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Minireview

Growth factor-dependent signaling and cell cycle progression

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Abstract There are three central ideas contained within this review. Firstly, growth factor-stimulated signaling is not restricted to a 30–60 min window, but occurs at a much later time as well. Secondly, the second wave of signaling overlaps temporally with the cell cycle program and may be directly responsible for engaging it. Thirdly, 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 drives 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.

Key words: PDGF; Cell cycle progression; Signal transduction

1. The cell cycle

1.1. 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 (Fig. 1). Each of these phases is regulated by the coordinated action of kinases and proteases [1,2]. When deprived of serum, cells continue to cycle until they complete mitosis, whereupon they exit into the G0 state [3,4]. 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 (Fig. 1) which is several hours prior to the transition between G1 and S [4,5]. 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. Furthermore, growth factors are not needed at later times to complete the other stages of the cell cycle.

Fig. 2 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 [5]. 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 [6]. 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.

1.2. 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 (Fig. 2B). For instance, growth factor-dependent activation of the Ras/Erk pathway increases cyclin D1 mRNA [7–13]. Furthermore, the PI3K/Akt pathway stabilizes the cyclin D1 proteins. At least in some cell types, activation of Akt inhibits glycogen synthase kinase 3β (GSK3β)-dependent phosphorylation of cyclin D1, and thereby prevents its degradation via the proteasomal pathway [14–17]. 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 [18]. Accumulation of cyclin D1 results in the assembly of cyclin D1/Cdk4,6 complexes [7]. 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 S phase [18–20]. Recent studies indicate that Akt acts downstream of PI3K to phosphorylate members of the forkhead family of transcription factors such as AFX/FKHR [21–25]. When phosphorylated these transcription factors move out of the nucleus and thereby cease driving transcription of $p27^{Kip1}$ [26]. Hence growth factors promote Cdk activity by increasing 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 (Fig. 2A). 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 (Fig. 2B). Firstly, it acts in collaboration with cyclin D1/Cdk4,6 to titrate $p27^{Kip1}$ levels. Secondly, cyclin E/Cdk2 phosphorylates $p27^{Kip1}$ and hence targets it for ubiquitination and degradation via the proteasome [27,28]. Thirdly, cyclin E/Cdk2 further phosphorylates Rb, which fully activates the E2F family (Fig. 2A).

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

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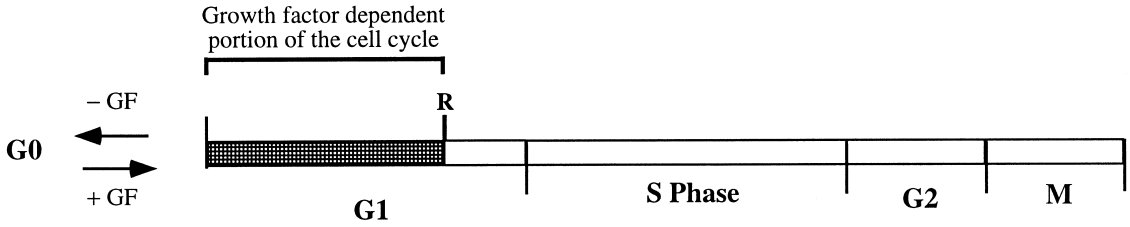

Fig. 1. Only a small portion of the cell cycle is regulated by growth factors. Cells that have been deprived of serum or growth factors exit the cell cycle and enter into the G0 state. Growth factors promote exit from G0, and cells will commit to one round of the cell cycle if growth factors are present up to the R point. An important component of the R point is phosphorylation of Rb, which is further outlined in Fig. 2. 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.

assembly of the cyclin D1/Cdk complexes [29,30] (Fig. 2). Furthermore, $p21^{Cip1}$ and $p27^{Kip1}$ are components of the active cyclin D1/Cdk4,6 enzyme [30]. 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 [31,32]. 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 [33] 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 elements of the cell cycle program that appear capable of receiving input from growth factor-dependent signaling. 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.

2. Most of the well-studied growth factor-initiated signaling events occur many hours before the cell cycle program

2.1. 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 [34,35]. 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 [36–38]. 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 [39,40]. 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 [41–43]. 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 [44]. 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 [45]. 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 [46]. 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.

2.2. 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 S phase. However, fibroblasts require 8–10 h of continuous exposure to growth factor to get past the R point [4,47,48]. 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.

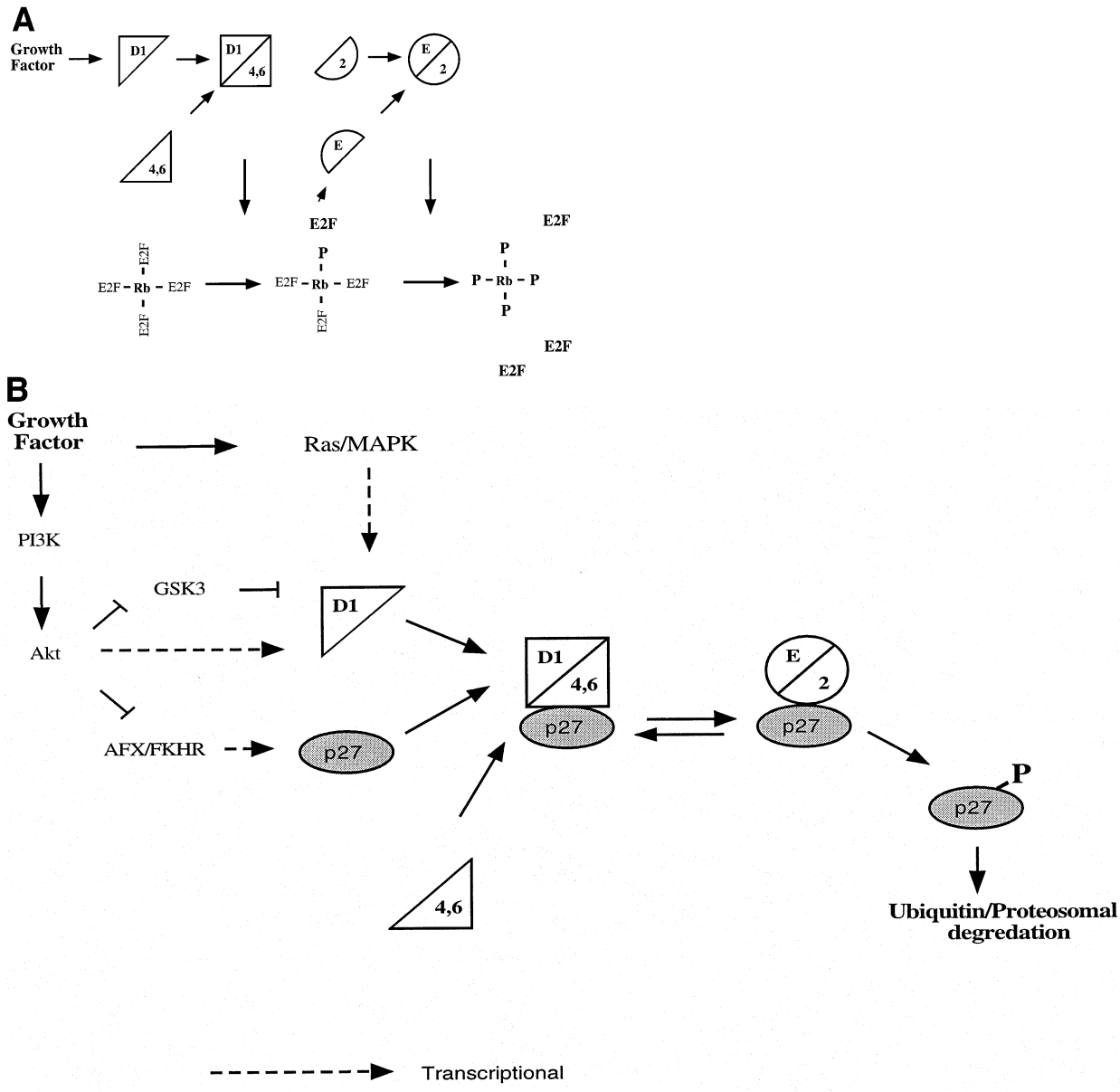

Fig. 2. The G1 cell cycle program. A: Full phosphorylation of Rb requires the coordinated action of two Cdks, and results in the release of the E2F family of transcription factors. Growth factors promote the accumulation of cyclin D1, which forms a complex with either Cdk4 or 6. The cyclin D1/Cdk4,6 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 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 towards 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 $p27^{Kip1}$. GSK3 is glycogen synthase kinase 3β; AFX/FKHR is the forkhead transcription factor.

3. Growth factor-stimulated signaling beyond the first 60 min

3.1. 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 S phase [49]. These studies were performed by pre-injecting cells with the antibody, and then stimulating with the mitogen. They 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 entry into S phase [50–54]. These studies strongly suggested that signaling enzymes are important for mitogenic signaling at times beyond the initial burst of signaling.

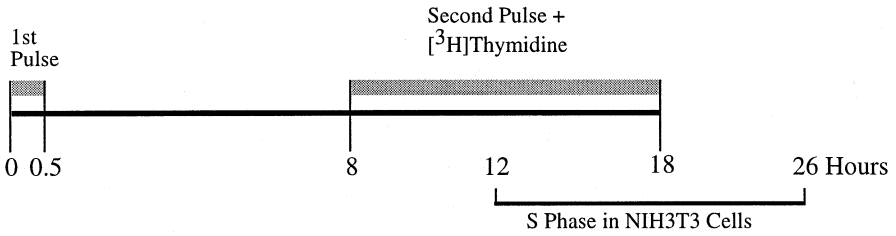

Fig. 3. The discontinuous stimulation assay. Serum-arrested NIH 3T3 cells were pulsed with PDGF for 30 min, the cells were then acid-washed and placed into medium containing 0.1% FBS. 7.5 h later PDGF and [^{3}H]thymidine were added and the cells were harvested at the 18 h time point. S phase, as measured by an increase in the incorporation of [^{3}H]thymidine, is between 12 and 26 h in these cells.

3.2. 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 [18,55]. 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 [42,56]. These studies demonstrate that there are two waves of activity for a variety 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 awaits further investigation.

3.3. 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 [20,42,54,56].

Some of the signaling enzymes make unequal contributions to the mitogenic response during the first and second waves of signaling. In the case of PI3K, and certain PKC family members, only the second wave of activity was required for PDGF-dependent entry into S phase [42,56]. 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 [57–59].

4. The 'two-wave' hypothesis for how signaling and cell cycle progression are linked

4.1. 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

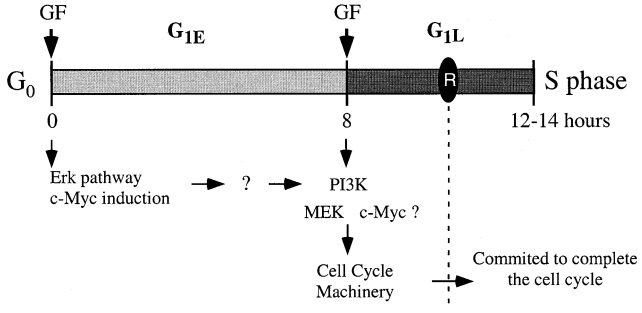

Fig. 4. The two-wave hypothesis for how signaling and cell cycle progression are linked. Exposure of quiescent (G0) cells to growth factors initiates many signaling events. Of these, activation of MEK/Erk and elevation of c-Myc are sufficient to drive cells out of G0 and through the early portion of 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 Fig. 2, 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.

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 (Fig. 3) to evaluate the possibility that a late phase of signaling is responsible for engaging the cell cycle program [60].

Two 30 min pulses of PDGF are sufficient to drive NIH 3T3 cells into 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 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 Fig. 4, we are proposing the terms early G1 ($G1_E$), and late G1 ($G1_L$) for these portions of G1.

4.2. 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 $G1_L$ [60]. 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 non-identical 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.

4.3. 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 $G1_L$ [60]. 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 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 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 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 $G1_E$ [60]. 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 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 [61]. 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 $G1_L$ [42,56]. Hence it is likely that there will be additional enzymes identified that are capable of accessing this common mitogenic cascade.

4.4. 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 [62–64]. Growth factors such as PDGF made the cells competent, but did not drive them into S phase. A second type of growth factor, such as insulin, was required for progression of the competent cell into S phase. Once competent, the cells remained in this state for many hours, and such a cell entered S phase 12–14 h after the addition of a progression factor [4].

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 S phase. In contrast, PDGF followed by insulin during the second pulse triggered robust DNA synthesis [60]. 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 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 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 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 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 [60]. 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 S phase by discontinuous instead of continuous exposure to growth factors.

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