The Cell Cycle, Signal Transduction, and Immunophilin–Ligand Complexes

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Why are scientists without formal training in immunology turning their attention to this field? Certainly, immunology has led to the development of powerful research tools that are utilized across departmental boundaries and require little knowledge of the field itself. Antibodies have had a significant impact in medicine (therapeutics, diagnostics) and basic research (protein detection/purification, enzymology). However, immunology is a vast study that extends far beyond these advances. Immunology is the study of a multicellular system designed to eliminate foreign invaders.[1] The immune system accomplishes this task by processes that cross many disciplines: chemistry and molecular recognition (antibody/antigen, MHC/TCR), genetics (somatic recombination/mutation), molecular biology (transcription of lymphokine, MHC, immunoglobin genes), and cell biology (lymphocyte differentiation/activation). Discoveries in these areas yield new meaning and relevance not only to immunology but to neighboring disciplines as well.

The cells of the immune system are first faced with the significant challenge of recognizing foreign entities. These cells then undertake their other fundamental task, destroying the invaders. In order to accomplish this latter task, a sophisticated response occurs involving the growth and proliferation of cells that recognize and destroy the foreign intruder. A component of this process will be the subject of this Account: the molecular details of the signals that instruct a cell to grow and divide.

Many studies of signal transduction, the process by which an extracellular molecule influences intracellular events, have involved cells of the immune system.[1] For example, the molecular events associated with signaling pathways in T lymphocytes have been illuminated with remarkable clarity during the past several years. As immunologists and cell biologists have now identified many of the molecules that regulate cell function, scientists with chemical training are well-positioned to achieve the next level of understanding. Chemistry is now able to address a level of complexity that extends beyond the systems studied in classical biochemistry. Thus, the living cell poses a great challenge for biological and organic chemists in the future. How can chemists uniquely approach this problem?

Natural products provide chemists with powerful tools to study molecular aspects of cellular function.[2] Many natural products interfere with specific cellular processes, including growth and division. By studying how the natural products interfere with these processes, the molecular details of the processes themselves can be illuminated. Why haven't cell biologists more regularly adopted this approach? The primary reason is that natural products-based studies of cellular function invariably require the methods of synthetic chemistry, with which most biologists are unfamiliar. On the other hand, cell biologists have other powerful tools at their disposal that are unfamiliar to many chemists.[3] This Account will focus on a natural products-based study of signal transduction pathways that regulate early events in the cell cycle. We hope to illustrate that the study of natural products together with one simple technique from cell biology, flow cytometry, is a powerful combination for unraveling some of the mysteries of complex cellular processes.

The Cell Cycle. The immune response consists of two arms: a humoral response consisting predominantly of antibodies and a cellular response consisting predominantly of killer T lymphocytes. The helper T lymphocyte orchestrates both responses by secreting proteins that stimulate antibody production from B lymphocytes and promote proliferation of the B lymphocytes, killer T lymphocytes, and helper T lymphocytes that recognize the foreign invader. Thus, an important component of the immune response is the regulation of cellular proliferation, i.e., regulation of the cell cycle. The cell cycle is the universal program carried out by cells in order to divide.[4] As diagramed in Figure 1, it has been divided into several phases defined by the state of the genome.

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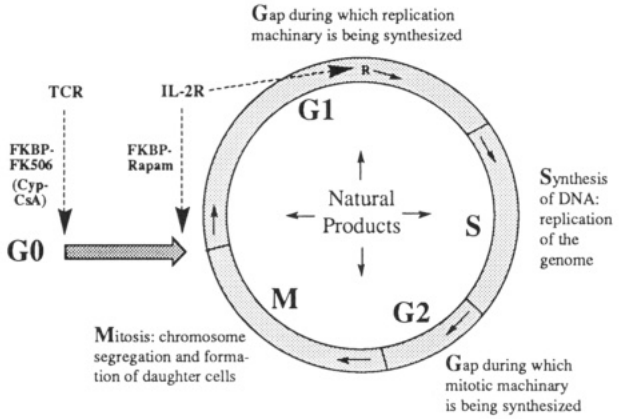

Figure 1. Natural products can be used to study the signaling pathways that drive the cell cycle.

Most cells in the body, such as the T lymphocytes of the immune system, are in a resting, nonproliferating state G0 (Figure 1). Cells are activated to enter the cell cycle by responding to specific extracellular stimuli. Following activation of a T lymphocyte through the T cell receptor (TCR) by an antigen presenting cell, a Ca^{2+}-dependent signal is transmitted through the cytoplasm and into the nucleus, where the interleukin-2 (IL-2) gene is transcribed. An essential role for Ca^{2+} has long been appreciated (activation can in part be achieved with the Ca^{2+} ionophore ionomycin); however, its function was unknown. When IL-2 is synthesized and secreted, it can bind to its own receptor (the IL-2R) on the surface of the same cell (an autocrine action). The IL-2R-mediated signaling pathway is much more complex than the earlier TCR pathway. For example, receptor occupancy is required for several hours in order for the growth factor IL-2 to drive the cell past a "point of no return", called the restriction (R) point,[5] in the G1 phase (the first *g*ap phase) of the cell cycle. Once this step has been passed, the cell will continue through the cell cycle independent of external signals. Although a molecular characterization has not been forthcoming, evidence indicates that passage through this regulatory point involves the accumulation of one or more labile proteins, which will be discussed later. In G1 the cytoplasmic and nuclear factors required for chromosome replication are synthesized. Chromosomes are replicated in S phase (the phase where DNA is *s*ynthesized). A second gap phase, G2, is followed by mitosis (M phase), which leads eventually to the production of two daughter cells.

Natural Products and Cell Cycle Signaling Pathways. Understanding the etiology of proliferative disorders such as cancer requires a definition of the molecular events that drive the cell cycle. Immunologists have turned to the study of T cell activation and proliferation as these processes are essential to the immune response and to autoimmune and other immune disorders. Several years ago, we initiated studies of three immunosuppressants—cyclosporin A (CsA), FK506, and rapamycin—that block T cell activation. Figure 2 summarizes mechanistic studies of the immunosuppressants, which uncovered a number of surprising findings.[6] Despite their different structures,

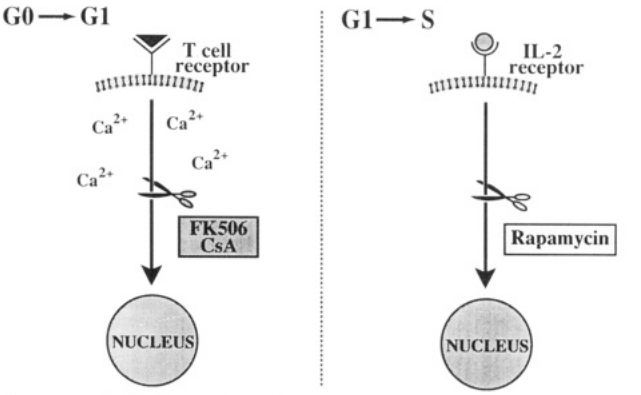

Figure 2. Ca^{2+}-dependent, CsA- and FK506-sensitive and Ca^{2+}-independent, rapamycin-sensitive signaling pathways.

FK506 and CsA were shown to block the same step in a family of Ca^{2+}-dependent signaling pathways, including the pathway emanating from the TCR. Despite its similarity in structure to FK506, rapamycin does not interfere with FK506- and CsA-sensitive signaling pathways. Instead, it blocks a family of Ca^{2+}-independent signaling pathways that emanate from growth factor receptors, including the IL-2R. Another surprising result was that FK506 and rapamycin interfere with the two pathways by first binding to the same intracellular receptor, the immunophilin FKBP12.

Studies of the nonnatural immunophilin ligand 506BD have shed light on how a single protein mediates the actions of two natural products on two distinct pathways (Figure 3).[7] 506BD was designed to have the structural elements shared by FK506 and rapamycin. Since FKBP12 was shown to bind to both of these compounds with high affinity, it seemed reasonable that these common structural elements were contacting the mutual receptor. Although 506BD also binds to FKBP12 with high affinity, it does not block FK506- and rapamycin-sensitive signaling pathways. It does, however, block the actions of both FK506 and rapamycin. Thus, it seemed that the binding of FK506 and rapamycin to FKBP12 was necessary, but not sufficient, for signaling inhibition. Furthermore, it seemed that the FKBP12–FK506 complex was interacting with cell cycle machinery necessary for activation of resting cells (the G0–G1 transition), whereas the FKBP12–rapamycin complex was interacting with cell cycle machinery necessary for progression through the G1 phase of the cell cycle (the G1–S transition). CsA, which binds to a second family of immunophilins called cyclophilins, appeared to contact the same target of the FKBP12–FK506 complex.

The molecular basis for this gain in immunophilin function that follows CsA and FK506 binding was revealed by affinity experiments using tissue and cell lysates. These experiments revealed that cyclophilin A–CsA and FKBP–FK506 complexes bind to the Ca^{2+}-regulated protein phosphatase calcineurin.[8] The specificity of these interactions and requirement for prior immunophilin–immunosuppressant association are evident in the data shown in Figure 4. Neither FKBP12, FK506, rapamycin, cyclophilin A, or CsA alone, nor the FKBP12–rapamycin and FKBP12–506BD com-

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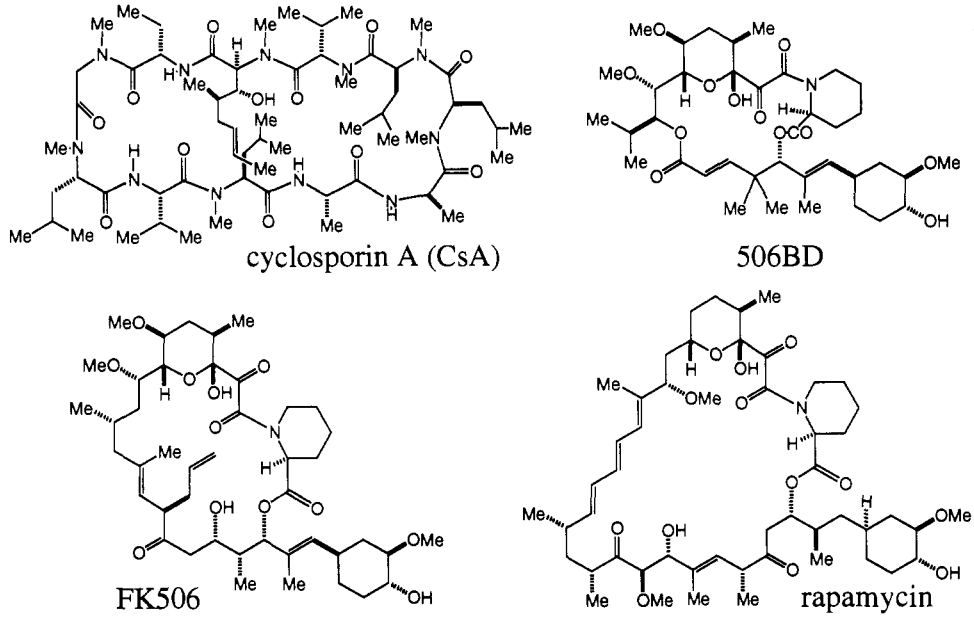

Figure 3. Structures of immunophilin ligands.

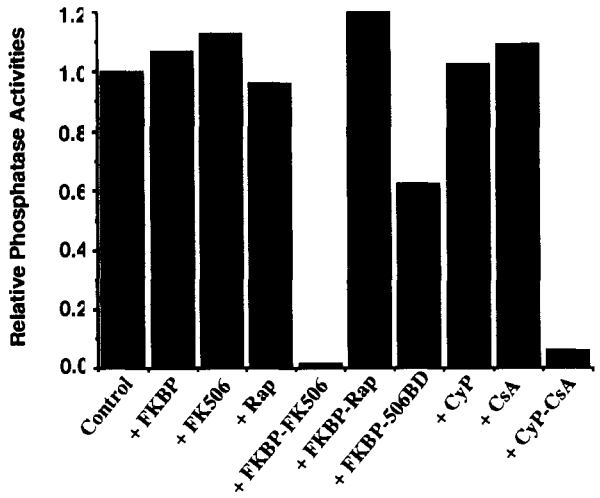

Figure 4. Immunophilin–drug complexes inhibit calcineurin phosphatase activity.[8] The phosphatase activity of calcineurin toward a ^{32}P-labeled phosphoserine-containing peptide under specified conditions is shown (normalized to 1) in the control column. The effects of a 5-fold addition of different additives are seen in columns to the right.

plexes have a significant effect on the phosphatase activity of calcineurin. In contrast, both cyclophilin A–CsA and FKBP12–FK506 are potent inhibitors of calcineurin's phosphatase activity.

The cellular relevance of these interactions was illustrated in studies that correlated the ability of CsA and FK506 analogs to bind to cyclophilin A or FKBP12, to inhibit TCR-mediated signal transduction, and, as their immunophilin complexes, to inhibit the Ca^{2+}-regulated protein phosphatase calcineurin (Figures 5 and 6).[9] Thus, these studies revealed the elusive role of Ca^{2+} in the TCR signaling pathway (for more discussion, see below).

Structural studies of immunophilins and immunophilin–ligand complexes have helped to explain the unusual properties of the illustrated immunosuppressant analogs.[10] CsA, FK506, and rapamycin can be viewed as "molecular glue" that mediates the interactions of immunophilins and calcineurin, since immunophilins and calcineurin do not bind in the absence of the natural products. In their presence, receptor–ligand–receptor complexes form with high affinity. Structural modifications of CsA and FK506 were identified (Figure 7A,B) that have little effect on immunophilin binding, yet abrogate both calcineurin binding and the ability of the analogs to inhibit signal transduction. FKBP12–rapamycin (Figure 7C) doesn't bind to calcineurin since the rapamycin component of the complex is not suitably configured to bind to the immunophilin–ligand binding site on calcineurin. (The FKBP12 components of the two FKBP12 complexes (Figure 7B,C) are nearly identical.) That a composite surface comprising both immunophilin and immunosuppressant residues is necessary for binding has been most clearly revealed in studies of the FKBP12–FK506 complex. Mutagenesis of FKBP12 led to several mutant FKBPs that, although able to bind FK506 with high affinity, were not able to bind to calcineurin as their FK506 complexes (Figure 7D).[11,12] Two residues on FKBP12, glycine 89 and isoleucine 90, were identified as being essential to mediate the high-affinity interaction with calcineurin. The FKBP13–FK506 complex, which binds weakly to calcineurin (K_i = 1.5 μM), can be transformed into a high-affinity ligand for calcineurin (K_i = 13 nM) by changing its corresponding residues to the two residues present in FKBP12.[13]

T Cell Receptor Signaling. The discoveries resulting from studies of FK506 and CsA can now be integrated with other findings in TCR signaling. In Figure 8 we present a detailed, albeit speculative, sequence of molecular events that constitute the TCR signaling pathway. There are admittedly gaps in our

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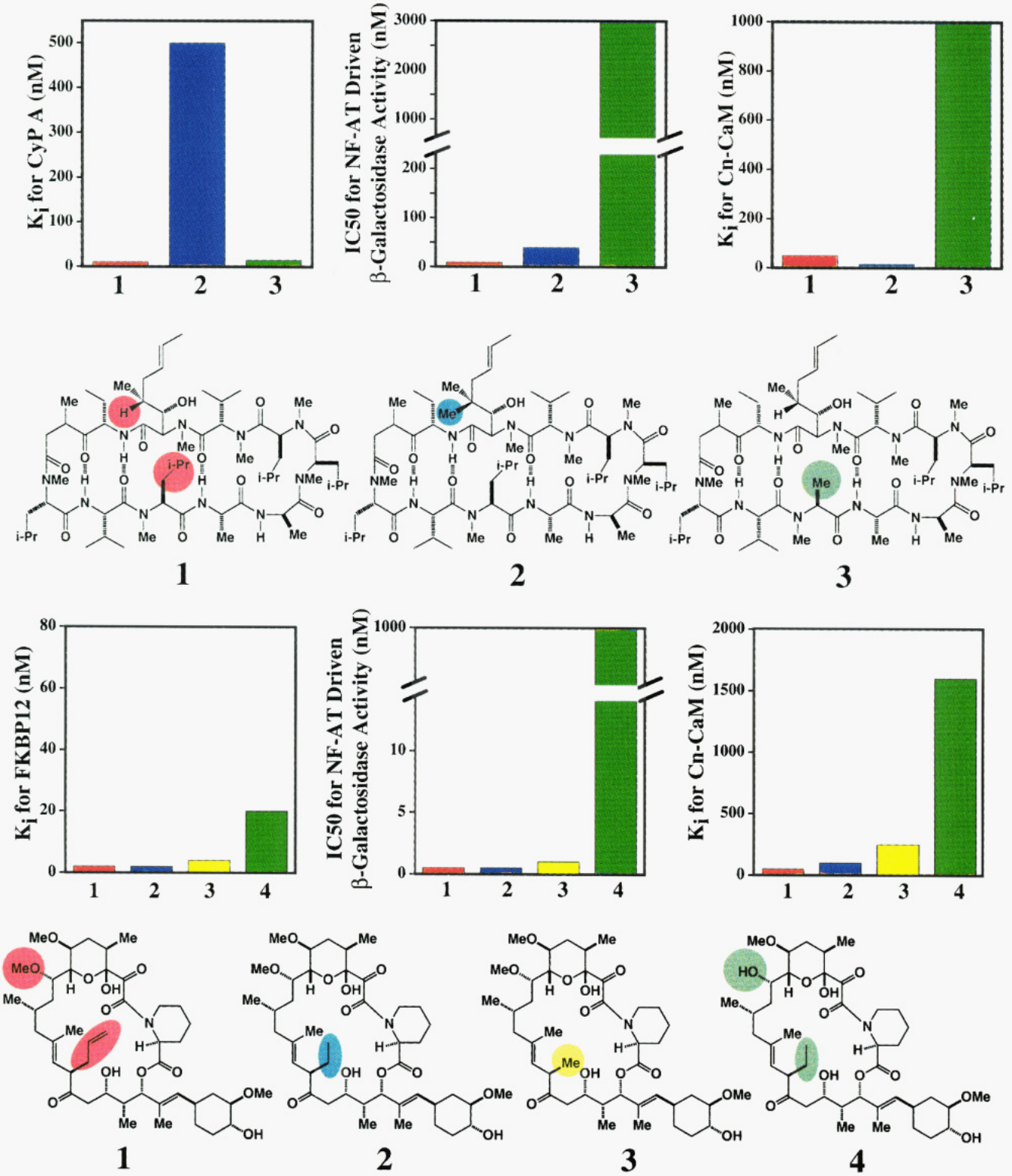

Figure 5. (Top) Structural modifications (**2**, **3**) of cyclosporin A (**1**) and an analysis of cyclophilin A binding, signal transduction inhibition, and calcineurin binding (of cyclophilin A–ligand complexes).

Figure 6. (Bottom) Structural modifications (**2**–**4**) of FK506 (**1**) and an analysis of FKBP12 binding, signal transduction inhibition, and calcineurin binding of FKBP12–ligand complexes.[9]

understanding of this pathway, and several of the hypothesized signaling molecules may be out of sequence. However, what has emerged from these studies is one of the best understood signal transduction pathways, particularly with respect to the generally more elusive later events of the signaling pathway (i.e., post Ca^{2+}).

In addition to its immunoglobulin-like α and β chains, which are responsible for binding to peptide–HLA complexes, the TCR comprises of several largely cytoplasmic chains. At least one of these, the ζ-chain, is important for propogating the signal through the cytoplasm.[14] Cross-linking of the TCR results in the phosphorylation of key tyrosine residues on ζ, probably

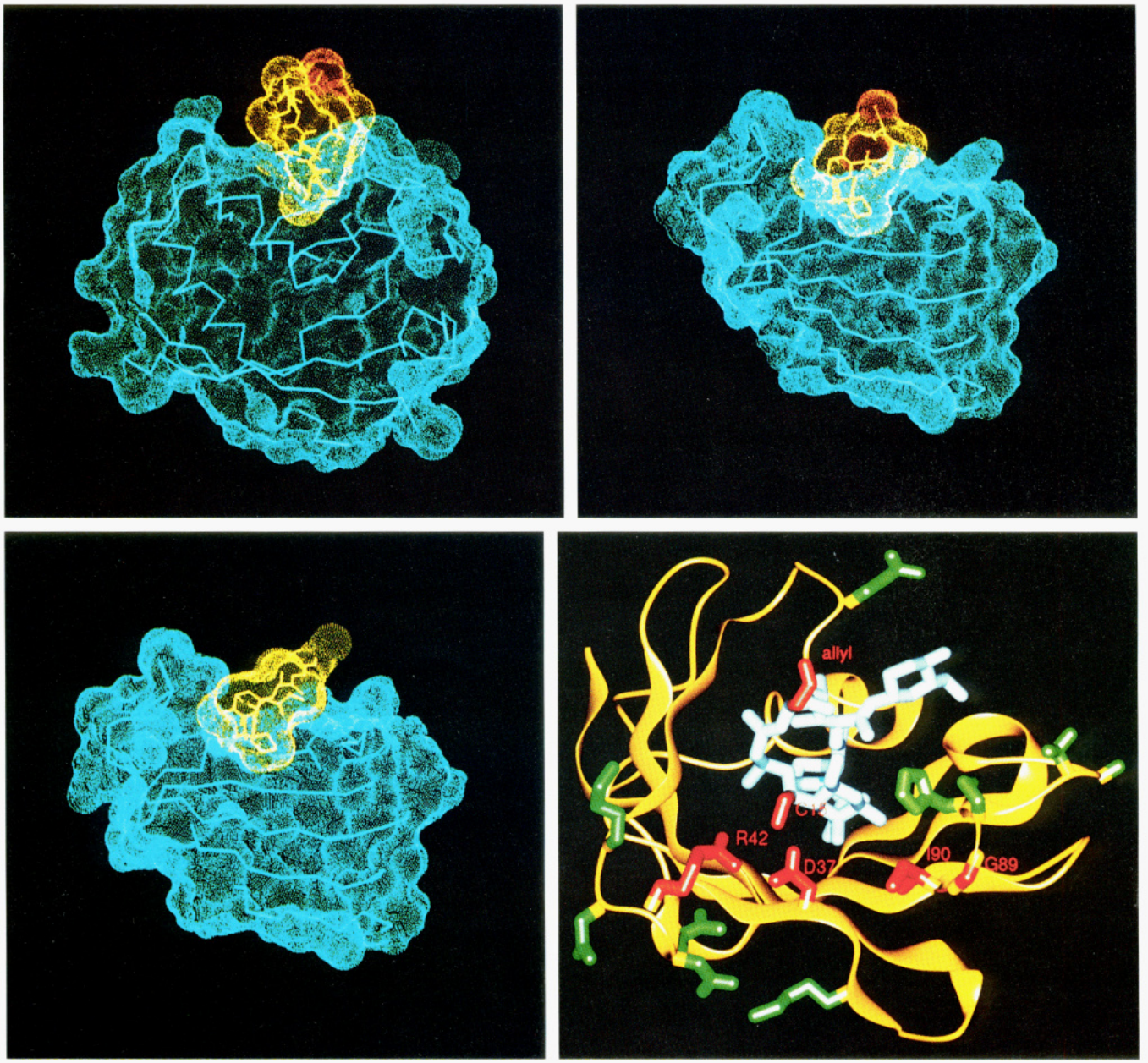

Figure 7. Structures of the immunophilin–ligand complexes. Upper left (A), upper right (B), and lower left (C): Cross sections of immunophilin (blue)–ligand (yellow) complexes. Orange dot surfaces indicate sites on CsA (N-Me-Leu6) and FK506 (21-allyl and 15 methoxyl) that upon modification result in ligands that bind their immunophilin with high affinity yet do not form an immunophilin complex capable of binding calcineurin.[9,35] A: Cyclophilin–CsA.[36,37] B: FKBP12–FK506.[38,39] C: FKBP12–rapamycin.[39,40] Lower right (D): FKBP12 (gold ribbon)–FK506 (blue licorice) color coded to display residues that influence calcineurin but not FK506 (red) and residues that influence neither binding event.[11,14] The results reveal that a composite surface comprising both FKBP12 and FK506 is recognized by calcineurin.

through the actions of one or both of the Src family member tyrosine kinases Lck and Fyn. Phosphorylated ζ is found to bind to the non-Src family tyrosine kinase ZAP-70.[15] As ZAP-70 was found to contain two phosphotyrosine-binding SH2 domains, it is likely that the ζ–ZAP-70 association involves these domains. This recruitment of ZAP-70 to the inner leaflet of the plasma membrane is thought to result in the activation of the lipase PLCγ1. By generating the "second messengers" diacyl glycerol and inositol trisphosphate, PLCγ1 leads to the activation of protein kinase C (PKC) and to the increase in the concentration of intracellular Ca^{2+}.

The discovery that immunophilin–ligand complexes target a Ca^{2+}-regulated protein phosphatase suggested that Ca^{2+}'s role is to activate this enzyme.[8] It appears that activated calcineurin then dephosphorylates the constitutive, cytoplasmic subunit (NF-AT$_c$)[16] of a T cell restricted transcription factor named NF-AT.[6] Resting T cells are found to have NF-AT$_c$ in the phosphorylated state and localized in the cytoplasm whereas activated T cells are found to have the subunit in a dephosphorylated state and localized in the nucleus.[17] Nuclear NF-AT$_c$ combines with its newly synthesized partner chains Jun and Fos (the Jun–Fos

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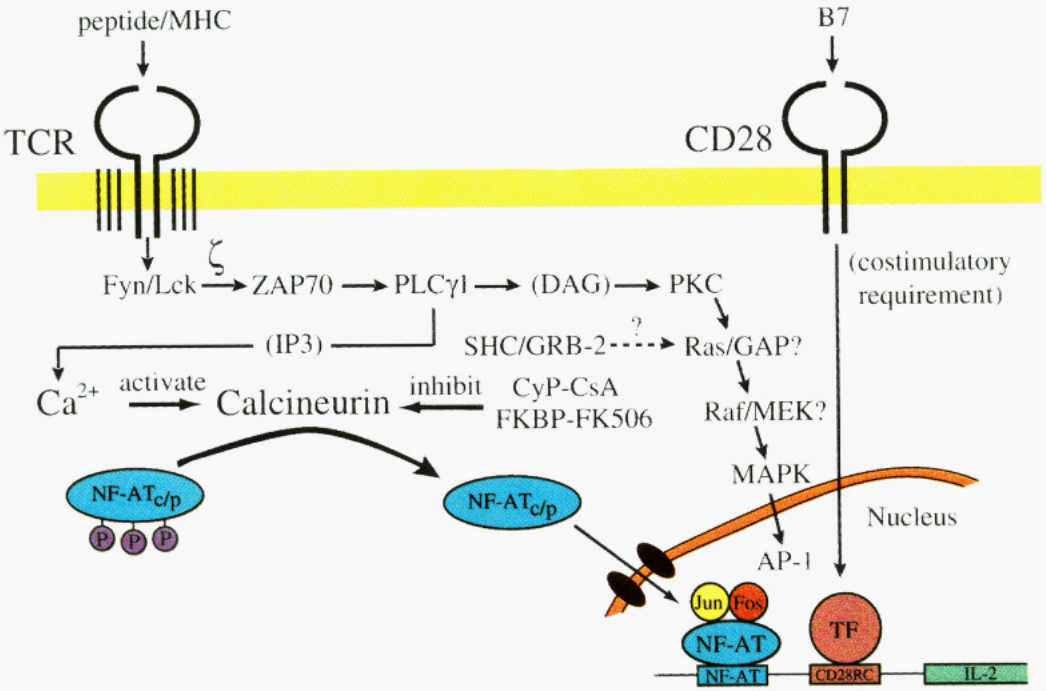

Figure 8. T cell activation via the TCR and CD28 signaling pathways. TCR = T cell receptor, HLA = human leukocytic antigen, Fyn/Lck = Src family member tyrosine kinases, ZAP70 = ζ activation protein, PLCγ1 = phospholipase C, DAG = diacyl glycerol, PKC = protein kinase C, Ras = Guanine nucleotide binding protein, GAP = GTPase activating protein, Raf = serine, threonine protein kinase, MEK = MAPK/Erk kinase, MAPK = mitogen activated protein kinase, AP-1 = Jun/Fos heterodimer, IP3 = inositol trisphosphate, Cyp = cyclophilin, CsA = cyclosporin A, FKBP = FK506 and rapamycin binding protein, $NF\text{-}AT_{c/p}$ = cytoplasmic, preexisting form of nuclear factor of activated T cells, TF = transcription factor(s), CD28RC = CD28 response complex, IL-2 = interleukin-2, B7 = ligand that activates CD28 signaling pathway.

heterodimer is known as AP-1) to create NF-AT. Apparently, AP-1 is rapidly synthesized following TCR activation by a CsA- and FK506-insensitive pathway involving PKC and Ras, which activate MAP kinase. NF-AT binding to its cis element in the IL-2 enhancer is necessary, but not sufficient, for IL-2 gene transcription. The T cell has a built-in fail-safe device referred to as the costimulatory requirement. Briefly, a second signaling pathway must also be activated by an antigen presenting cell in order for IL-2 transcription to proceed. This pathway involves the CD28 receptor, which transmits a signal that results in the appearance of additional IL-2 enhancer binding activity. Like most other signaling pathways, very few details concerning this pathway are currently available.

A remarkable lesson learned from studies of the TCR signaling pathway is that the signal serves as a switch that controls the cellular compartmentalization of a transcription factor. They also illustrate a cell biological control mechanism for transcriptional regulation. There has been considerable interest in the development of a whole new class of therapeutic agents that are envisioned to interfere with specific signaling pathways in cells. CsA and FK506 are the first illustrations of drugs that operate via this mechanism. They were not of course discovered with this goal in mind, yet they serve as the paradigm for the anticipated new class of drugs that might be rationally discovered by using our growing knowledge of signaling pathways.

An unsolved mystery in immunophilin research is the mechanism by which the FKBP–rapamycin complex is able to block G1 cell cycle progression. The remainder of this Account will detail the studies we have undertaken to shed light on the molecular events that are both necessary for G1 progression and targeted by the FKBP–rapamycin complex. First, we will describe a widely used technique in cell biology that we feel has great promise in organic chemistry. The technique, flow cytometry, can be used as a general tool to investigate the properties of the large class of natural products that interfere will cell cycle pathways.

Flow Cytometry: The Cell Biologists' Counterpart to NMR. Flow cytometry is a powerful analytical tool that can rapidly quantitate many properties of a population of cells, such as cell size, DNA and protein content, expression of a particular protein, intracellular Ca^{2+} concentration, and pH.[17,19] The technique involves staining a population of cells with a fluorescent dye. Either the intrinsic property of a dye or the coupling of the dye to a molecule that confers specificity, such as an antibody or high-affinity ligand, determines which parameter is measured. The stained cells flow through a capillary tube perpendicular to the path of an Ar laser beam tuned to 488 nm. Numerous mirrors, photomultiplier tubes (PMTs), and optical filters are arrayed around the capillary tube to detect photons emitted from the dyes. The data for each cell are collected by the PMTs and analyzed using a microcomputer.

As stated above, the parameter measured depends on the staining of the cells. Propidium iodide (PI) is commonly used in order to quantitatively determine the double-stranded nucleic acid content of cells and carry out studies related to the cell cycle. Following

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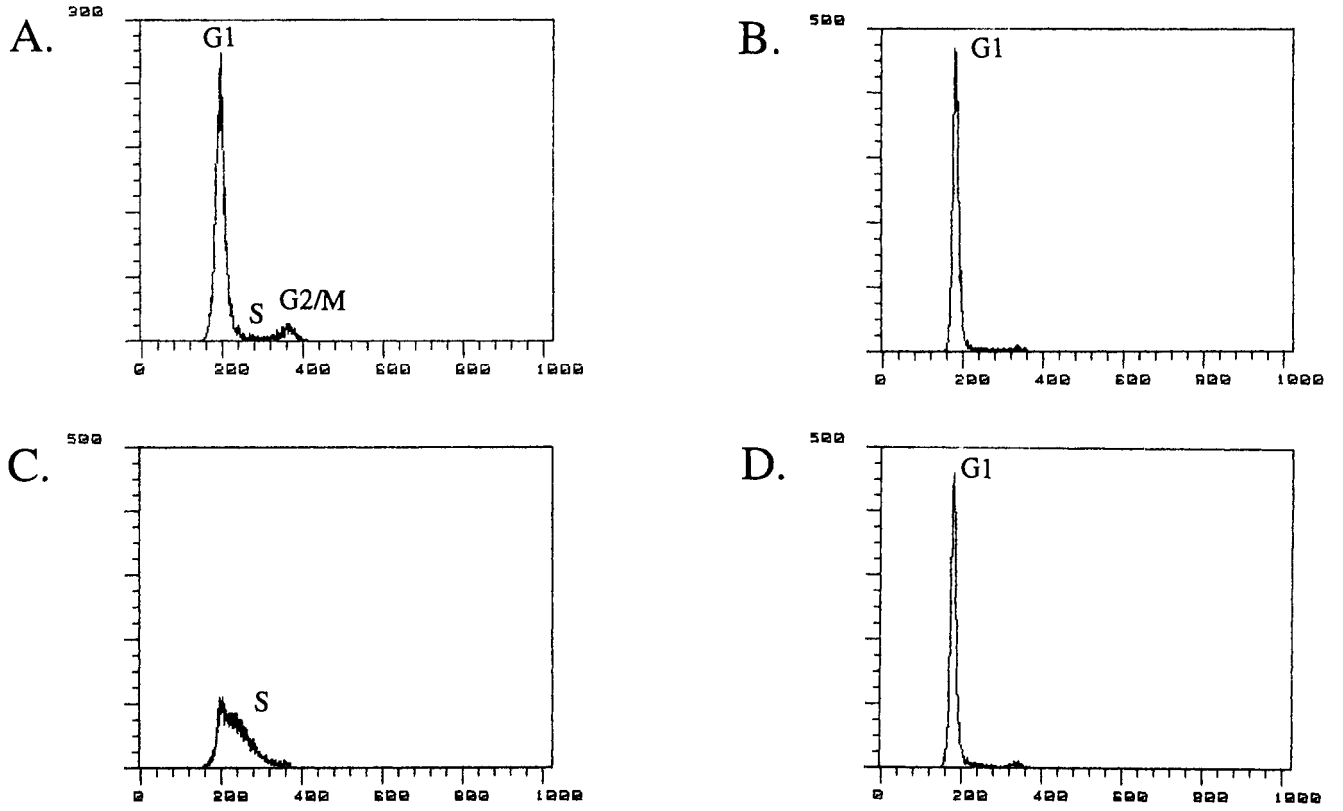

Figure 9. Flow cytometric analysis of MG-63 cells. (A) Asynchronous MG-63 cells. Each histogram is number of cells versus fluorescence intensity (amount of DNA). G1, S, and G2/M phases are indicated. (B) Synchronized MG-63 cells after 48 h of serum (growth factor) deprivation. (C) Synchronized cells 18 h following serum readdition. (D) Synchronized cells 18 h following serum readdition in the presence of 50 nM rapamycin. These data indicate that rapamycin arrests MG-63 cells in G1.

hydrolysis of the cellular RNA by RNase, PI intercalates into DNA exclusively, rendering the dye fluorescent with an excitatory λ_{max} of 493 nm. As cells pass through the Ar laser beam, PI emits photons (λ_{max} = 630 nm) proportional to the amount of DNA present in the cell. In a standard experiment, 10^4–10^5 cells are analyzed, and the data are plotted as a histogram: number of cells vs amount of emitted light. In this basic experiment, the cell cycle is divided into three components: G0/G1, S, and G2/M (using PI staining alone, G0 and G1 cells as well as cells in G2 and M are not distinguishable, but there are two-dimensional experiments to distinguish each phase of the cell cycle). A second method of staining is the conjugation of an antibody or other high-affinity ligand with a fluorescent dye for analysis of the presence of its cognate antigen or receptor on the surface of cells. In addition, cells can be stained with dyes whose fluorescent properties are sensitive to calcium concentration or membrane potential.

Rapamycin Inhibits Progression through the G1 Phase of the Cell Cycle. We will now illustrate the use of flow cytometry to determine the effects of a natural product on cell cycle progression using rapamycin as a test case. Moreover, as will become evident, this type of information is useful for quickly focusing subsequent biochemical experiments that are designed to elucidate the molecular mechanism of rapamycin's action and establish something about the signaling pathways necessary for G1 progression.

Rapamycin prevents T lymphocytes from responding to IL-2,[20] a stimulus known to cause division by allowing cells to progress through the G1 phase of the cell cycle.[21] While rapamycin is known to block a step prior to duplication of the genome, further characterization of the arrest may provide insights into the target of rapamycin as well as signal transduction pathways present in G1.

Isolation of cells arrested at G0 or early G1 is required in order to study rapamycin's effects on the cell cycle. Such a homogeneous population can be attained by removing growth factors, allowing cycling cells to accumulate at the beginning of the growth factor dependent stage G1. Unfortunately, normal lymphocytes die relatively soon after IL-2 is withdrawn. In contrast to lymphocytes, osteosarcoma (bone cancer) cell line MG-63 can be deprived of serum growth factors for 48 h without loss of viability.[22] Synchronized MG-63 cells progress together through G1 and into S phase once serum growth factors are reintroduced.

With synchronized cells, flow cytometric analysis was used to investigate the sensitivity of MG-63 cells to rapamycin.[23] The DNA content of an asynchronous population of MG-63 cells in the presence of growth factors is illustrated in Figure 9A: the abscissa represents increased DNA staining while the ordinate indicates the number of cells. In this population all the phases of the cell cycle are represented. When deprived of serum, cells gather at the beginning of G1, before DNA synthesis has begun (Figure 9B). If serum growth factors are reintroduced for 18 h, most of the cells have

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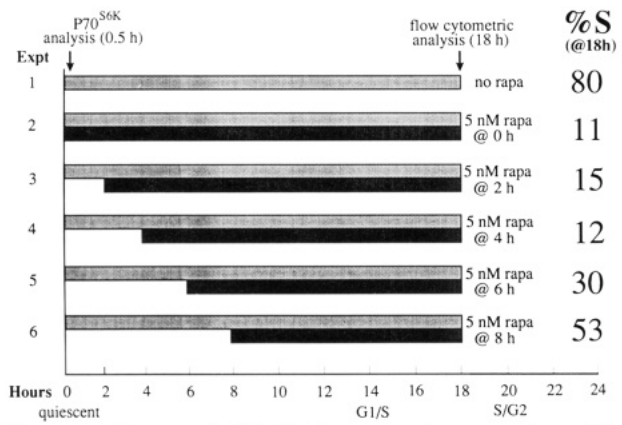

Figure 10. Rapamycin blocks G1 progression even when added 4 h after activation.[22] The lower bars in experiments 1–6 indicate the time of addition of rapamycin relative to the addition of serum (at $T = 0$). Earlier studies of rapamycin's effects on $p70^{S6K}$ focused on events that occur within 30 min of activation. Flow cytometric analysis occurred at 18 h. See text for an analysis of these experiments.

progressed through G1 and entered S phase and G2 (Figure 9C). Rapamycin at a concentration of 5 nM prevented synchronized cells from exiting G1 following 18 h of serum stimulation (Figure 9D). Furthermore, excess FK506 reversed the inhibitory effects of rapamycin treatment, indicating that rapamycin also functions in a complex with an FKBP. Synchronized MG-63 cells and flow cytometry were then used to determine when cells become refractory to rapamycin treatment following serum growth factor stimulation (Figure 10). Addition of rapamycin at 2 and 4 h after serum stimulation clearly inhibited S phase entry while addition at later times had only partial effects. Rapamycin seems to block a critical event that occurs some time after 4 h of serum stimulation.

Rapamycin Inhibits Two Protein Kinases Active in G1. Biochemical characterization of this "event" remains incomplete, but two protein kinases, $p70^{S6K}$ [24–27] and a cyclin-dependent kinase (cdk), are inhibited by rapamycin. Although current data indicate that these kinases are not the direct target of rapamycin action, further characterization of rapamycin's effects on these proteins not only may lead to the target but also will fundamentally increase our understanding of G1 regulation.

$p70^{S6K}$ was first characterized for its ability to catalyze the phosphorylation of a single protein of the small ribosomal subunit (S6), part of the machinery involved in protein synthesis. The kinase has a molecular weight of 70 000 and is phosphorylated and activated within minutes following growth factor stimulation.[28] Activation of $p70^{S6K}$ can be reversed within 2 min by rapamycin treatment[24] which transforms a phosphorylated $p70^{S6K}$ to an underphosphorylated form. In osteosarcoma cells $p70^{S6K}$ remains active and phosphorylated for at least 8 h after serum stimulation.[29] Both the activation and phosphorylation of $p70^{S6K}$ can be reversed at any time within these first 8 h of G1 phase by adding rapamycin. According to the flow cytometry data summarized in Figure 10, if $p70^{S6K}$ activity is required for G1 progression, it must be at some time after the first 4 h of serum stimulation. In other words, $p70^{S6K}$ activity in the first 4 h is not sufficient to allow progression through the R point to S phase.

Interestingly, the "window" within G1 where rapamycin is effective correlates with the activity of a cyclin-dependent protein kinase. Cdk's are the catalytic subunits of a complex consisting of at least two proteins, cdk's and regulatory subunits called cyclins.[30,31] The best studied member of a growing family of cdk's is $p34^{cdc2}$ (*c*ell *d*ivision *c*ontrol 2 refers to the name of the gene that encodes this 34 000 MW protein).[32] The kinase activity of $p34^{cdc2}$ oscillates during the cell cycle, whereas the levels of the cdk's remain relatively constant throughout the cell cycle. The kinase activity is necessary for yeast and mammalian cells to undergo the G2 to M transition, corresponding to the point in the cell cycle where the kinase is most active, and may play key roles in other phases of the cell cycle. Recently, evidence has been accumulating that another family member, $p33^{cdk2}$, mediates, at least in part, transition through the R point, and hence the G1 to S transition, in mammalian cells.[31]

As their name implies, cyclins oscillate throughout the cell cycle in a well-defined pattern.[30] These proteins bind to one or more cdk's and modulate their kinase activity, thus accounting for the oscillating nature of the kinase activity throughout the cell cycle. Starting in early G1, the temporal pattern of expression in mammalian cells is as follows: cyclins D, C, and E in G1; cyclin A in late G1, S, G2, and M; and cyclin B in S, G2, and M. The cyclins are labile proteins, and it has been postulated that the levels of one or more cyclins must exceed a threshold for a cell to pass the R point in late G1. In addition, a popular view held among biologists is that the actions of one cyclin lead to the expression and/or activation of downstream cyclins, one of the mechanisms to ensure directionality to the cell cycle.

Cdc2 kinase activity increases 4–6 h after the addition of serum in MG-63 cells, and this activity can be inhibited by treating cells with rapamycin.[23] Rapamycin treatment has no effect on the expression of $p34^{cdc2}$ or $p33^{cdk2}$, a related kinase. Working together with Richard T. Williams and Frederick L. Hall at the Childrens Hospital of Los Angeles, University of Southern California School of Medicine, we have found that rapamycin inhibits the expression of cyclins. Cyclins E and A are induced greater than 8 h after serum stimulation, and this increase is completely inhibited by rapamycin. In contrast, cyclin D levels increase after only 4–6 h of growth factor stimulation; rapamycin causes a relatively small decrease in this induced cyclin D expression. As described above, cyclins that are expressed early may be involved in the

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expression of later cyclins. It appears that the induction of cyclins E and A relies on a biochemical step after the production of cyclin D. The correlations between cyclin D expression, the loss of cdk activity, and the "window" of rapamycin sensitivity suggest that cyclin D may in some way participate in the "event" that we have defined to occur some time after 4 h of stimulation.

P9 is a protein that binds three cdk's (cdc2, cdk2, and cdk3) with high affinity; presumably, any cyclins that associate with the p9-conjugated sepharose beads do so through at least a cdk intermediary.[33] Cyclin D was retained by p9-sepharose when it was incubated with extracts from serum-stimulated cells.[23] Using extracts prepared from cells pretreated with rapamycin, ~90% less cyclin D associated with the p9-sepharose, suggesting that cyclin D can no longer directly or indirectly bind any of the three cdk's, Figure 11. Since cyclin association with cdks is required for activation of the holoenzyme,[34] rapamycin may inhibit cdk activity by blocking association with cyclins. Whether rapamycin affects cyclins other than D and how rapamycin prevents cyclin–cdk association are currently under investigation.

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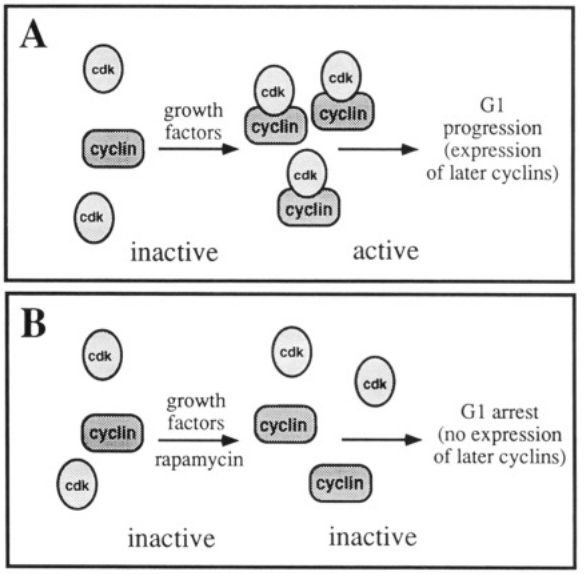

Figure 11. FKBP–rapamycin blocks cyclin–cdk association and kinase activity.

Summary

Here we have illustrated the use of two natural products to explore the signaling processes involved in G0 to G1 and G1 to S phase transitions. Through their ability to specifically inhibit cellular processes, natural products serve as molecular probes to define critical components of complicated biochemical events. Thus, natural products chemistry in alliance with tools such as flow cytometry provide an excellent opportunity for chemists to meet the challenge of deciphering biological processes at a molecular level.