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Inositol lipids and DNA replication

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Control of DNA synthesis by growth factors seems to depend upon the generation of intracellular mitogenic signals, which are responsible for initiating the sequence of events leading to the onset of DNA synthesis. Many growth factors have tyrosine kinase activity suggesting the proteins phosphorylated on tyrosine might be likely candidates as intracellular signals. Other candidates are the calcium and hydrogen ions whose concentrations change dramatically during the action of most growth factors, many of which also stimulate the hydrolysis of inositol lipids. In particular, certain growth factors stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate to give the two second messengers diacylglycerol and inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$). The former stimulates protein kinase C, which is responsible for increasing intracellular pH by switching on an $Na^+–H^+$ exchanger. The water-soluble $Ins1,4,5P_3$ released to the cytosol can be metabolized along two separate pathways: it can either be dephosphorylated to free inositol or it can be converted into additional inositol polyphosphates such as $Ins1,3,4,5P_4$ and $Ins1,3,4P_3$. These inositol phosphates seem to play a key role in regulating intracellular calcium, with $Ins1,4,5P_3$ functioning to release internal calcium, whereas $Ins1,3,4,5P_4$ may function to regulate the entry of external calcium.

There is evidence to suggest that these internal messengers may converge on certain key processes responsible for initiating the programme of cell growth. It is argued that an increase in intracellular calcium might be an important intracellular signal for activating both the transcription of a family of early genes, typified by *fos*, as well as the enzyme S6 kinase, which phosphorylates the ribosomal protein S6 which may regulate protein synthesis. The increase in pH seems to play a permissive role and may create the necessary ionic milieu for S6 phosphorylation and protein synthesis to occur. The onset of RNA and protein synthesis, which occur within the first few minutes after the arrival of a growth factor, represent the initial events of the programme of cell growth which culminates in DNA synthesis and cell division.

Introduction

A major challenge in cell biology is to understand how mitogenic signals arriving at the cell surface trigger the initiation of DNA synthesis, which culminates in cell division. Attempts to understand how cell growth is controlled is beset by both spatial and temporal problems that have obscured the nature of the mitogenic signal pathway. The temporal aspect concerns the fact that DNA synthesis occurs many hours after the mitogenic signal first arrives at the cell surface. As we shall see later, many growth factors trigger a whole host of early events which then set in motion an orderly sequence of biochemical reactions that finally trigger DNA replication. The problem is to uncover the way in which this orderly programme of growth unfolds during the G_1 period.

The spatial problem refers to the fact that although mitogenic signals bind to cell-surface receptors, they act ultimately to stimulate DNA replication within the nucleus.

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Somehow the information received at the periphery must be translated into internal signals, which act within the nucleus. The search is on for the identity of these internal signals. Some of the growth factor receptors have tyrosine kinase activity suggesting that they may phosphorylate key proteins which then function to initiate DNA synthesis. However, no such second messenger role has been ascribed to any of the protein substrates that are phosphorylated on tyrosine residues. Another action of many of these growth factors is to stimulate the hydrolysis of inositol lipids to generate a variety of messengers, some of which have been implicated in the control of cell growth.

Receptors that act through the inositol lipids also stimulate an increase in intracellular calcium and have thus been referred to as calcium-mobilizing receptors. There has been rapid development in our understanding of this receptor mechanism and here we examine its proposed role in transmitting mitogenic signals from the surface into the nucleus. The first indication that inositol lipids might play a role in mitogenesis was the discovery that phytohaemagglutinin (PHA) stimulated a rapid increase in the turnover of phosphatidylinositol (PtdIns) when added to human lymphocytes (Fisher & Mueller 1968). This initial observation was substantiated in several other tissues (Michell 1982) and the impression grew that certain mitogenic agents might use inositol lipids as part of a transduction mechanism for controlling cell growth.

Inositol lipid metabolism

The key feature of this receptor mechanism is that an inositol lipid located within the inner leaflet of the plasma membrane is hydrolysed to generate second messengers (Berridge 1984; Hokin 1985; Downes & Michell 1985). In comparison with other membrane lipids, PtdIns is unique in that its inositol headgroup is further phosphorylated to form the polyphosphoinositides. First, PtdIns is phosphorylated to phosphatidylinositol 4-phosphate (PtdIns4P) which, in turn, accepts another phosphate on the 5-position to give phosphatidylinositol 4,5-bisphosphate ($PtdIns4,5P_2$). It is the latter that is hydrolysed during signal transduction to generate the second messengers diacylglycerol (DG) and inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$). This hydrolysis is done by a membrane-bound $PtdIns4,5P_2$ phosphodiesterase (PDE), which cleaves the bond linking the hydrophilic headgroup ($Ins1,4,5P_3$) to the hydrophobic lipid component (DG) embedded within the bilayer. The activity of this phosphodiesterase is controlled not only by the occupation of an appropriate receptor but also on the presence of a GTP-binding protein (G_p), which serves as a coupling agent to relay information from the receptor to the PDE (Haslam & Davidson 1984; Cockroft & Gomperts 1985; Taylor & Merritt 1986).

Cell membranes have a family of G-proteins all involved in transducing information across the plasma membrane. The classic example is the stimulatory G-protein (G_s), which couples receptors to adenylate cyclase. Because the putative G-protein of the inositol lipid receptors seems to differ from that described previously, it has been abbreviated to G_p with the subscript referring to phospholipid (Cockroft & Gomperts 1985). In an unstimulated state, G_p is probably bound to GDP but once a receptor is occupied it undergoes a conformational change which is transmitted to G_p causing a loss of GDP and an enhanced affinity for GTP acting from within the cell. The arrival of GTP heralds the so-called on-reaction which has two major effects: it reduces the affinity of the receptor for its ligand while simultaneously activating the PDE to initiate the hydrolysis of $PtdIns4,5P_2$. The off-reaction occurs when this activated

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transduction process is terminated by the hydrolysis of GTP to GDP by the GTPase activity associated with G_p.

The identity of G_p remains to be determined but there are intriguing suggestions that it may be the product of the *ras* oncogene (Chiarugi *et al.* 1985; Fleischman *et al.* 1986; Wakelam *et al.* 1986). When NIH 3T3 cells were transformed with the N-*ras* proto-oncogene there was no change in the basal rate of inositol phosphate formation but the stimulatory effect of bombesin was enormously enhanced (Wakelam *et al.* 1986). If *ras* does turn out to be G_p, then it is easy to envisage how the activated *ras* oncogene, which has lost its GTPase activity, might lead to a continuous generation of second messengers independently of growth factors.

Figure 1 summarizes inositol lipid metabolism.

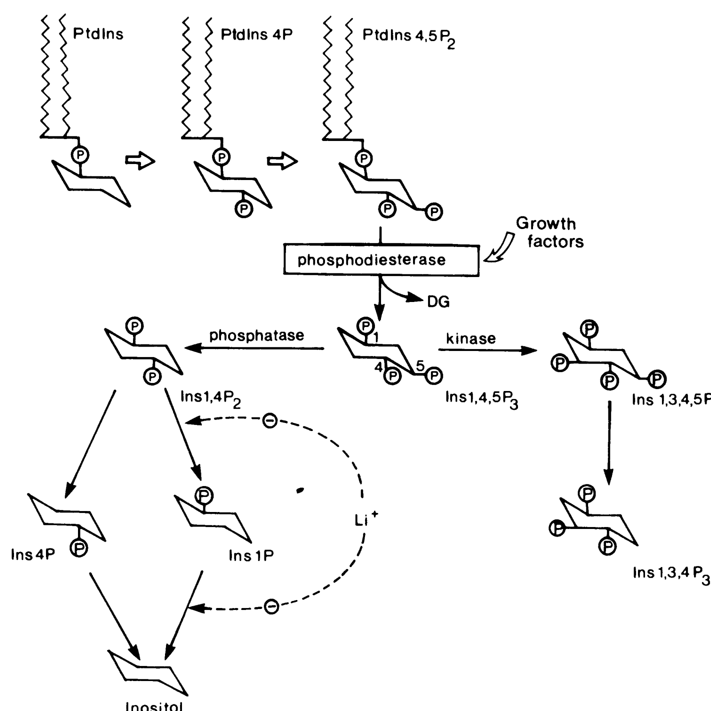

FIGURE 1. Summary of inositol lipid metabolism. Certain growth factors stimulate a phosphodiesterase to hydrolyse PtdIns4,5P_2 to Ins1,4,5P_3 and DG. The former can be metabolized via two pathways. In one pathway it is sequentially dephosphorylated to free inositol and the other pathway converts it into additional inositol polyphosphates. The proposed role of DG and the inositol polyphosphates is outlined in figure 2.

SECOND MESSENGER METABOLISM

Each of the initial second messengers (DG and Ins1,4,5P_3) formed by hydrolysing PtdIns4,5P_2 can be metabolized by two separate pathways. The DG can either be recycled back to reform PtdIns or it can be the substrate of the DG lipase that represents one of the pathways responsible for releasing the arachidonic acid, which is used to synthesize eicosanoids such as the prostaglandins and leukotrienes. Because some of these products, e.g. prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), are potent growth factors, the release of arachidonic acid by the inositol lipid receptor mechanism may play an important role in regulating cell growth through both autocrine and paracrine mechanisms.

The Ins1,4,5P_3 released from the membrane can also be metabolized along two separate

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pathways. Along one pathway it is sequentially dephosphorylated to free inositol which can be reused to synthesize PtdIns. The alternative pathway results in the generation of additional inositol polyphosphates. It begins with an inositol trisphosphate kinase, a soluble Mg^{2+}-requiring enzyme, which phosphorylates Ins1,4,5P_3 to inositol 1,3,4,5-tetrakisphosphate (Ins1,3,4,5P_4). This enzyme was originally described in *Xenopus* oocytes and rat brain (Irvine *et al.* 1986) but now appears to be ubiquitous because it has been described in many other cell types (Biden & Wollheim 1986; Hansen *et al.* 1986; Stewart *et al.* 1986; Hawkins *et al.* 1986). The significance of Ins1,3,4,5P_4, which was first discovered in experiments on cerebral cortical slices, is that it is the precursor of Ins1,3,4P_3 (Batty *et al.* 1985), the new inositol trisphosphate isomer found in most cells (Irvine *et al.* 1984; Burgess *et al.* 1985). As these new inositol polyphosphates (Ins1,3,4,5P_4 and Ins1,3,4P_3) might function as second messengers (as discussed later), it becomes of some interest to determine whether there is any control over which way Ins1,4,5P_3 flows down its two alternative metabolic pathways. The dephosphorylation pathway is unlikely to yield additional messengers, whereas the phosphorylation pathway may yield other important signal molecules. It is of some interest, therefore, to find that an increase in the intracellular level of calcium appears to favour the metabolism of Ins1,4,5P_3 along the phosphorylation pathway, resulting in an enhanced formation of Ins1,3,4,5P_4 and Ins1,3,4P_3 (Biden & Wollheim 1986; Lew *et al.* 1986).

Second messenger action

The second messengers spawned by this inositol lipid receptor mechanism have a variety of effects within the cell. The DG functions to activate protein kinase C with both calcium and phosphatidylserine (PS) functioning as cofactors in the stimulatory process (Nishizuka 1984, 1986). As part of this activation process there appears to be a physical translocation of the enzyme from the cytosol into the membrane. The increase in intracellular calcium may be responsible for this translocation, which then serves to prime the enzyme to the stimulatory effect of DG (Wolf *et al.* 1985). Once inserted into the membrane, protein kinase C becomes susceptible to calcium-activated proteases that cleave the enzyme to release a 50 kDa fragment (Kishimoto *et al.* 1983). This proteolytic cleavage is thought not to be of physiological relevance but part of a degradation process. However, the 50 kDa fragment is constitutively active even in the absence of DG and calcium, and could thus function in cellular control assuming sufficient quantities are formed *in vivo*. Guy *et al.* (1986) claim that the 50 kDa fragment is formed during mitogenic stimulation of B-lymphocytes. An important observation with regard to the proposed role of this receptor mechanism in the control of cell growth was the observation that tumor-promoting phorbol esters are potent activators of protein kinase C (Castagna *et al.* 1982). Indeed, the putative cellular receptors for the phorbol esters turn out to be protein kinase C (Ashendel 1985).

Most previous models have considered that protein kinase C acts at the plasma membrane where DG is formed, but it is feasible that this enzyme might also act within the nucleus. On the basis of sequence information derived from cloning the protein kinase C gene (Knopf *et al.* 1986; Parker *et al.* 1986), it is apparent that the N-terminal region of the molecule contains a number of cysteine residues organized into an internal repeat of 50 amino acids. Each repeat has a set structure of $-C-X_2-C-X_{13}-C-X_2-C-$, which is very similar to the arrangement of the DNA-binding loops described in the protein transcription factor IIIA of *Xenopus* oocytes

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(Miller *et al.* 1985). There is no direct evidence yet that protein kinase C is bound to DNA, or how it might be activated within the nucleus. One possibility is that after proteolytic cleavage of protein kinase C at the plasma membrane, the N-terminal fragment containing the DNA-binding domains might migrate into the nucleus to serve some regulatory role. *In vitro* experiments have already shown that protein kinase C can phosphorylate DNA topoisomerase II resulting in a marked increase in its enzymic activity (Sahyoun *et al.* 1986).

The water-soluble Ins1,4,$5P_3$ released to the cytosol functions as a second messenger to mobilize calcium from the endoplasmic reticulum (ER) (Berridge & Irvine 1984). Calcium is constantly cycling across the ER membrane with Ins1,4,$5P_3$ acting to stimulate the passive efflux component without altering the calcium pump. The most likely model is that there is a calcium channel within the ER membrane, which is opened when Ins1,4,$5P_3$ binds to its putative receptor. In addition to promoting the release of internal calcium, this inositol lipid mechanism can also bring about an entry of external calcium. Just how the calcium permeability of the plasma membrane is enhanced is somewhat of a mystery but there are suggestions that the new inositol polyphosphates, particularly Ins1,3,4,$5P_4$, might play a role (Hansen *et al.* 1986; Irvine & Moor 1986). The Ins1,4,$5P_3$ formed first would act initially to mobilize internal calcium whereas the entry of external calcium would occur later after the conversion of Ins1,4,$5P_3$ to Ins1,3,4,$5P_4$.

Second messengers and control of macromolecular synthesis

At present we have very little idea of how growth factors acting during the protracted G_1 period finally initiate DNA replication at the beginning of the S-phase. Growth factors set in motion a genetic programme for growth which gradually unfolds during G_1. Two early components of this programme are a rapid induction of mRNA transcription occurring within minutes of growth factor addition, with an increase in protein synthesis. The latter must continue throughout the G_1 period before reinitiation of DNA synthesis can occur (Pardee *et al.* 1978). These macromolecular events are part of the growth programme because inhibition of either RNA transcription or protein synthesis prevents growth.

Within minutes of adding a growth factor to quiescent Balb/c3T3 cells there is a rapid increase in the appearance of new mRNA species, which reach a peak in 40–90 min and then decline (Lau & Nathans 1985). There is some temporal variation between the different species but the overall impression is that a specific set of genes are being activated for a relatively short period early in G_1, perhaps by the same internal signals. The identity of some of these early genes is known, and include actin (Riddle *et al.* 1979; Elder *et al.* 1984), *myc* (Kelly *et al.* 1983) and *fos* (Greenberg & Ziff 1984; Kruijer *et al.* 1984; Muller *et al.* 1984). The *fos* gene is of particular interest because it can induce cellular transformation when introduced by retroviruses, suggesting that it may have a key role in controlling cell growth. Further evidence has come from studies showing that a c-*fos* antisense RNA inhibits the proliferation of 3T3 cells (Holt *et al.* 1986). I shall thus use *fos* as a reference gene to identify how cell surface receptors might act to stimulate these early transcriptional events.

Another event occurring early in G_1 is the phosphorylation of ribosomal protein S6, an integral component of the 40*S* ribosomal subunit. This S6 subunit is located close to the RNA binding site and its phosphorylation seems to enhance the rate of protein synthesis by increasing the affinity of the 40*S* ribosome for mRNA and may enhance the preferential

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translation of the early growth associated mRNA transcripts discussed earlier (Duncan & McConkey 1982; Glover 1982; Thomas *et al.* 1982). The phosphorylation of S6 is done by a specific S6 kinase which thus provides another marker to assess what second messengers are being generated by growth factors during early G_1.

Studies on a variety of cell types has revealed that there are numerous similarities in the types of growth factors and pharmacological agents capable of activating both *fos* and S6 kinase (table 1). One way of approaching this problem is to identify whether there is any common

Table 1. Summary of the stimuli that activate transcription of the proto-oncogene c-*fos* and phosphorylation of the ribosomal protein S6

stimulus	*fos*	S6 phosphorylation
PDGF	+[1,2,3,4]	+[8–10]
EGF	+[2,3,5,6]	+[10,11]
Con A	+[7]	?
insulin	0[4]	partial[11]
phorbol ester	+[2–7]	+[10–12]
A23187	+[5–7]	+[13]
src	?	+[12]

References: 1, Cochran *et al.* (1984); 2, Kruijer *et al.* (1984); 3, Muller *et al.* (1984); 4, Greenberg & Ziff (1984); 5, Bravo *et al.* (1985); 6, Milbrandt (1986); 7, Moore *et al.* (1986); 8, Wettenhall *et al.* (1983); 9, Chambard *et al.* (1983); 10, Thomas (1986); 11, Martin-Perez *et al.* (1984); 12, Blenis *et al.* (1984); 13, I. Novak (personal communication).

theme to the way in which these various agents act to identify which second messenger pathways are being employed. The fact that phorbol esters (TPA and PMA) and calcium ionophores can stimulate these macromolecular events suggests a role for the inositol lipid pathway that would thus account for the action of PDGF. Addition of PDGF to cultured cells stimulates the hydrolysis of PtdIns4,5P_2 resulting in the formation of Ins1,4,5P_3 and DG (Habenicht *et al.* 1981; Berridge *et al.* 1984; Hasegawa-Sasaki 1985). These two second messengers are responsible for initiating the early ionic signals, an increase in calcium and pH, which appear to be important in triggering ionic responses (Berridge 1984; Rozengurt 1986). During the action of PDGF, there is a large increase in the level of intracellular calcium (Moolenaar *et al.* 1984; Hasegawa-Sasaki 1985; McNeil *et al.* 1985) much of which is derived initially by release of calcium from internal reservoirs which can lose as much as 50% of their stored calcium (Frantz 1985). Insulin, EGF and phorbol esters cause no such depletion, which occurs only in response to those growth factors capable of generating the Ins1,4,5P_3 that mobilizes intracellular calcium. However, EGF is known to elevate the intracellular level of calcium by promoting the entry of external calcium (Hesketh *et al.* 1985). A characteristic of many of the stimuli shown in table 1, therefore, is to increase the intracellular level of calcium. A particularly clear example is the induction of *fos* transcription in PC 12 cells by agents or treatment which open voltage-dependent calcium channels (Greenberg *et al.* 1986; Morgan & Curran 1986). Stimulation with calcium ionophores (table 1) also suggests that calcium is likely to be one of the intracellular signals responsible for regulating both *fos* transcription and S6 phosphorylation (figure 2).

Because both events are also activated by phorbol esters (table 1), we are left to consider the role of protein kinase C. One important action of this kinase is to stimulate the Na^+–H^+

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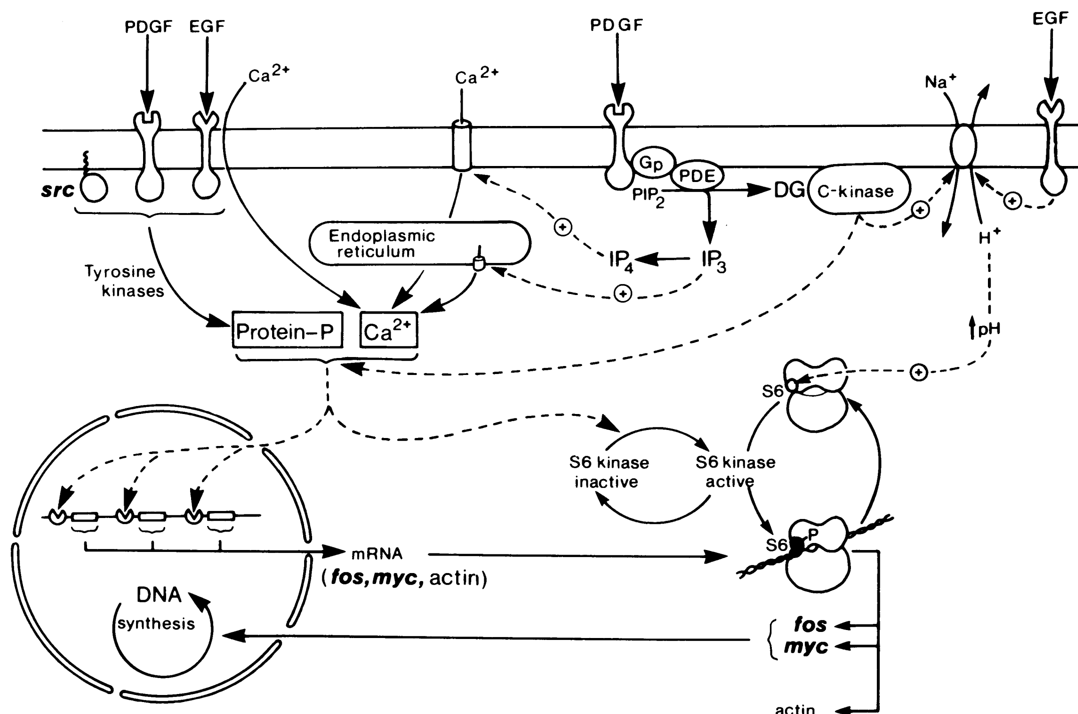

FIGURE 2. Proposed role of mitogenic signals derived from the cell membrane in the control of RNA transcription and protein synthesis. There appear to be two major types of internal messenger, phosphorylated proteins derived from protein kinases such as protein kinase C (C-kinase) and the tyrosine kinases associated with receptors and oncogenes (e.g. *src*) and the inorganic ions (calcium and hydrogen). Stimulation of inositol lipid hydrolysis plays a major role in generating these ionic signals. These internal mitogenic signals are then responsible for activating RNA transcription and S6 kinase. The latter phosphorylates the ribosomal subunit S6, which stimulates protein synthesis and may enhance the translation of the early gene products that initiate the growth control programme that culminates in DNA synthesis.

exchanger to bring about the increase in cytoplasmic pH, which is a key event for the onset of DNA synthesis (Rozengurt 1986). Because the induction of *fos* can occur independently of this increase in cytoplasmic pH (Bravo & McDonald-Bravo 1986; Moore *et al.* 1986), the latter is unlikely to regulate gene transcription and has been linked more with the onset of S6 phosphorylation (figure 2). Increasing pH by itself did not increase the level of S6 phosphorylation, which depended on some other aspect of receptor activation (Chambard & Pouyssegur 1986). Even though the enzyme can be activated at low pH values, it is incapable of phosphorylating S6 (I. Novak, personal communication). The increase in pH, therefore, does not act through the S6 kinase directly but instead it may alter the conformation of the substrate S6 making it more susceptible to phosphorylation (I. Novak, personal communication).

How else might protein kinase C act? One possibility is that it acts indirectly to enhance the primary stimulatory pathways based on either calcium or tyrosine phosphorylation. For example, protein kinase C may act to increase the sensitivity of various calcium responses such that they begin to respond even at resting levels of calcium. Such a modulatory role would help to explain how cell growth can be induced even when protein kinase C has been down-regulated. Prolonged phorbol ester treatment results in a down-regulation of protein kinase C such that there is no DNA synthesis after further addition of PMA whereas the mitogenic action

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of growth factors such as PDGF and FGF are unaffected (Coughlin *et al.* 1985; Tsuda *et al.* 1985; Olashaw *et al.* 1986). Because growth factors can stimulate DNA synthesis independently of protein kinase C, the latter is unlikely to be part of the primary mitogenic pathway, which again stresses the possible significance of either the Ins1,4,5P_3/calcium pathway or tyrosine phosphorylation. Although both PDGF and EGF can act by raising intracellular calcium, albeit via different mechanisms, they also have tyrosine kinase activity which must be considered as another possible mechanism for triggering growth (figure 2). Isolation of S6 from stimulated cells reveals that all the phosphate is on serine residues, which indicates that the tyrosine phosphorylation cascade must act indirectly by first of all stimulating the S6 kinase (figure 2).

The existence of such a tyrosine phosphorylation cascade may help to explain how transformation by v-*src* can bring about an increase in S6 phosphorylation (Blenis & Erikson 1986). In the case of *src*, however, we are faced with an ambiguity because, like EGF and PDGF, it may also be able to alter the intracellular level of calcium. Cells transformed with v-*src* have an enhanced inositol lipid turnover (Sugimoto *et al.* 1984). Cells transformed with polyoma virus also have elevated levels of Ins1,4,5P_3 thought to arise through the interaction of middle T with $pp60^{c\text{-}src}$ (Kaplan *et al.* 1986). Fry *et al.* (1985) described an increase in inositol lipid turnover that follows transformation of cells with the *abl* oncogene, which also has tyrosine kinase activity. It is likely, therefore, that the *src* and *abl* oncogenes might bring about an increase in intracellular calcium through an elevation in inositol lipid hydrolysis. Therefore the action of these oncogenes is still uncertain, whereas they may function through tyrosine phosphorylation there is indirect evidence that they could also act by increasing intracellular calcium.

The next question to consider is how these putative mitogenic signals stimulate transcription of genes such as *fos*? The family of early genes that respond to growth factors can also be switched on by cycloheximide, suggesting that they may normally be regulated by an unstable repressor that is turning over rapidly. Just where such a repressor might act is still unclear. Recent studies have identified a sequence within the 5′-flanking region of c-*fos* that is characteristic of a typical enhancer element and contains a region of hyphenated dyad symmetry (Treisman 1985). Nuclear extracts from HeLa cells contain a protein(s) that binds specifically to this 5′ promotor region and thus may play a role in regulating transcription in response to growth factors (Treisman 1986). Treisman (1986) has referred to this activating protein as SRF (Serum Response Factor). At present, it is possible only to speculate how the messenger pathways discussed earlier will interact with elements such as SRF. Activity of the latter might be modulated by protein kinase C (Treisman 1986). Alternatively, calcium may act through its intracellular receptor calmodulin to stimulate a protein kinase to phosphorylate SRF (Morgan & Curran 1986). SRF could also represent the site of action of the tyrosine phosphorylation cascade. If these different signal pathways converge on a common substrate, then one might expect it to be phosphorylated on both tyrosine and serine residues. It is of some interest, therefore, that after addition of mitogens there is a rapid phosphorylation of a minor cytoplasmic protein on both tyrosine and serine (Cooper & Hunter 1985). Similarly, the p36 protein, which is a substrate for tyrosine kinases, is also a substrate for protein kinase C (Gould *et al.* 1986). Such experiments clearly reveal that individual protein substrates can be phosphorylated by separate protein kinases and may represent the point of convergence for the mitogenic signal pathways. The S6 kinase may represent another focal point where the different signal pathways converge to control protein synthesis (figure 2).

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A key question for the future, therefore, is to identify these sites of convergence. So far, I have considered the tyrosine phosphorylation cascade and the calcium pathway converging on the final effectors but another possibility is that the tyrosine kinase pathway may converge earlier onto the calcium pathway by phosphorylating calmodulin. In Rous sarcoma virus-transformed cells, which are known to have elevated S6 phosphorylation levels, there was an increase in the phosphorylation of calmodulin on tyrosine residues (Fukami *et al.* 1986). Another example is protein p36, which appears to alter its calcium-binding properties after tyrosine phosphorylation (Soric & Gordon 1986). The drug cyclosporine, which has been used as an immunosuppressant, may provide a mechanism for distinguishing between these signal pathways. In lymphocytes, cyclosporine inhibits those growth factors that operate through calcium while having no effect on the action of phorbol esters or lipopolysaccharides, which do not depend on an elevation in intracellular calcium (Bijsterbosch & Klaus 1985). The fact that cyclosporine can bind to calmodulin (Colombani *et al.* 1985) is consistent with calcium being one of these signal pathways. Whatever the final pathway turns out to be, it must at some stage modify a protein that functions like an SRF to bind to the various promotor regions to trigger the transcription of a family of early genes simultaneously. The same intracellular signals may also be responsible for activating the kinase that phosphorylates S6 (figure 2). These concerted increases in RNA and protein synthesis are the first steps in the growth programme that culminates in DNA synthesis.

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