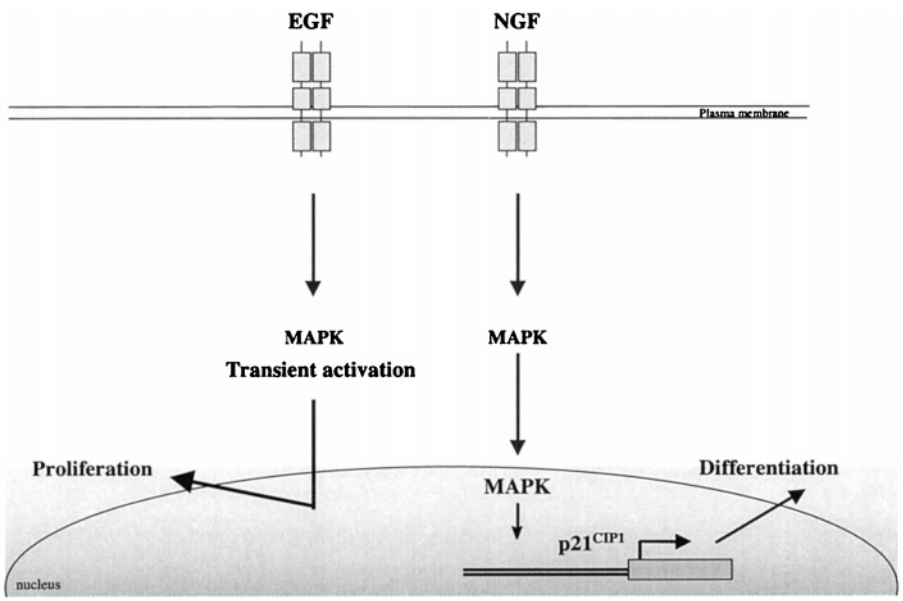

Figure 8. MAPK activation: differentiation versus proliferation. Epidermal growth factor (EGF) and nerve growth factor (NGF) both activate the MAPK cascade, but EGF has a mitogenic effect, whereas NGF usually causes cell differentiation. The activation of MAPK by EGF is transient, whereas the activation by NGF is sustained. The sustained activation of MAPK is accompanied by the induction of the CKI p21.

tants of ras and raf can convert the EGF-induced cell proliferation to a cell cycle arrest and induce neurite outgrowth [113, 114]. Furthermore, inhibition of MAPKK by the MEK inhibitor PD098059 completely blocks NGF-induced neurite formation in PC12 cells, indicating that the MAPK pathway is absolutely required for NGF-induced neuronal differentiation [115].

Although it is still not completely understood how NGF induces differentiation in PC12 cells, the mechanism by which NGF induces cell cycle arrest appears to resemble the G1 arrest in starfish eggs and the pheromone pathway in budding yeast. Instead of the yeast CKI FAR1, however, the mammalian CKI $p21^{CIP1}$ is supposed to be involved. The expression of $p21^{CIP1}$ is induced by MAPK after NGF treatment of PC12 cells [116], presumably by activation of the Sp1 transcription factor [117]. Treatment of PC12 cells with EGF does not result in induction of $p21^{CIP1}$ (8). Moreover, overexpression of $p21^{CIP1}$ has been shown to be sufficient for the induction of growth arrest and activation of differentiation in myelomonocytic cells [118], whereas treatment with transforming growth factor-β resulted in growth arrest caused by MAPK induced upregulation of $p21^{WAF1/CIP1}$ [119]. However, depending on the cell type, other CKIs may be involved in differentiation as well [120].

Nuclear translocation of MAPK is observed both in cells stimulated with various growth factors and in continuously cycling cells. In CHO cells that are grown in the continuous presence of serum, phosphorylation of $p42^{MAPK}$ occurs as soon as 10 min after mitosis. This phosphorylation occurs both in adherent cells and in cells that are incubated in suspension in the presence of growth factors [121], suggesting that the phosphorylation of MAPK primarily originates from growth factor signaling. As in the stimulation of quiescent cells, the activation of MAPK in continuously cycling cells appears to correlate with expression of cyclin D. When growth factors are removed immediately after mitosis, MAPK is not phosphorylated, and the expression of cyclin D decreases rapidly [122]. Expression of another G1 cyclin, cyclin E, was found to be dependent on both growth factors and cell attachment and did not seem to be directly correlated with the activation of MAPK.

However, although the expression of cyclin E appears to be mediated by integrin signaling, the exact mechanism of regulation is not quite clear. Some proteins of the integrin signal transduction pathway, like Grb2 and Sos, appear to decrease when cells are cultured in suspension, and at later times in G1, the phosphorylation of $p42^{MAPK}$ also appears to decline (E. Hulleman et al., unpublished results). This may influence in some way the activity of cyclin D in suspension cells, leading to an insufficient inactivation of the retinoblastoma protein and consequently to lack of expression of cyclin E. In fact, it has been shown that activation of the MAPK

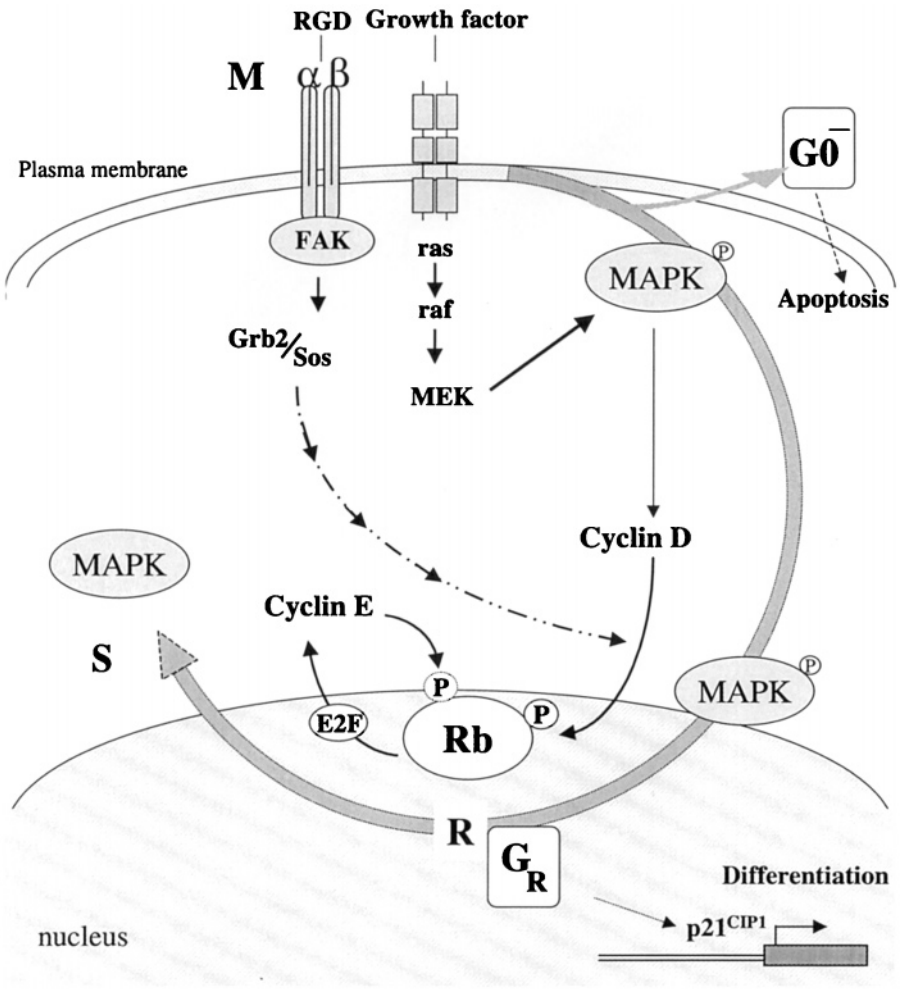

Figure 9. Model representing the spatiotemporal regulation of cell cycle progression by integrin- and growth-factor signaling. MAPK is phosphorylated immediately after mitosis (M), via the ras-raf-MEK pathway. This phosphorylation step is localized in the cytoplasm and is required for passing an early restriction (R) point that possibly leads to apoptosis ($G0^-$). In addition, this phosphorylation is required for the sustained expression of cyclin D. At the end of the G1 phase, phosphorylated MAPK is translocated to the nucleus where can activate nuclear targets. In the presence of growth factors. MAPK is subsequently exported out of the nucleus, a process that is probably correlated with passage through R. In the absence of growth factors MAPK will remain nuclear, leading to an increased expression of $p21^{CIP1}$ and a stop at R. This stop at R, or G_R phase, is suggested to be associated with cell differentiation. Passage through R is correlated with the phosphorylation of retinoblastoma (Rb) by the cyclin D/cdk4,6 complex. Initial phosphorylation of Rb results in expression of cyclin E, which itself is required for full phosphorylation of Rb and entry into the S phase. For expression of cyclin E, integrin signaling appears to be required. Although the precise coupling of the integrin receptors and cyclin E induction is not yet known, cyclin D appears to be involved in this feature.

signaling pathway facilitates the assembly of cyclin D into catalytically active complexes [100], and that expression of cyclin E is partially dependent on the activation of cyclin D [122]. Alternatively, other pathways, like the PI3 kinase signal transduction cascade may be involved in integrin-mediated expression of cyclin E, since the transcription factor E2F has been reported to be regulated by this pathway [123, 124]. Moreover, the induction of MAPK activity at the G1/S transition in cells grown in suspension was shown not to be sufficient for induction of DNA synthesis, suggesting that at least one other adhesion-dependent signaling event is required for G1/S progression [125].

Although the expression of cyclin D decreases rapidly when MAPK is not activated at the M/G1 transition (i.e. in the absence of growth factors), dephosphorylation of MAPK at later times in G1 does not seem to influence the expression of cyclin D in CHO cells. It was demonstrated that CHO cells react differently on serum withdrawal, depending on their position in the cell cycle [121]. When growth factors are removed immediately after mitosis, cells appear to enter a G0-like state: signal

transduction cascades are not activated, the expression of cyclin D and retinoblastoma rapidly decreases and no DNA synthesis takes place. In addition, expression of $p27^{KIP1}$ is upregulated when cells are serum starved for longer periods of time. When cells are depleted from serum later in G1, however, also no DNA synthesis takes place, but MAPK is still phosphorylated after overnight starvation, cyclin D is still present and there is no significant increase in the levels of $p27^{KIP1}$. These findings suggest that in CHO cells (and possibly in other transformed cells as well), at least two serum-dependent restriction points exist that regulate progression through the cell cycle. The first restriction point is found to be located within 10–15 min after mitosis and may correlate with the point described by Gurley and co-workers [126] that leads to apoptosis upon treatment with Br-cAMP. In fact, CHO cells that are incubated in suspension in the absence of growth factors die within 2 h after replating [E. Hulleman et al., unpublished results]. Moreover, cells grown as a monolayer that are depleted from serum immediately after mitosis were found not to be able to resume cell cycle progression after longer starvation times, and cells appeared to alter their morphology. This change in morphology appeared to be irreversible, and upon longer starvation periods or drug treatment, these cells will probably go into apoptosis [121].

The second serum-dependent point is located at the end of mid-G1 and appears to represent the 'classic' restriction point R [1]. Interestingly, this point was found to correlate with the nuclear translocation of MAPK that was originally described for cells that were grown in the continuous presence of serum [103]. Since nuclear translocation of $p42^{MAPK}$ at the end of mid-G1 was now found to occur in the absence of serum, the actual trigger for the translocation appears to function independent of the presence of growth factors, although serum seems to be required during the early times after mitosis. As opposed to the relatively short nuclear localization of $p42^{MAPK}$ that was observed in cells cultured in the presence of serum, MAPK appeared to localize in the nucleus for longer periods of time in cells deprived of serum early in G1 (nuclear localization is observed after overnight starvation). These serum-deprived cells did not pass the G1/S transition, suggesting that not only nuclear entry but also nuclear exit is essential for progression through the cell cycle. Since a prolonged nuclear localization of MAPK has been shown to correlate with the upregulation of $p21^{CIP1/WAF1}$ and differentiation in PC12 cells [116, 127], the sustained MAPK activity in the nucleus at the second restriction point may be linked to cell differentiation.

Together, these data can be used to design a model in which the position in the cell cycle and the localization of the proteins are integrated (fig. 9). During the M phase, growth factor receptors and integrin receptors (at the plasma membrane) are not activated. Immediately after mitosis, however, signal transduction initiates, resulting in the phosphorylation of MAPK, continued expression of cyclin D (dependent on growth factor signaling) and expression of cyclin E (both integrin and growth factor signaling). At the end of G1 both cyclins are supposed to coordinately phosphorylate the retinoblastoma protein, which triggers progression through R [40]. Apart from R, another serum-dependent restriction point can be distinguished, early in G1, at which cells arrest when growth factors are depleted. This first point leads to entry into a G0-like state, which may be linked with apoptosis, whereas the second point may lead to up regulation of $p21^{CIP1/WAF1}$ and subsequently to cell differentiation. Activation of MAPK early in G1 is important for passage of the first restriction point, expression of cyclin D and nuclear translocation of $p42^{MAPK}$ at the end of mid-G1. The presence of growth factors during the remainder of G1 is important for the duration of nuclear MAPK localization and passage of the second restriction point. A short nuclear MAPK localization would lead to cell cycle progression, whereas a prolonged nuclear localization induces differentiation of cells.

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