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Cell Cycle: Regulatory Events in $G_1 \rightarrow S$ Transition of Mammalian Cells

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Abstract A cell divides into two daughter cells by progressing serially through the precisely controlled G_1, S, G_2, and M phases of the cell cycle. The crossing of the G_1/S border, which is marked by the initiation of DNA synthesis, represents commitment to division into two complete cells. Beyond this critical point no further external signals are required. We now have more comprehensive knowledge of the temporal sequence of systems at this key transition from G_1 to S—growth factor responses, a cascade of kinase reactions, activation of cyclins and their associated kinases, and oncogene and tumor suppressor gene products. Furthermore, we know that the absolute requirement for calcium and the timing of events associated with calmodulin and the 68 kDa calmodulin-binding protein are consistent with overall Ca^{++}/calmodulin control of all steps from the response to growth factors in G_1 to DNA replication in S phase. We now have to sort out the inter-relationships of myriad control proteins and their relation to the Ca^{++}/calmodulin-dependent controls—Which are causes? Which are effects? And which are parallel processes? The answers will be important, as they represent both a much deeper understanding of this key process of life and an important opportunity for improving therapeutic medicine. © 1994 Wiley-Liss, Inc.

Key words: growth factors, calmodulin, calmodulin-binding proteins, cyclins, cyclin-dependent kinases, oncogenes, tumor suppressor genes, replitase

The process by which one cell divides into two is fundamental to all growth, whether we are talking about developmental growth of an embryonic cell or uncontrolled proliferation of a cancer cell. It was found some twenty years ago that the initiation of DNA synthesis, as the cell traverses the boundary between the presynthesis state G_1 and DNA synthesis period S, represents the commitment to go through all subsequent states of cell division. Cellular commitment to S phase is, in turn, controlled by a series of inter-related regulatory events during G_1 phase of cell cycle [Baserga, 1985; Pardee, 1989]. Starting from the quiescent (G_0) state, progression of cells through G_1 and into S is absolutely dependent on the availability of specific growth factors and a suitable extracellular environment and is mediated by transcription, translation, and intracellular redistribution of a variety of signaling proteins and the enzymes of DNA biosynthesis. This short essay will focus primarily on the controls associated with this all-important G_1 to S transition.

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CELL CYCLE AND G_1 EVENTS

The cell cycle is the process in which a single eukaryotic cell gives rise to two daughter cells in four serial biologically defined phases: first gap (G_1), DNA synthesis (S), second gap (G_2), and mitosis (M). Completion of the specific sequence of metabolic events in each phase prepares cells to proceed to the next. For example, the ability to enter into S phase is determined by the complete synthesis of the enzymes of DNA replication and other signal molecules during G_1. Similarly, cellular DNA must be completely duplicated in S phase for the cells to enter G_2 and M. In most tissues cells exist in a quiescent, nonproliferating, state termed "G_0." G_0 cells remain viable and metabolically active, performing tissue-specific functions, for an extended period. Unlike terminally differentiated cells, G_0 cell retain the ability to enter the proliferative cell cycle, responding to extracellular factors, e.g., wound healing.

Based on the extracellular conditions, the "decision" for the G_0 cell to enter the cell cycle or for the cycling cells to exit into G_0 is made shortly before S in late G_1 period. Following a successful factor-dependent progression of cells from G_1 to S, the ensuing events responsible for the progression of S phase cells into G_2 and G_2 phase cells into M are controlled by internal signals triggered by the completion of biosynthetic process unique to S and G_2, respectively [Murray and Kirschner, 1989], and are rather insensitive to external conditions. Thus, the extracellular factor-dependent commitment of late G_1 cells to enter S phase is the ultimate rate limiting step in determining the overall ability of the cells to complete cell cycle and give rise to two daughter cells.

GROWTH FACTOR REQUIREMENTS IN PROGRESSION OF THE CELLS FROM G_0/G_1 TO S PHASE

Growth factors in serum specific to cell types are essential for the progression of G_0 and G_1 cells into S phase. Most of the experimental evidence for growth factor-dependent progression of mammalian cells from G_0/G_1 into S phase has been obtained from the studies with 3T3 and other fibroblast cells. However, related studies with cytokines point to a similar regulatory process taking place during proliferative stimulation of hematopoietic cells [Crabtree, 1989]. Although some of the growth requirements and molecular events in G_1 phase of epithelial cells have been observed to be different from those of fibroblasts [Clement et al., 1990], a fuller understanding of these processes in epithelial cells is limited by the difficulties to culturing these cells in vitro.

The overall progression of cells from G_0 to S is controlled by a series of specific growth factors which determine the ability of cell to proceed from one regulatory point to the next. As the first step towards proliferation quiescent cells initially require growth factors (see Fig. 1), such as platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF), for initiating early regulatory processes, referred to as reaching "competence" [Pledger et al., 1977; Scher et al., 1979]. In the presence of epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), competent cells can progress to the next stage in G_1, termed the "V" point [Leof et al., 1983]. Cells at the "V" point exhibit a rapid rate of protein synthesis [Rossow et al., 1979] and soon reach the "restriction" point "R" [Pardee, 1974]. In the presence of IGF-I or insulin, these cells will then commence DNA synthesis without any additional requirement for growth factors, tran-

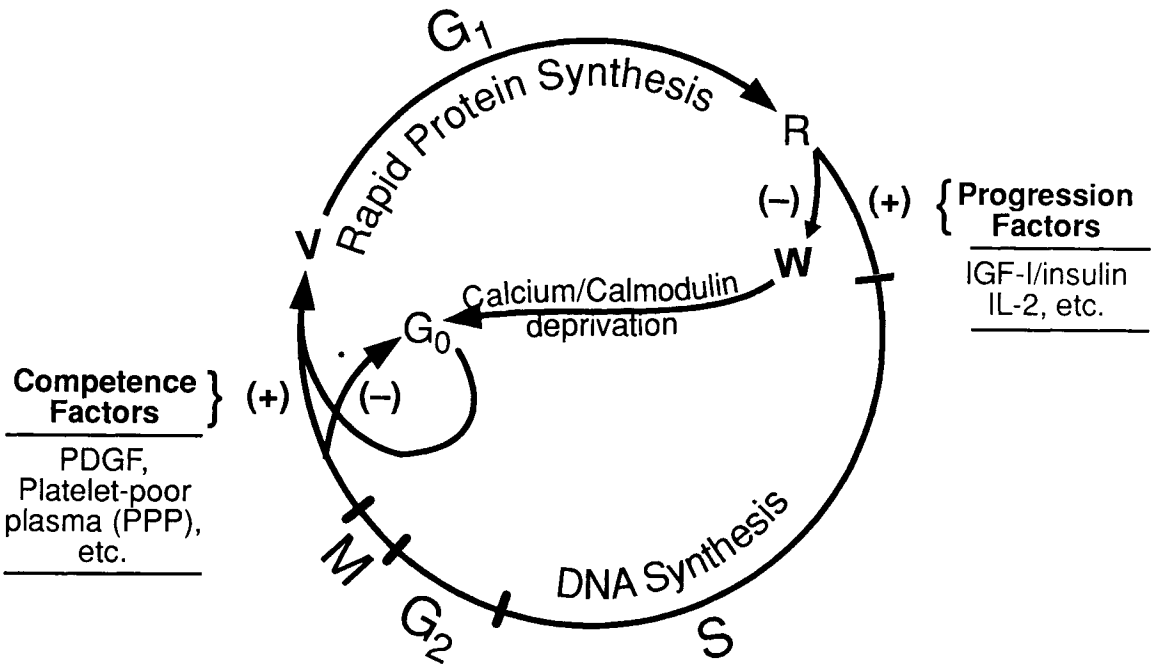

Fig. 1. Cell cycle model depicting growth factor-dependent control points in G_1 and the requirement of Ca^{++}/calmodulin for the transition of cells from G_1 to S.

scription, or protein synthesis [Rossow et al., 1979]. In the absence of IGF-I or insulin, cells in late G_1 reach the "W" point [Das, 1981; Pledger et al., 1978], and retain the ability to readily enter into S phase if IGF-I or insulin is provided within a short period; however, if these growth factors are not provided within 5–6 h they enter quiescence (G_0) [Campisi and Pardee, 1984]. Thus IGF-I action controls the terminal step for the onset of DNA synthesis [Olashaw et al., 1987].

METABOLIC EVENTS ASSOCIATED WITH THE PROGRESSION OF CELLS FROM G_1 TO S PHASE

Although the $G_1 \rightarrow S$ transition in mammalian cells is dependent primarily on the interaction between growth factors and their cognate receptors, the intracellular regulatory process(es) stemming from growth factor/receptor interaction on the membrane and culminating in the induction of nuclear DNA replication and cellular proliferation are not fully understood. As shown in Figure 2, the growth factor/receptor interaction is associated with the activation of membrane-bound phospholipase C, which catalyzes the conversion of phosphatidylinositol 4, 5-bisphosphate (PIP_2) to diacylglycerol (DG) and inositol 1, 4, 5 trisphosphate (IP_3). DG activates and redistributes phosphotidyl serine-dependent protein kinase C [Fleishman et al., 1986; Wolfman and Macara, 1987], which phosphorylates target proteins on serine and/or threonine residues. Target proteins include adenylate cyclase [Sibley et al., 1988]. IP_3 is known to release Ca^{++} from intracellular stores, leading to the activation of several proteins and enzymes, among them, a calcium-receptor protein, calmodulin (CaM). While the involvement of protein kinase C and cyclic nucleotides (cAMP and cGMP) in cellular proliferation is widely implicated [Whitfield et al., 1987; Persons et al., 1988], their causal relationship to and their specific mechanism of action in the growth factor-dependent transition of cells from G_1 to S phase are controversial [Besterman et al., 1986; Jacob and Cuatrecasas, 1986].

Cyclin-dependent protein kinases (CDKs) have emerged in recent years as key regulators of cell cycle progression both in yeast and in mamma-

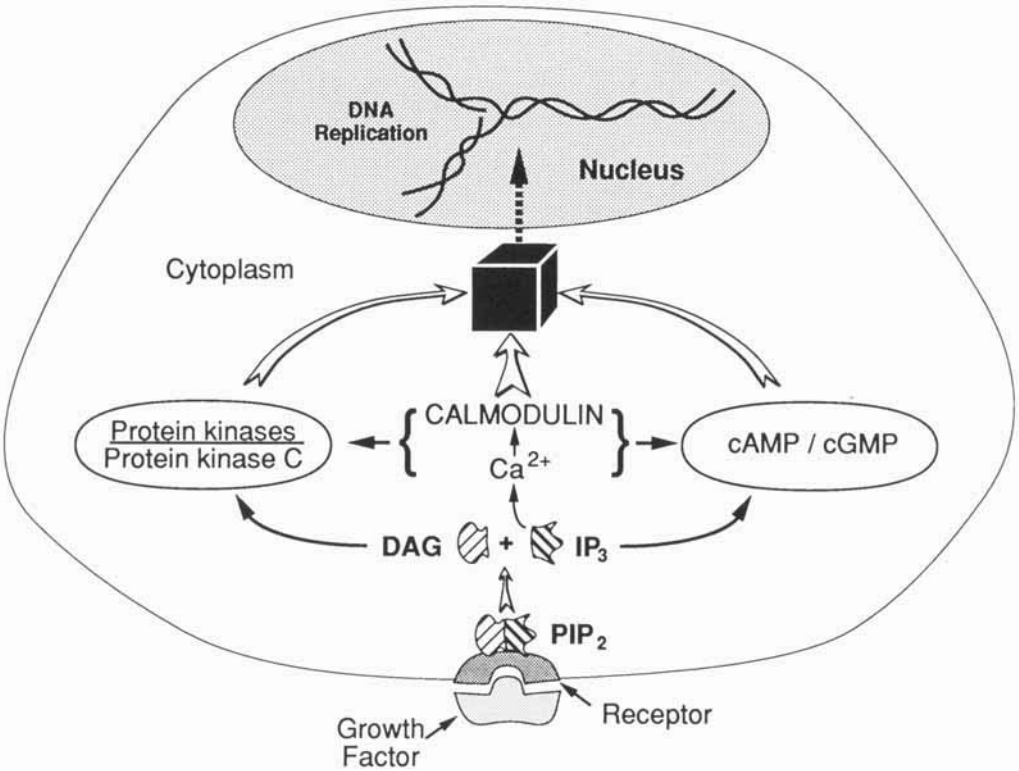

Fig. 2. An overview of signal transduction pathways stemming from growth factor/receptor interaction. The black box represents yet unknown final event(s) in the onset of DNA synthesis, landmark of cellular entry into S phase.

lian cells [Lee et al., 1988]. In addition to the changes in cyclin levels, as shown in Figure 3, the activation of CDKs and their interaction with cyclins are associated with the ability of cells to progress from one phase to the next [Pines and Hunter, 1990; Sherr, 1993]. Cyclin E, and perhaps D or A, in association with CDKs seem to function in the transition of cells from G_1 to S [Otsubo and Roberts, 1993; Sherr, 1993]. Furthermore, both cyclin E and A exhibit properties unique to the restriction protein whose increase to a critical level at R point in late G_1 period was suggested to induce the transition of cells from G_1 to S phase [Dou et al., 1993]. Based on the studies with $P34^{cdc2}$ in yeast, phosphorylation and dephosphorylation of specific tyrosine and threonine residues of CDKs are responsible for their activation and interaction with cyclins. In addition to growth factors that stimulate proliferation, there are other factors that inhibit cell growth of mammalian cells in mid- to late-G_1. One such growth inhibition factor, transforming growth factor-β (TGF-β), was shown to arrest the cell cycle in middle- to late-G_1 phase by limiting cyclin E interaction with CDK2 [Koff et al., 1993].

In yeast, formation of a cyclin B-cdc2 kinase complex associated with $G_2 \rightarrow M$ transition is regulated by a specific phosphatase responsible for tyrosine dephosphorylation of cdc2 kinase [Gautier et al., 1991], and, in *Aspergillus nidulous,* such phosphatase activity is suggested to be regulated by Ca^{++}/calmodulin (CaM) or specific calmodulin-binding proteins (CaM-BPs) [Lu and Means, 1993]. However, as indicated in Figure 3, it is not yet known which specific kinases and phosphatases and whether CaM or CaM-BPs are involved in activation of the CDKs responsible for $G_1 \rightarrow S$ transition of mammalian cells. Furthermore, the specific targets of activated CDKs that might determine the onset of DNA replication (S phase) remain elusive.

Another signal transduction pathway activated by growth factor/receptor interaction involves mitogen-activated protein (MAP) kinase. Some of the regulatory proteins phosphorylated by MAP kinases are also known to be phosphorylated by cyclin-dependent kinases [Davis, 1993; Izumi and Maller, 1991]. Since the EGF receptor is a major cell membrane substrate for MAP kinase [Takishima et al., 1991], it is possible that MAP kinase signal transduction pathway may play an important role in growth factor-dependent early- to mid-G_1 events.

A cascade of kinase and phosphatase reactions following growth factor/receptor interactions also result in the expression of some of the oncogenes, such as *c-fos, c-myc,* and *c-ras.* Expression of each of these gene products has been demonstrated to be critical for the progression of cells through G_1 and into S phase, and an overexpression or mutational changes in these

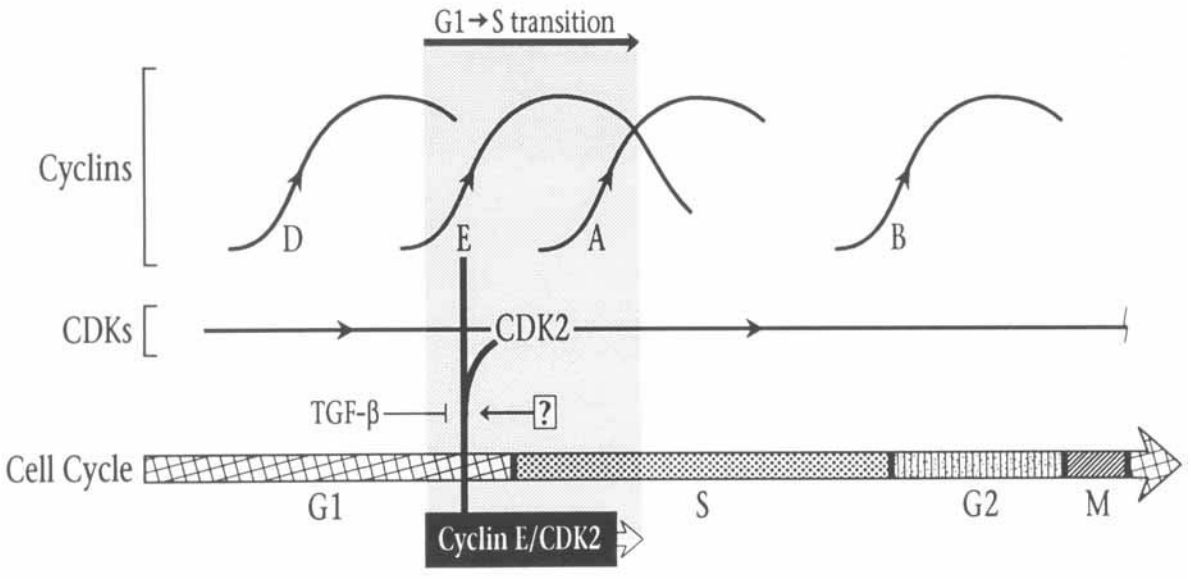

Fig. 3. A temporal relationship between approximate expression of cyclins and progression of cells through G_1, S, G_2, and M. The question mark indicates unknown regulators of interaction between cyclins and cyclin-dependent kinases in mammalian cells.

gene products was associated with uncontrolled proliferation characteristic of cancer cells. While *c-fos* and *c-myc* are expressed in response to PDGF and EGF in early-G_1, *c-ras* is expressed in response to IGF-I that triggers the transition of cells from G_1 to S [McCaffrey et al., 1987; Lu et al., 1989]. Introduction of antisense oligonucleotides or monoclonal antibodies specific to *c-ras* into cycling cells prevented them from entering into S phase [Mulcahy et al., 1985]. Although these observations point to the pivotal role for the oncogene products, such as that of *c-ras,* in cellular proliferation, the specific mechanism of their action in transition of cells from G_1 to S phase remains sketchy.

We now know from genetic studies that there are negative controls in mammalian cells that can counter the positive signals and block progression from G_1 to S [Goodrich et al., 1991; Weinberg, 1991; Ullrich et al., 1992]. These genes are called anti-oncogenes or tumor suppressor genes. Classical examples of such negative regulatory proteins are retinoblastoma gene product (pRb), a pRb-like protein known as p107, and p53. Lack of their expression or mutations in their genes result in uncontrolled proliferation of the cells. They seem to prevent the progression of cell cycle by binding to the transcription factors necessary for the expression of genes essential for DNA synthesis and cellular proliferation. Tumor suppressor proteins are underphosphorylated during their interaction with transcription factors in late-G_1 and hyperphosphorylated as cells enter S phase [Cooper and Whyle, 1989]. Phosphorylation of pRb and other tumor suppressor proteins by cyclin-dependent kinases seem to remove their inhibitory capacity, releasing the transcription factors and allowing their functional expression [Hinds et al., 1992; Lees et al., 1992].

ROLE OF CALCIUM AND CALMODULIN IN CELL PROLIFERATION

The progression of cells from G_1 to S is dependent on Ca^{++} [Means and Rasmussen, 1988]. As indicated in Figure 1, in the absence of Ca^{++}, cells at G_1/S boundary enter quiescence, as do those that are deprived of growth factor itself [Campisi and Pardee, 1984]. This would imply that growth factor action at G_1/S boundary is governed by Ca^{++}-regulated events. Lowering of the Ca^{++} levels in the medium, when mammalian cells have progressed to the G_1/S boundary, prevents initiation of DNA synthesis by stopping the expression and/or activation of ribonucleotide reductase, and the synthesis of four deoxynucleotides [Whitfield et al., 1982]. Furthermore, Ca^{++}-deprived cells seem to undergo dismantling of prereplicative structures and enter a state of quiescence [Whitfield et al., 1982]. Inositol 1, 3, 4, 5-tetrakisphosphate (IP_4), formed by phosphorylation of IP_3, has been demonstrated to stimulate the initiation of DNA synthesis by release of Ca^{++} [Hill et al., 1989]. A role for the Ca^{++} regulatory system in the control of cell cycle is also evident from the observation that the PSTAIR peptide (conserved 16 amino acid sequence of cyclin-dependent kinases from yeast and higher eukaryotes) triggers a specific increase in the concentration of intracellular free Ca^{++} by inducing its release from intracellular stores [Picard et al., 1990].

Since most of the effects of Ca^{++} are known to be mediated by the activation of calmodulin (CaM), changes in the intracellular CaM levels could also regulate Ca^{++}-medicated events. An integrated role of Ca^{++} and CaM in cellular proliferation is evident from the following observations: a) CaM levels are elevated 2 to 3 fold at the G_1/S boundary [Chafouleas et al., 1984; Rasmussen and Means, 1987]; b) there is a temporal coincidence between the increases in CaM levels and the progression of cells into S phase, regardless of the length of G_1 [Chafouleas et al., 1984]; c) there is a direct correlation between intracellular CaM levels and the ability of cells to replicate DNA [Chafouleas et al., 1987]; d) CaM stimulates DNA replication in isolated liver cells [Boynton et al., 1980]; e) progression of cells from G_1 to S phase and the expression of DNA polymerase-α activity during cell cycle are sensitive to CaM antagonists [Hidaka et al., 1981; Lopez-Girona et al., 1992]; f) there is a stimulating effect of Ca^{++} on DNA synthesis in rat hepatocytes, which is mimicked by the addition CaM and is blocked by CaM antagonists and anti-CaM antibody [Boynton et al., 1980; Jones et al., 1982]; and g) many solid tumors in liver and regenerating normal liver contain extremely high levels of CaM [MacManus et al., 1981]. Thus, CaM, serving as Ca^{++}-receptor, appears to play a central role in the control of cell proliferation.

In addition, the insulin receptor, which is known to stimulate transition of a variety of cells from G_1 to S phase [Pledger et al., 1978; Campisi and Pardee, 1984], contains a calmodulin binding domain, and CaM is shown to en-

hance insulin-mediated receptor kinase activity [Groves et al., 1985, 1986]. It was suggested that the Ca^{++} and CaM participate in early post-receptor events in the cellular mechanism of insulin action [Sacks and McDonald, 1988].

CALMODULIN-BINDING PROTEINS (CaM-BPs) IN CELL PROLIFERATION AND IN TRANSITION OF CELLS FROM G_1 TO S PHASE

Most of the observations discussed above, relating to the signalling mechanisms in transition of cells from G_1 to S phase, were made by monitoring the intracellular events that immediately followed growth factor/receptor interaction on cell membrane. However, in order to identify the signalling molecule(s) that link early events generated by growth factor/receptor interaction on the membrane to the ultimate nuclear events responsible for the onset of DNA replication (S phase), we took a "reverse approach." In this approach, we first identified metabolic events associated with the onset of DNA replication, characteristic of S phase, and then monitored their modulation in response to growth factor-dependent transition of cells from G_1 to S phase. We first observed that as the synchronized fibroblasts proceed from G_1 to S phase, several of the enzymes associated with DNA replication translocate into nuclei and assembled into a mega complex called "replitase" [Reddy and Pardee, 1980; for a review see Reddy and Fager, in press]. Consistent with the involvement of Ca^{++} and CaM in the transition of cells from G_1 to S, replitase complex assembled at G_1/S boundary is associated with CaM and a specific CaM-BP of 68 kDa [Subramanyam et al., 1990]. Similarly, specific CaM-BPs are also found to be associated with immuno-purified preparations of DNA polymerase-α from a variety of mammalian cells [Hammond et al., 1988]. An intracellular-Ca^{++}-dependent nuclear localization and nuclear matrix association of CaM was also observed during proliferative stimulation of rat liver cells [Serratosa et al., 1988; Pujol et al., 1989].

The significance of the interaction of CaM and specific CaM-BPs with the enzymes of DNA synthesis was investigated in the context of growth factor-dependent transition of cells from G_1 to S phase. It was observed that the nuclear localization and interaction with the enzymes of DNA replication of both CaM and the 68 kDa CaM-BP were associated with insulin-dependent progression of cells from G_1 to S phase [Subramanyam et al., 1990]. A similar growth factor-dependent modulation of the 68 kDa CaM-BP was observed in hematopoietic progenitor cells, particularly, as they transit from G_1 to S phase [Reddy et al., 1992a]. Furthermore, CaM-specific monoclonal antibodies inhibit DNA replication in permeabilized S phase cells [Reddy et al., 1992b] and purified 68 kDa CaM-BP stimulates DNA replication in permeabilized hematopoietic progenitor cells (Reddy et al., manuscript submitted). In toto, these observations point to a second messenger role for CaM and the 68 kDa CaM-BP in linking the signal generated by growth factor/receptor interaction on the membrane to the nuclear events in transition of cells from G_1 to S phase. These nuclear events include the assembly of a multienzyme complex for DNA synthesis and the onset of DNA replication.

PROSPECTIVE STUDIES

As indicated by the black boxes in Figures 2 and 3, although the cascade of events generating the second messengers and activating specific kinases stem from known growth factor/receptor interactions, the precise mechanism by which these initial events lead to the final specific biological response, i.e., DNA synthesis, remains elusive. Further characterization of the 68 kDa CaM-BP, which exhibits both growth factor-dependent modulation and an interaction with the enzymes of DNA replication, will help to bridge this gap in our knowledge. Such studies must also attempt to account for the relation to kinases, such as CDKs, and oncogene products, such as *c-ras,* whose expression and/or activation immediately precede the transition of cells from G_1 to S. It is possible that, as second messengers, the CaM and specific CaM-BPs associated with the enzymes of DNA synthesis could play an integrated role with CDKs and other regulatory proteins in determining the transition of cells from G_1 to S. Currently available data on the role of CaM and specific CaM-BPs in cellular proliferation, particularly at G_1/S boundary, strongly encourages a more extensive characterization of these proteins in order to gain in depth knowledge about signal transduction pathways leading to the growth factor-dependent transition of cells from G_1 to S.

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