Growth Factor-Dependent Signaling and Cell Cycle Progression

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I. Introduction

A widely used model system to investigate cell proliferation is stimulation of serum-arrested cells with growth factors. Recent data suggest that there are two waves of growth factor-dependent signaling events required for a proliferative response. One is an acute burst of signaling, which occurs immediately after growth factor stimulation and lasts for 30−60 min. The other occurs in a different time frame (8−12 h post stimulation) and involves activation of cyclin-dependent kinases (Cdks). In addition to a general overview of growth factor-dependent signaling, we present our "two wave" hypothesis for how signaling and cell cycle progression are linked.

II. Three Steps in Growth Factor-Dependent Signaling

A. The First Step: Binding of Growth Factor to the Transmembrane Receptor

A recurring theme for growth factor receptors is ligand-induced dimerization of the receptor. There are a variety of ways in which this occurs. Some ligands, such as platelet-derived growth factor (PDGF), are dimeric and their receptors encode a single ligand-binding domain. Receptor dimerization results from the binding of one receptor to each half of the dimeric ligand. The PDGF ligand is a member of a family of cysteine knot-containing proteins, and many other growth factors that share this structural feature appear to interact with their receptors in a comparable fashion.[1] Other growth factors are monomeric and manage dimerization of their cognate receptors by a different strategy. Members of the fibroblast growth factor (FGF) family have two binding sites for the receptor within a single molecule of FGF. Resolution of the crystal structure of the FGF ligand−receptor complex indicated that each receptor molecule also has two binding sites for the ligand.[2−4] Hence, the ligand−receptor complex consists of two molecules of receptor and two molecules of FGF. An additional feature of the FGF family is that high-affinity binding of FGF to its receptor requires heparan sulfate proteoglycans, which spatially organize the ligands to functionally associate with the receptor. These are only two examples of how growth factors dimerize their receptors; a number of reviews cover additional well-studied cases.[5,6]

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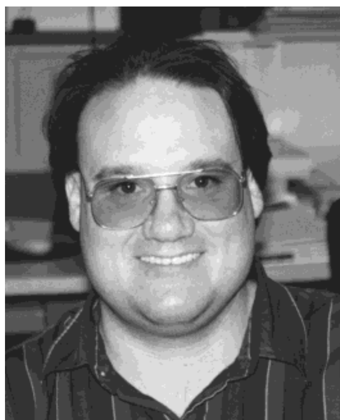

Steven M. Jones was born in Washington, D.C., He received his B.A. degree in Biology from Boston University in 1988. Under the direction of Kathryn Howell, he earned his Ph.D. degree in Cell and Developmental Biology from the University of Colorado. His thesis research focused on identifying molecules that are required for the formation of exocytic transport vesicles from the trans Golgi network. After receiving his Ph.D. in 1997, he moved to Boston, MA, to join the laboratory of Andrius Kazlauskas at the Schepens Eye Research Institute and Harvard Medical School. During his postdoctoral training in the Kazlauskas lab (1997–2000), he studied the signals that connect growth factor signaling to cell cycle progression. Steven is currently a postdoctoral fellow in the laboratory of Shiela Thomas at Beth Israel Deaconess Medical Center and Harvard Medical School in Boston, MA. His current research focuses on how the cytoskeleton regulates complex biological processes such as cell growth, differentiation, migration, and survival. His work is also investigating the role cytoskeletal regulation plays in disease using in vitro cell culture systems and inducible gene knockouts in the mouse.

Andrius Kazlauskas was born in Cleveland, OH, in 1959. He graduated from Cleveland State University with his Ph.D. degree in Chemistry in 1986. He studied signaling by tyrosine kinases as a postdoctoral fellow in Jonathan A. Cooper's lab at the Fred Hutchinson Cancer Research Center from 1986 to 1990. He has held an independent research position from 1991, first at the National Jewish Center and then at the Schepens Eye Research Institute and Harvard Medical School. His research interests are elucidating signal transduction pathways used by receptor tyrosine kinases to drive cell proliferation. He has been married to Lione Bradunas since 1982 and is raising two children.

The theme of ligand-induced dimerization of receptors is not universal. The insulin receptor is a dimer even before binding of insulin, which is a monomer. In addition, polymerized collagen is a ligand for the discoidin receptor tyrosine kinases,[7,8] and it is not obvious whether this sort of ligand is able to induce dimerization or oligomerization of it receptor. In summary, many, although not all, receptor tyrosine kinases are dimerized in response to binding of ligand and there are a variety of mechanisms by which this event proceeds.

B. The Second Step: Activation of the Receptor's Kinase Activity

Binding of ligand results in an elevation of the receptor's kinase activity. Activation of a kinase involves a reconfiguration of various domains, including the activation loop, as well as the orientation of the upper and lower lobes of the kinase.[9] Tyrosine kinases are often phosphorylated in the activation loop, and this event probably contributes to the conformational changes leading to activation of the kinase. For receptor tyrosine kinases, ligand-induced dimerization brings the kinase domains into close proximity and facilitates transphosphorylation at the activation loop tyrosines.[6,10] Whether ligand binding promotes additional events that contribute to activation of the kinase is still not fully understood.

While the ligand promotes activation of a receptor tyrosine kinase, there are also factors that suppress the receptor's kinase activity. Cells express many types of phosphotyrosine phosphatase (PTPs) that have the potential to functionally repress the receptor's kinase activity. For instance, addition of inhibitors of PTPs leads to a rapid increase in the phosphorylation of receptor tyrosine kinases in the apparent absence of ligand. In addition, growth factors trigger a burst of hydrogen peroxide production, which has the potential to inhibit PTPs. In some cell types, inhibition of peroxide production blunts PDGF-dependent tyrosine phosphorylation of the PDGFR,[11] whereas in other it does not.[12] Finally, there are proteins that prevent activation of receptor tyrosine kinases. Kek-1 is a transmembrane protein that physically interacts with the drosophila EGFR and antagonizes the effect of EGFR.[13] Whether functionally similar proteins exist in mammalian systems as well as what step of the activation mechanism they impact remain open questions. In summary, the balance between positive and negative factors determines the activity of a receptor tyrosine kinase.

C. The Third Step: Recruitment and Activation of Signaling Enzymes

One of the consequences of tyrosine phosphorylation of the receptor is to enable the receptor to recruit a variety of signaling enzymes.[14,15] The interaction of signaling enzymes with the activated receptor is dependent on tyrosine phosphorylation of the receptor (i.e., conditional). This interaction is also specific, and the specificity is determined by both the receptor and the signaling enzyme.[14,15] The amino acid context surrounding the tyrosine phosphorylation site is the receptor's contribution to specificity, whereas the PTB or SH2 domain of the signaling enzymes has an intrinsic preference for binding partners and hence makes a contribution to the specificity of the interaction. There is a long and growing list of proteins that associate with the βPDGFR,[14,15] and these same signaling enzymes can associate with many other activated receptor tyrosine kinases as well. In this

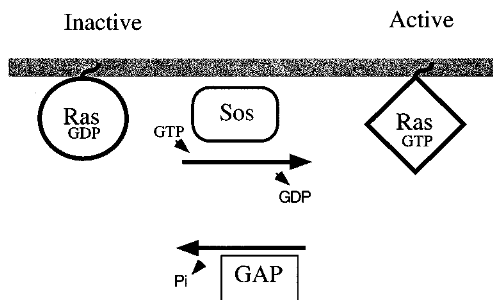

Figure 1. Activation of Ras. Activation of Ras is catalyzed by the nucleotide exchange factor Sos, which promotes the release of GDP. Although Ras has intrinsic GTPase activity, the hydrolysis of GTP to GDP is enhanced by GAP, and this event converts Ras back to its inactive state. (Reprinted with permission from *Oncogene* **2000**, *19*, 5558–5567.)

review we will focus on several well-studied examples and refer the interested reader to other reviews for additional information.[14,15]

One of the signaling proteins activated in a growth factor-stimulated cell is Ras.[16] Activated Ras functions as a cofactor for a variety of signaling enzymes.[17] The nucleotide exchange factor, Sos, promotes the exchange of GDP for GTP on Ras, which converts Ras to its active state (Figure 1). Inactivation of Ras proceeds by a distinct chemical reaction, i.e., hydrolysis of the GTP to GDP, and is promoted by GTP-ase activating proteins called GAPs. Sos is a constitutively active cytoplasmic enzyme, whereas Ras is anchored to the membrane. Consequently, activation of Ras requires translocation of Sos from the cytoplasm to the membrane, which is mediated by adapter proteins. There are several ways by which Ras can be activated in growth factor-stimulated cells, and they all appear to involve a change in the subcellular localization of the Grb2/Sos complex. Grb2 is an SH3–SH2–SH3 adapter protein that mediates binding of Sos to activated receptors. The Grb2/Sos complex is constitutive and in resting cells is primarily cytoplasmic. Tyrosine phosphorylation of the receptor enables the SH2 domain of Grb2 to stably associate with receptor tyrosine kinase receptors. This relocalizes Sos to the membrane, the cellular compartment in which its substrate, Ras, resides (Figure 2). Alternative scenarios include association of the Grb2/Sos complex with other adapter proteins such as Shc. Many growth factors induce tyrosine phosphorylation of Shc, leading to its association with Grb2/Sos. This trimeric complex can relocate to the membrane via Shc's SH2 or PTB/PH domains. The functional consequence of these changes is the same as when Grb2/Sos associates with a tyrosine-phosphorylated growth factor receptor: Sos gains access to Ras and activates it.

Phosphoinositide 3 kinase (PI3K) is one of the SH2 domain-containing signaling enzymes that are activated in growth factor-stimulated cells. The p85 subunit of PI3K contains a number of domains, and it is via the SH2 domains that p85 and its tightly associated p110 catalytic subunit are recruited to activated receptors. Unlike the Grb2/Sos complex, localizing the PI3K holoenzyme to the membrane by its association with the PDGFR is not sufficient to activate the enzyme.[18] Ras must be activated, which directly binds to the catalytic subunit of PI3K[19,20] and provides the necessary second input for activation in growth factor-stimulated cells (Figure 3). PI3K is being increasingly appreciated as a family of enzymes instead of a single entity, and the individual members of the PI3K family may have unique functions in promoting growth factor-dependent responses.[21,22]

Tyrosine phosphorylation of signaling enzymes is an additional component of their activation in growth

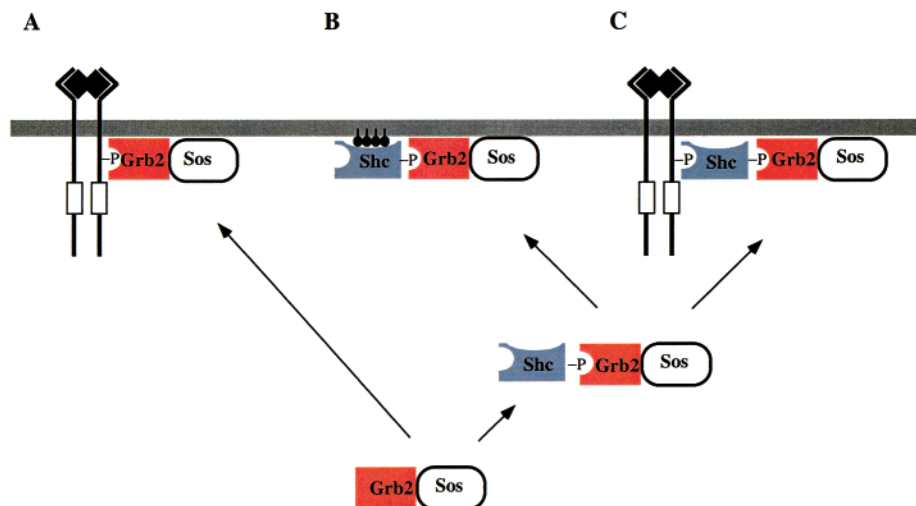

Figure 2. There are multiple ways to recruit Sos to the membrane. Grb2 constitutively associates with Sos and mediates its recruitment to the membrane in one of three ways. (A) The SH2 domain of Grb2 binds directly to a tyrosine-phosphorylated receptor. (B,C) The growth factor promotes tyrosine phosphorylation of Shc and hence its association with Grb2 via the SH2 domain of Grb2. The resulting trimer is recruited to the membrane in one of two ways. The SH2 domain of Shc mediates binding to a tyrosine-phosphorylated receptor (C). Alternatively, the PTB/PH domain of Shc interacts with lipids in the membrane and hence translocates the Shc/Grb2/Sos complex to the membrane. These membrane lipids include products of PI3K, which is activated in response to growth factor stimulation. The three recruitment scenarios are not mutually exclusive and may be occurring simultaneously in growth factor-stimulated cells. As noted in Figure 1, the functional consequence of recruitment of Sos to the membrane is activation of Ras. (Reprinted with permission from *Oncogene* **2001**, *19*, 5558–5567.)

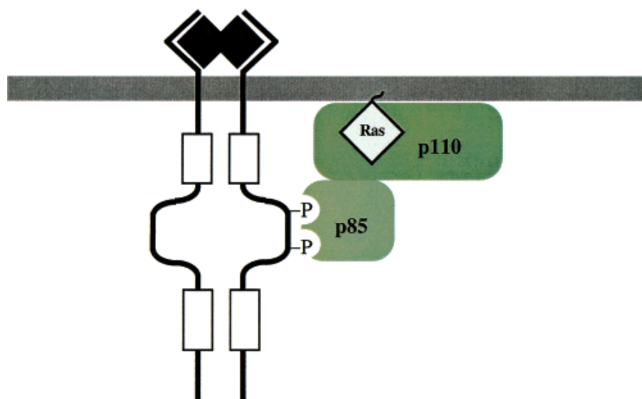

Figure 3. Activation of PI3K in PDGF-stimulated cells. Two types of changes are required for activation of PI3K in response to PDGF. One is activation of the enzyme and the second is permitting access to its substrate. Both of these events occur within minutes of exposure to PDGF, and it is not clear whether one needs to precede the other. Gaining access to its membrane-localized substrates involves ligand-induced phosphorylation of the receptor at tyrosine residues that are central to the binding site for the SH2 domains of the p85 subunit of PI3K. In addition, the interaction between activated Ras and the p110 catalytic domain may also contribute to localization and/or proper orientation of PI3K in the membrane. Activation of PI3K involves both the engagement of the SH2 domains within the p85 subunit and occupancy of the Ras binding site in the catalytic subunit. The figure depicts the complex that results from these events. Once it is activated, PI3K phosphorylates PI-4-P and PI-4,5-P2 to generate PI-3,4-P2 and PI-3,4,5-P3, respectively, which are potent second messengers that engage a variety of signaling cascades. (Reprinted with permission from *Oncogene* **2000**, *19*, 5558−5567.)

factor-stimulated cells. While some of these proteins are direct substrates of the receptor, they may also be phosphorylated by the kinases activated by the receptor. For instance, Src family kinases (SFKs) are activated in PDGF-stimulated cells[23−25] and phosphorylate many proteins, including phospholipase C γ (PLCγ).[26] PDGF-dependent tyrosine phosphorylation of PLCγ is dramatically compromised in cells expressing PDGFRs that fail to activate SFKs.[27,28] However, the same kinase may not be phosphonylating PLCγ in all cell types. Comparable PDGF-dependent tyrosine phosphorylation of PLCγ was observed in cells that lacked SFKs versus cells in which Src had been reexpressed.[29] Similarly, blocking SFKs with a newly developed inhibitor did not prevent PDGF-dependent tyrosine phosphonylation of PLCγ.[107] Hence, tyrosine phosphorylation of signaling enzymes may proceed via more than one PDGF-activated kinase, and there appears to be differences between cell types regarding which kinases phosphorylate a given substrate.

D. Converting Enzymology into a Cellular Response

In comparison to the first three steps of growth factor-dependent signaling, our understanding of how these steps culminate in cellular responses is very limited. The eventual consequence of exposing cells to growth factors such as PDGF can be cell movement, proliferation, differentiation, or protection from death. How the cell selects among these possible responses and the alteration in the signaling cascades necessary to mediate the appropriate response remain poorly understood. However, significant progress has been made in identifying which of the many signaling enzymes are required for cellular responses such as mitogenesis. Using a variety of approaches, most investigators conclude that PI3K and to a lesser extent PLCγ are contributing to PDGF-dependent mitogenesis.[14,15,30,31] Furthermore, these two enzymes appear to be functionally redundant, as PDGF-dependent DNA synthesis can be rescued in a mitogenically incompetent receptor mutant when either the PI3K or PLCγ/PKC pathway is activated.[32] Such findings suggest the existence of a common mitogenic signaling cascade that can be accessed by various signaling enzymes. This issue will be further addressed below.

There is some controversy regarding the relative contribution of some of the other receptor-associated signaling proteins in PDGF-dependent mitogenesis.[33] For instance, SFKs have been clearly shown to be required for the mitogenic response of cells to PDGF[34,35] whereas in other cases they are dispensable.[27,29,36] It is possible that the differences relate to the choice of experimental approach and/or the model system in which the experiments were performed. The experimental approaches in which SFKs were essential for DNA synthesis were ones where SFKs were inhibited globally. In contrast, PDGF-dependent mitogenesis was unimpeded in settings where the SFK was selectively interrupted at the level of the receptor. Since SFKs make an essential contribution to integrin-dependent responses,[37,38] which are essential for mitogenesis,[39] it is possible that the critical contribution of the SFKs comes at the level of the integrin instead of the receptor.

III. Growth Factors Are Not the Only Extracellular Cue Needed for Cell Proliferation

A. Integrins

Integrins are a family of cell surface receptors consisting of heterodimers between α and β subunits that mediate attachment of cells to extracellular matrix (ECM) proteins such as fibronectin and collagen.[37,38] The intracellular domains of integrin molecules not only physically link the plasma membrane to the cytoskeleton, but also initiate signaling cascades. Evidence that integrins synergize with growth factors to drive a mitogenic response includes the observations that integrins and activated receptor tyrosine kinases coprecipitate.[40] In addition, the nature of the ECM onto which cells are plated (and hence the types of integrins that are engaged) can enhance or diminish the mitogenic potency of growth factors.[40,41] Finally, compromising the contribution of integrins by suspending a population of adherent cells alters some of the signaling pathways triggered by growth factors and prevents cell cycle progression.[37,38,42] Thus, the combined inputs of integrins and growth factors are required for cell cycle progression.

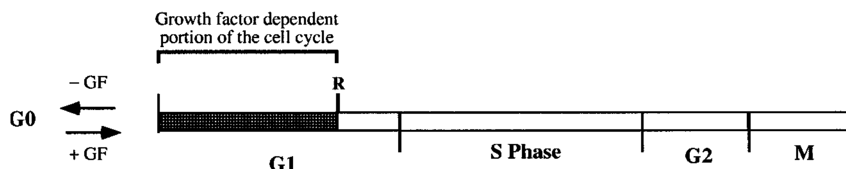

Figure 4. Only a small portion of the cell cycle is regulated by growth factors. Cells that have been deprived of serum or growth factors exit the cell cycle and enter into the G0 state. Growth factors promote exit from G0, and cells will commit to one round of the cell cycle if growth factors are present up to the R point. An important component of the R point is phosphorylation of Rb, which is further outlined in Figure 5. Once past the R point, most cells will continue through the other stages of the cell cycle, even if the growth factor is removed from the culture medium. (Reprinted with permission from *FEBS Lett.* **2001**, *490*, 110−116 and *Oncogene* **2000**, *19*, 5558−5567.)

B. Cell Shape

Cell cycle progression is also controlled by cell shape and cytoskeletal tension.[43] Under conditions where the growth factor and total cell-ECM contact is kept constant, cell shape determines whether the cells do or do not move into the S phase.[44,45] These studies showed that if cells were prevented from spreading, they failed to make the G1/S transition, despite engagement of integrins and the presence of growth factors. The following types of observations support the idea that cytoskeletal tension is also a regulator of cell cycle progression. Cell proliferation within a tissue is localized to regions where the ECM of the basement membrane is thinner than the surrounding regions, and the change in ECM content alters the tensional force on cells.[44−47] In addition, pharmacological disruption of the cytoskeleton (and hence the tractional force) prevents cells from moving into the S phase. Finally, activation of Rho A, a small GTPase protein that modulates the integrity of the cytoskeleton, promotes degradation of $p27^{Kip1}$ and eliminates the anchorage requirement for S-phase entry.[48,49] These findings demonstrate that cell proliferation is not only regulated by growth factors and the ECM. The shape of the cell and cytoskeletal tension are also important variables, and $p27^{Kip1}$ is at least one of the points at which they interface with the cell cycle program. The cell shape and tractional force variables may be particularly relevant to in vivo settings as compared with tissue culture system that is usually used to study regulation of cell proliferation.[43]

IV. The Cell Cycle

A. The G0 to S Interval Is the Only Portion of the Cell Cycle That Is Regulated by Growth Factors

When plated at low cell density in serum-containing medium, cultured cells move through four phases of the cell cycle: G1, S, G2, and M (Figure 4). Each of these phases is regulated by the coordinated action of kinases and proteases.[50,51] When deprived of serum, cells continue to cycle until they complete mitosis, whereupon they exit into the G0 state.[52,53] These cells can be reintroduced into the cell cycle by the re-addition of serum or purified growth factor. The mitogen must be present until the R point, which is several hours prior to the transition between G1 and S.[53,54] Thus, in serum-deprived cells, all of the growth factor-stimulated events that are necessary for completion of one round of the cell cycle occur before the R point (Figure 4). Furthermore, growth factors are not needed at later times to complete the other stages of the cell cycle.

Figure 5 outlines the cell cycle events that constitute the G1 cell cycle program. Phosphorylation of the retinoblastoma (Rb) protein is the current molecular definition of the R point.[54] At least two classes of G1 cyclin-dependent kinases (Cdks) collaborate to fully phosphorylate Rb, which results in the release of the E2F family of transcription factors.[55] This class of transcription factors initiates subsequent events necessary for transition through the other phase of the cell cycle, even in the absence of serum. Consequently, the mitogenic potential of growth factors is intrinsic to their ability to promote phosphorylation of Rb.

B. Elements of the Cell Cycle Program That Are Regulated by Signaling Enzymes

Growth factors promote phosphorylation of Rb by regulating the activity of the G1 Cdks. This involves promoting the synthesis and stability of cyclin subunits as well as decreasing the levels of Cdk inhibitors (Figure 5B). For instance, growth factor-dependent activation of the Ras/Erk pathway increases cyclin D1 mRNA.[56−62] Furthermore, the PI3K/Akt pathway stabilizes the cyclin D1 proteins. At least in some cell types, activation of Akt inhibits glycogen synthase kinase 3 β (GSK3β)-dependent phosphorylation of cyclin D1 and thereby prevents its degradation via the proteasomal pathway.[63−66] Others have also implicated PI3K/Akt in cyclin D1 accumulation, although the mechanism of action does not appear to be in stabilization of the cyclin D1 protein and appears to involve transcriptional activation of cyclin D1.[67] Accumulation of cyclin D1 results in the assembly of cyclin D1/Cdk4, 6 complexes.[56] Growth factor-dependent elimination of Cdk inhibitors such as $p27^{Kip1}$ proceeds through a PI3K-dependent pathway and is essential for transition through G1 and into the S phase.[67−69] Recent studies indicate that Akt acts downstream of PI3K to phosphorylate members of the forkhead family of transcription factors such as AFX/FKHR.[70−74] When phosphorylated, these transcription factors move out of the nucleus and thereby cease driving transcription of $p27^{Kip1}$.[75] Hence, growth factors promote Cdk activity by increasing the levels of cyclins and decreasing the levels of Cdk inhibitors.

Active cyclin D1/Cdk4, 6 partially phosphorylates Rb, which begins to release the E2F family members

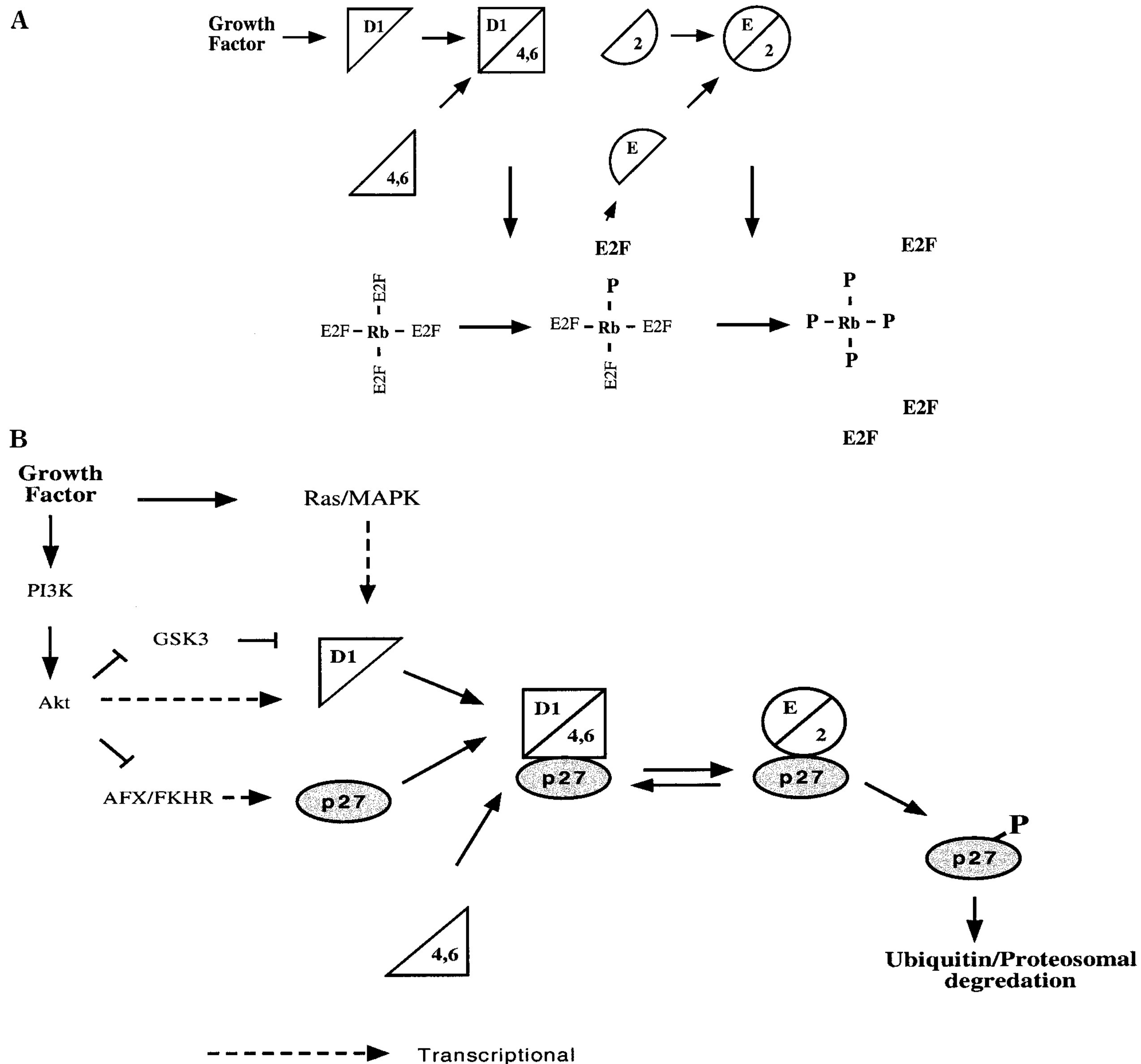

Figure 5. G1 cell cycle program. (A) Full phosphorylation of Rb requires the coordinated action of two Cdks and results in the release of the E2F family of transcription factors. Growth factors promote the accumulation of cyclin D1, which forms a complex with either Cdk4 or 6. The cyclin D1/Cdk4, 6 complex phosphorylates Rb, releasing a small amount of E2F, which in turn drives the formation of cyclin E. The cyclin E/Cdk2 complex further phosphorylates Rb, releasing more E2F. The E2F family of transcription factors promotes transcription of genes that initiate the transition into the S phase. (B) Role of $p27^{Kip1}$ in the cell cycle program $p27^{Kip1}$ and $p21^{Cip1}$ (not shown) promote assembly of the cyclin D/Cdk4, 6 complex. $p27^{Kip1}$ inhibits the kinase activity of both of the cyclin/Cdk complexes and appears to be more potent toward cyclin E/Cdk2. There are at least three ways by which $p27^{Kip1}$ is neutralized. Growth factors suppress the synthesis of $p27^{Kip1}$ protein; cyclin E/Cdk2 phosphorylates $p27^{Kip1}$ and targets it for degradation; cyclin D/Cdk4, 6 sequester $p27^{Kip1}$. Akt acts to inhibit GSK3β (glycogen synthase kinase 3β) and thereby stabilize cyclin D1 protein. Phosphorylated cyclin D1 is targeted for ubiquitination and degradation. In some systems, Akt also promotes cyclin D1 accumulation by a transcriptional mechanism. A second way by which Akt promotes cell cycle progression is by phosphorylating members of the forkhead transcription factor family such as AFX/FKHR. Phosphorylation of AFX/FKHR relocates it to the cytoplasm and hence prevents it from driving transcription of $p27^{Kip1}$. (Reprinted with permission from *FEBS Lett.* **2001**, *490*, 110–116 and *Oncogene* **2000**, *19*, 5558–5567.)

(Figure 5A). Free E2F promotes the transcription and consequent accumulation of a second cyclin, cyclin E, which couples with the Cdk2 kinases. The appearance of cyclin E/Cdk2 has at least three functional consequences (Figure 5B). First, it acts in collaboration with cyclin D1/Cdk4, 6 to titrate $p27^{Kip1}$ levels. Second, cyclin E/Cdk2 phosphorylates $p27^{Kip1}$ and hence targets it for ubiquitination and degradation via the proteasome.[76,77] Third, cyclin E/Cdk2 further phosphorylates Rb, which fully activates the E2F family (Figure 5A).

The role of $p27^{Kip1}$ and a second Cdk inhibitor $p21^{Cip1}$ has become more complicated with the appreciation of an additional function for these proteins. They not only block Cdk activity, but $p27^{Kip1}$ and $p21^{Cip1}$ are also instrumental in the assembly of the cyclin D1/Cdk complexes[78,79] (Figure 5). Furthermore, $p21^{Cip1}$ and $p27^{Kip1}$ are components of the active cyclin D1/Cdk4, 6 enzyme.[79] Finally, there appears to be a difference between cyclin D1/Cdk4, 6 and cyclin

E/Cdk2 in the way they are regulated by p27^{Kip1}. In contrast to the readily detectable kinase activity of the cyclin D1/Cdk4, 6/p27^{Kip1} complex, when p27^{Kip1} joins the cyclin E/Cdk2 complex, it extinguishes kinase activity.[80,81] Thus, the Cdk inhibitors promote activation of cyclin D1/Cdk4, 6 and inhibit the activity of cyclin E/Cdk2. Further studies will be required to resolve the apparent paradox regarding how the Cdks both promote and inhibit[82] cyclin D1/Cdk4, 6 activity.

In summary, activation of the Ras/Erk or PI3K/Akt pathways results in an increase in cyclin D1 mRNA, and some investigators find that the PI3K/Akt pathway stabilizes the cyclin D1 protein. In addition, growth factors suppress p27^{Kip1} levels in a pathway that also involves PI3K. Thus, cyclin D1 and p27^{Kip1} are two points in the cell cycle program at which growth factor-stimulated signaling has an input. Since full phosphorylation of Rb, i.e., passing the R point, requires the coordinated input of several distinct components of the cell cycle program, there may be additional points of the cell cycle program at which growth factor-dependent signaling makes other essential contributions.

V. Most of the Well-Studied Growth Factor-Initiated Signaling Events Occur Many Hours before the Cell Cycle Program

A. Growth Factor-stimulated Signaling Is Transient

In acutely stimulated cells there are two themes to the signaling events: phosphorylation/dephosphorylation and changes in the subcellular location. For instance, tyrosine phosphorylation of the PDGFR at the appropriate tyrosine residues enables stable association with PI3K.[14,83] While this relocates PI3K from the cytoplasm to a juxtamembrane location, the location of its lipid substrate, accumulation of active Ras is also needed for activation of PI3K[18−20] (Figure 3). PI3K generates second messengers (PI-3, 4-P2 and PI-3, 4, 5-P3), which are the activators for downstream signaling enzymes such as Akt and PKC family members.[22,84] These ser/thr kinases are some of the enzymes capable of relaying the mitogenic signal along a cascade that appears to be part of the network that integrates signals which emanate from integrins and the sensors of cell shape and cytoskeletal integrity.

While the exact nature of this overall signaling network is far from understood, it is clear that the initial phase of growth factor-stimulated signaling events does not persist much longer than 60 min. For instance, PI3K products accumulate within minutes of PDGF stimulation and then return to near basal levels by 30 min.[85−87] There appears to be a variety of reasons why signaling subsides, one of which relates to the half-life of the growth factor receptor. One of the proteins that is phosphorylated in response to growth factors is c-Cbl, a protein that promotes internalization and/or degradation of growth factor receptors.[88] Enzymes such as PTEN, a phosphatase capable of dephosphorylating and hence metabolizing the PI3K lipid products, may also contribute to the decline of cellular PI3K lipid products.[89] Other well-characterized mechanisms to extinguish signaling include the rapid expression of new genes that counteract the signaling enzymes. MKP-1 is a phosphatase that dephosphorylates and hence inactivates Erk family members.[90] In resting cells, MKP-1 levels are low and then rise quickly following mitogenic stimulation.

In summary, growth factors trigger a rapid burst of signaling events that subsides even in the continuous presence of growth factor. Receptor internalization and degradation as well as the appearance of enzymes, which antagonize the signaling enzymes, are some of the ways in which the cell silences the growth factor-initiated signaling cascade.

B. How Do Growth Factor-stimulated Signaling Events Engage the Cell Cycle Program?

If the first wave of growth factor-dependent signaling is complete within 60 min, then what triggers the cell cycle program, which begins roughly 7−9 h after exposure to PDGF? Since the early signaling events induce the expression of many new genes, including those that are involved in cell proliferation, perhaps it is the products of these genes that are responsible for engaging the cell cycle program. If this were indeed the case, then exposure to growth factor for 1−2 h, which is sufficient to induce the immediate early genes, would also be sufficient to drive cells into the S phase. However, fibroblasts require 8−10 h of continuous exposure to growth factor to get past the R point.[53,91,92] Hence, the early burst of signaling is insufficient for cell cycle progression and there must be additional inputs that the growth factor makes at latter time points. Insight into this long-standing question has come from a number of labs, demonstrating that there are requirements for signaling enzymes and/or signaling events well beyond the well-studied early burst of signaling.

VI. Growth Factor-Stimulated Signaling Beyond the First 60 min

A. Microinjection Studies Indicate That Signaling Enzymes Are Needed well beyond the First 60 min

One approach to investigate the importance of a signaling enzyme for growth factor-dependent mitogenesis is to eliminate it by microinjection of a neutralizing antibody directed against the signaling enzyme and assay the effect on S-phase entry. The Stacey lab used this approach and learned that activated Ras is required for entry into the S phase.[93] These studies were preformed by preinjecting cells with the antibody and then stimulating with the mitogen. This experimental protocol indicated that Ras was important at some point but did not specify when. By injecting the antibody after exposing cells to the growth factor, it has been possible to begin to assess when the signaling enzyme is contributing to mitogenic signaling. Blocking Ras, SHP-2, or PI3K hours after the initial wave of growth factor-dependent signaling prevented growth factor-dependent

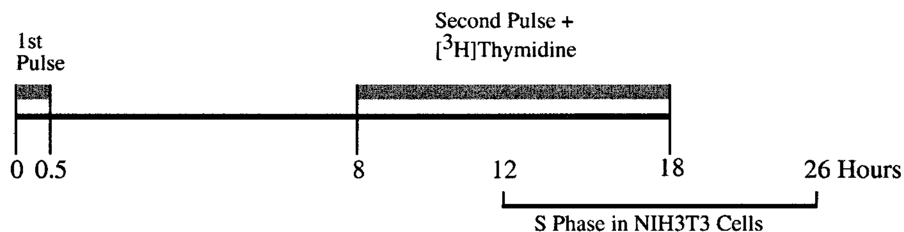

Figure 6. Discontinuous stimulation assay. Serum-arrested NIH 3T3 cells were pulsed with PDGF for 30 min; the cells were then acid washed and placed into medium containing 0.1% FBS. After 7.5 h, PDGF and [^{3}H]thymidine were added, and the cells were harvested at the 18 h time point. The S phase, as measured by an increase in the incorporation of [^{3}H]thymidine, is between 12 and 26 h in these cells. (Reprinted with permission from *FEBS Lett.* **2001**, *490*, 110−116 and *Oncogene* **2000**, *19*, 5558−5567.)

entry into the S phase.[30,31,94−96] These studies strongly suggested that signaling enzymes are important for mitogenic signaling at times beyond the initial burst of signaling.

B. Two Waves of Signaling in Cells Treated Continuously with Growth Factors

To directly investigate the idea that signaling is occurring at later time points, a number of investigators began to monitor signaling events in cells that had been treated with growth factors for longer times. For instance, several groups have reported biphasic activation of Ras in serum-stimulated NIH 3T3 cells.[67,97] The level of active Ras peaks within 10 or 30 min, recedes, and then peaks again 2−6 or 2−4 h later. Similarly, PDGF triggers two waves of PI3K and PKC activity in HepG2 cells, an early and then a late phase, the late phase being 3−7 h after the addition of growth factor.[86,98] These studies demonstrate that there are two waves of activity for a number of signaling systems and raise a number of interesting questions.

For instance, how are these two waves of activity regulated? The first wave of activity has been studied at length as it is the one observed in acutely stimulated cells. As outlined in the sections above, there is a wealth of information regarding the mechanism by which signaling enzymes such as Ras and PI3K are activated in acutely stimulated cells. In contrast, virtually nothing is known regarding the molecular events by which the second wave of activity appears. Whether the mechanisms by which the signaling enzymes are activated during the first and second wave of signaling are comparable await further investigation.

C. The Second Wave of Signaling Is Required for Cell Proliferation

An additional question that arises from the observation that there are two waves of enzymatic activity is the relative contribution of each wave to growth factor-driven mitogenesis. The second wave of signaling is required for S-phase entry, at least in the case of Ras and PI3K, since injecting neutralizing antibodies directed against these proteins blocked cell cycle progression. Additional approaches have also found that the second wave of PI3K, PKC, and Ras activity is essential for cells to respond mitogenically to growth factors.[69,86,96,98]

For some of the signaling enzymes the first and second waves of signaling make unequal contributions to the mitogenic response. In the case of PI3K and certain PKC family members, only the second wave of activity was required for PDGF-dependent entry into the S phase.[86,98] The addition of pharmacological inhibitors at times corresponding to the second wave of activity attenuated PDGF-dependent DNA synthesis. The inhibitors had no effect if they were used to block only the first wave of PI3K or PKC activity. Similarly, adding synthetic PI3K lipid products or diacylglycerol (DAG), an activator of certain PKC family members, rescued PDGF-dependent DNA synthesis but only when they were added at times corresponding to the second wave of activity. Adding the PI3K lipid products or DAG simultaneously with PDGF failed to promote PDGF-dependent DNA synthesis in this system. Hence, although PI3K and PKCs are activated during the first wave of PDGF-induced signaling, their activation at this time is dispensable for the DNA synthesis response. It is likely that they are contributing to other PDGF-dependent cellular responses such as chemotaxis and survival.[99−101]

VII. The "Two Wave" Hypothesis for How Signaling and Cell Cycle Progression Are Linked

A. Growth Factor-Dependent Signaling Is not Needed Continuously during the Interval between G0 and the R Point

Because the initial wave of signaling occurs so much in advance (7−9 h) of even the first element of the cell cycle program, these early signaling events do not appear to be directly responsible for engaging components of the cell cycle program. In contrast, the second wave of signaling overlaps with the cell cycle program and hence may be directly triggering the cell cycle program. This hypothesis has been difficult to test because the second wave of signaling requires prolonged exposure to PDGF, which probably triggers events other than those required for cell cycle progression. We have recently employed a discontinuous stimulation assay (Figure 6) to evaluate the possibility that a late phase of signaling is responsible for engaging the cell cycle program.[102]

Two 30 min pulses of PDGF are sufficient to drive NIH 3T3 cells into the S phase and through the rest of the cell cycle. Furthermore, the kinetics of S-phase entry as well as events of the cell cycle program

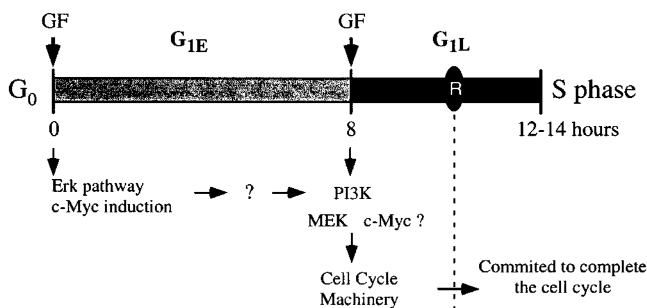

Figure 7. Two-wave hypothesis for how signaling and cell cycle progression are linked. Exposure of quiescent (G0) cells to growth factors initiates many signaling events. Of these, activation of MEK/Erk and elevation of c-Myc are sufficient to drive cells out of G0 and through the early portion of G1 ($G1_E$). Further progression through G1 requires a second input of growth factor. The timing of this requirement overlaps with the second wave of signaling and initiates the cell cycle program. This occurs in the late phase of G1 and is termed $G1_L$. PI3K is one of the signaling enzymes that are activated at this later time and capable of engaging the cell cycle program. As outlined in Figure 5, the cell cycle program results in phosphorylation of Rb, transition past the R point, and commitment to one round of the cell cycle. At least some of the events that occur in $G1_L$ have been well defined, i.e., the cell cycle program. In contrast, the molecular events that are necessary for transition through $G1_E$ are just beginning to be identified. (Reprinted with permission from *FEBS Lett.* **2001**, *490*, 110−116 and *Oncogene* **2000**, *19*, 5558−5567.)

proceeded comparably in cells treated continuously or discontinuously with PDGF. The first pulse defined the start of the experiment, and the ideal time for the second pulse was 8 h. Importantly, the first pulse was insufficient to initiate the cell cycle program, whereas that second pulse of growth factor rapidly engaged the cell cycle program, i.e., cyclin D1 protein was detectably elevated within 1 h of the second pulse. Hence, the early burst of signaling made the cells able to engage the cell cycle program, which was triggered by the second pulse of PDGF. As illustrated in Figure 7, we are proposing the terms early G1 ($G1_E$) and late G1 ($G1_L$) for these portions of G1.

B. Different Sets of Signaling Enzymes Mediate Progression through $G1_E$ and $G1_L$

The subdivision of the G0 to S interval is also supported by the finding that different sets of signaling enzymes mediate transition through $G1_E$ and $G1_L$.[102] Of the many signaling events triggered by the first pulse of PDGF, activation of MEK and elevation of c-Myc were sufficient for transition through $G1_E$. MEK activity was also necessary during the second pulse of growth factor-driven signaling. In contrast, synthetic PI3K lipid products failed to drive cells through $G1_E$ but were sufficient for transition through $G1_L$. These findings indicate that traversing the two segments of the G0 to S interval requires nonidentical sets of signaling enzymes. Finally, transition through $G1_E$ is a prerequisite for engaging the cell cycle program, which is the consequence of subsequent exposure to growth factor.

C. A Common Signaling Cascade Is Used by Many Mitogens

Many agents are mitogenic, and while they interact with specific and unique cell surface receptors, it is possible that they eventually engage a common cascade to promote cell cycle progression. This idea has been investigated with the discontinuous stimulation assay described above. Six mitogens were tested for their ability to substitute for PDGF during the first or second pulse, i.e., to drive cells through $G1_E$ or $G1_L$.[102] Four of the six agents (fetal bovine serum [FBS], FGF, PDGF, and lysophosphatidic acid [LPA]) were completely interchangeable. Any of the four agents given at the first pulse followed by any one of the four in the second pulse drove cells into the S phase. These findings imply that there is a common signaling cascade that can be accessed by a variety of receptor tyrosine kinases as well as G protein-coupled receptors.

Unlike the four mitogens described above, EGF and insulin failed to drive cells through $G1_E$. However, these agents were biologically active as EGF or insulin promoted progression through $G1_L$. Cells that had been brought through $G1_E$ by a pulse of FBS, bFGF, LPA, or PDGF were driven into the S phase when EGF or insulin was used for the second pulse. Hence, the cells have receptors for EGF and insulin and these receptors access the necessary events to engage the cell cycle program and propel the cells through $G1_L$ and into the S phase. The failure of EGF and insulin to promote transition through $G1_E$ could be because a 30 min pulse of these two growth factors triggers a much less robust activation of Erk and elevation of c-Myc as compared with the four agents that drive cells through $G1_E$. These findings indicate that there is a common signaling pathway that is utilized by many different agents. Furthermore, we predict that any agent capable of activating Erk and elevating c-Myc during the first wave of signaling followed by an elevation of PI3K products 8 h later will be sufficient to drive NIH 3T3 cells into the S phase.

Several lines of evidence indicate that the signaling pathways discussed above are not the only ones that are capable of engaging the mitogenic cascade. Cells that are nullizygous for *c-myc* are viable, although they proliferate more slowly than control cells.[103] Similarly, DAG is as effective as PI3K lipid products in rescuing PDGF-dependent DNA synthesis when added to cells at times that appear to correspond to $G1_L$.[86,98] Hence, it is likely that there will be additional enzymes identified that are capable of accessing this common mitogenic cascade.

D. Revisiting Competence and Progression

Using subsaturating concentrations of growth factors, Pledger, Stiles, Antoniades, and Sher demonstrated that in Balb/c 3T3s cell cycle progression required the input of two different types of factors.[104−106] Growth factors such as PDGF made the cells competent but did not drive them into the S phase. A second type of growth factor, such as insulin, was required for progression of the competent cell

into the S phase. Once competent, the cells remained in this state for many hours and such a cell entered the S phase 12−14 h after the addition of a progression factor.[53]

We were interested in determining how the discontinuous stimulation assay related to the competence/progression concept. To this end, we adapted the discontinuous stimulation assay to Balb/c 3T3 cells. When PDGF was used for the first and second pulses, there was very little DNA synthesis induced. Insulin at the first and second pulses or insulin first and then PDGF also failed to induce entry into the S phase. In contrast, PDGF followed by insulin during the second pulse triggered robust DNA synthesis. Thus, it appeared that we were observing the previously reported competence/progression phenomenon with the discontinuous stimulation assay.

Analyzing the time at which cells synthesized DNA further supported the idea that the Balb/c 3T3 cells were behaving along the competence/progression guidelines. When the competence and progression factors were used during the first and second pulses, respectively, the cells entered the S phase during the 22−32 h window. In contrast, DNA synthesis was observed in the 12−22 h window when a complete set of mitogens (PDGF and insulin or FBS) was used during both pulses. The delay in the onset of DNA synthesis was because the competent cells did not begin to traverse the G0 to S interval until receiving the progression factor at the 8 h time point. The presence of both the competence and progression factors during the first pulse enabled the cells to become competent and to begin traversing G1 right away. This suggests that under these experimental conditions, cells become competent very quickly.

Finally, we determined whether the competence/progression phenomenon was intrinsic to the first or second pulse in the discontinuous stimulation assay. Adding a complete set of mitogens during the first pulse was insufficient to drive cells into the S phase. When such cells were given a second pulse of either PDGF or insulin, they initiated DNA synthesis during the early window. This indicated that the complete mitogens drive cells only through the first segment of the G0 to S interval. From this point either a progression or competence factor promotes transition through the rest of G1 and into the S phase. Hence, it appears that competence is a component of the events that involve traversing the first segment of the G0 to S interval. We speculate that competence preceeds exit from G0 and $G1_E$ traverse because making cells competent does not shorten the G0 to S interval.

We have also applied the discontinuous stimulation assay to HepG2 cells. The maximal DNA synthesis response was observed when PDGF was given at the first and second pulses. Hence, PDGF was a complete mitogen instead of a competence factor and in this regard the HepG2 cells are more similar to the NIH 3T3 cells than the Balb/c 3T3s. These findings further support the idea that the competence/progression phenomenon is not universal to all cell types. Finally, there are at least three cell lines that can be efficiently driven into the S phase by discontinuous instead of continuous exposure to growth factors.

VIII. Summary

There are three central ideas contained within this review. First, growth factor-stimulated signaling is not restricted to a 30−60 min window but occurs at much later time as well. Second, the second wave of signaling overlaps temporally with the cell cycle program and may be directly responsible for engaging it. Third, the G1 to S interval appears to encompass two distinct phases of the cell cycle, during which the coordinated activation of distinct sets of signaling enzymes drive cell cycle progression. Each of these concepts is likely to initiate new investigation and hence provide additional insight into the fundamental question of how growth factors drive cell proliferation.

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X. References

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