Cyclin-dependent kinases: regulators of the cell cycle and more

Cyclin-dependent kinases determine the timing of key events in the cell cycle, and may also regulate other important cellular functions. Although some of the effects of activating these kinases are clear, the mechanisms by which the effects are produced are not; several types of chemical probes that might be enlightening can be imagined.

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Life is the most complicated chemical reaction. Since the mid-19th century, biologists have understood the growth and reproduction of living things in terms of the growth and division of the cells that compose them. The last ten years have revolutionized our understanding of the cell cycle, the coordinated set of changes that converts one cell into two daughter cells. Research on a wide range of organisms has revealed the remarkable evolutionary conservation of the biochemical engine that drives the critical events of the cell cycle, such as DNA replication and chromosome segregation. The key components of this engine are active protein kinases composed of two subunits: a catalytic cyclin-dependent kinase (Cdk) subunit, and a regulatory cyclin subunit. In this article I review what we know about the biological functions of these kinases and the biochemical reactions that control their activity, then discuss how the application of chemistry to biology could help solve the many unanswered questions about Cdk–cyclin complexes. I have referenced only work performed since the last comprehensive review of the cell cycle [1].

The biology of Cdk–cyclin complexes

The role of Cdk–cyclin complexes is best appreciated in the simplified cell cycles of early embryos. These cell cycles are short, consist of a rapid alternation of DNA replication and mitosis, and occur without any growth. The entry into mitosis is triggered by the activation of mitosis-promoting factor (MPF), a complex of Cdc2, the first Cdk discovered, and cyclin B, the first cyclin. Once active, MPF induces all of the events that characterize mitosis, including nuclear envelope breakdown, chromosome condensation, and the assembly of the mitotic spindle. The end of mitosis is triggered by the proteolysis of cyclin B, which leads to the inactivation of MPF, the reformation of interphase nuclei, and cytokinesis. Chromosome segregation does not require the destruction of cyclin B, but does require the destruction of an unknown protein. Both the unknown protein and cyclin B are tagged for destruction by conjugation to the protein ubiquitin. Once MPF has been inactivated, the destruction of cyclin B ceases, allowing the accumulation of the newly-synthesized cyclin B that will trigger the next round of mitosis. The levels of MPF thus follow a regular pattern of steady increase to a peak level, followed by dramatic decline (Fig. 1a); the beginning and end of mitosis correlate with the times at which MPF levels rise above and fall below a critical threshold.

Cdk–cyclin complexes also control DNA replication. In early embryos, the complex between Cdk2 and cyclin E is essential for DNA replication, although its kinase activity appears essentially constant during the cell cycle (Fig. 1a). Despite the fact that Cdk2–cyclin E activity is unregulated, DNA replicates only once in each cell cycle. This is because passage through mitosis is required to make chromosomes competent to replicate. After the early rapid cell cycles, the activity of Cdk2–cyclin E complexes becomes tightly regulated. The levels of Cdk2–cyclin E now rise and fall, like those of MPF (Fig 1b), and activation of this complex corresponds to a special transition early in the cell cycle, named Start, that leads to DNA replication. This change results in a mature cell cycle with rather different characteristics from the early embryonic one; when neither MPF nor Cdk2–cyclin E are active neither DNA replication nor mitosis occurs, and the cell is said to be in one of two 'gap' phases (G1 and G2).

Although multicellular eukaryotes have entirely separate Cdk–cyclin complexes for DNA replication and mitosis, unicellular eukaryotes use a single Cdk for both purposes. In the budding yeast Cdc2 (named Cdc28 in this organism) associates with one set of cyclins, the G1 cyclins, to induce passage through Start, and another set, the B-type cyclins, to induce mitosis. There are two extreme interpretations of the ability of one kinase subunit, Cdc28, to induce events as different as DNA replication and mitosis. One is that the cyclins are crucial in determining the substrate specificity of Cdk–cyclin complexes. Thus, the presence of different cyclins at different points in the cell cycle would lead to phosphorylation of different sets of substrate proteins. Alternatively, the kinase activity may be essentially the same, but the substrates available for phosphorylation may change during the cell cycle. In this hypothesis, the two

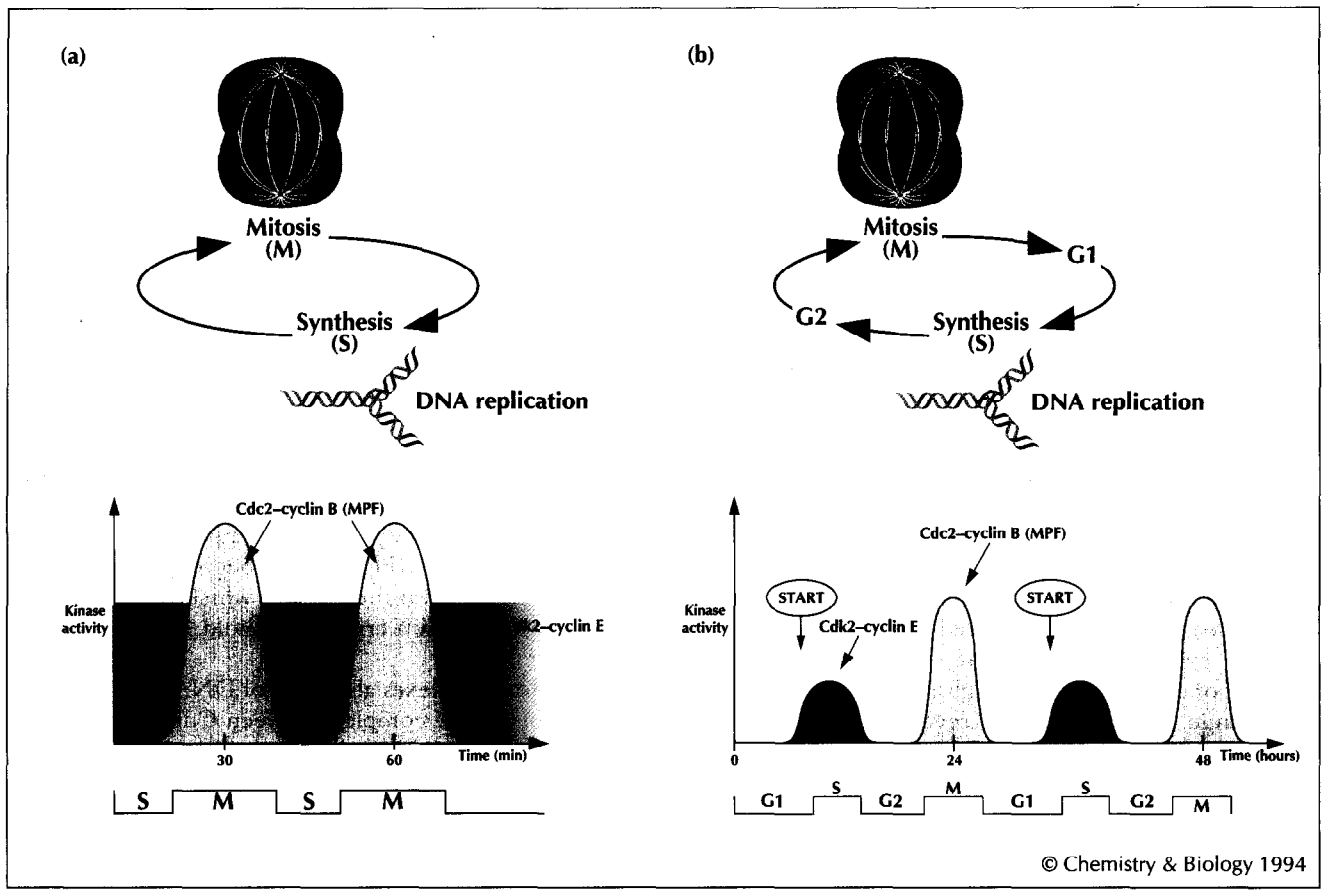

Fig. 1. Cdk–cyclin complexes in the cell cycle. **(a)** The embryonic cell cycle. Cdk2–cyclin E levels are constant, but Cdc2–cyclin B (MPF) levels vary. High levels of MPF trigger mitosis. **(b)** The post-embryonic cell cycle. Cdk2–cyclin E levels are now tightly controlled; increases in the level of this complex trigger DNA replication. When neither Cdk2–cyclin E nor Cdc2–cyclin B are active, there is a 'gap' in the cycle (G1 or G2); as a result, the post-embryonic cell cycle is much longer than that seen in the embryo.

different cyclins are required to allow independent control of two different transitions. There is circumstantial evidence for both of these hypotheses, so it may be that both mechanisms contribute to the dramatic difference between Start and mitosis.

As if the ability of different Cdk–cyclin complexes to trigger two distinct, complex events in the cell cycle weren't confusing enough, PCR cloning and genetic approaches have revealed a plethora of additional Cdks and cyclins. Many of these have yet to be assigned functions, but in at least one well-studied case it appears that evolution has also exploited Cdk–cyclin complexes for purposes other than control of the cell cycle. In budding yeast, low phosphate levels activate an inhibitor of the Pho85/Pho80 Cdk–cyclin complex. Because the transcription factor regulated by this complex is only active in the non-phosphorylated form, inhibiting the kinase activates the expression of genes involved in phosphate uptake [2,3]. In mammalian brains, Cdk5 is bound to a protein that by sequence homology at least is not a cyclin [4,5], suggesting that either cyclins have diverged beyond the point of recognition, or that some Cdks have found new partners during the course of evolution. Since this complex is highly expressed in post-mitotic neurons it seems likely that it has a function other than cell-cycle regulation.

The chemistry of Cdk–cyclin complexes

The ways of regulating Cdk–cyclin complexes are as diverse as their biological functions. The different layers of regulation are used to make the progress of the cell cycle dependent on a wide variety of signals from inside and outside the cell. Since the kinase activity of Cdks absolutely depends on cyclin binding, the simplest control is the regulation of cyclin abundance. For example, cells turn off MPF and exit from mitosis by destroying cyclin B. The second level of control is the phosphorylation of the Cdk subunits. One phosphorylation site (Tyr15) lies immediately adjacent to the ATP-binding site and acts to inhibit kinase activity, probably by altering the geometry of the ATP binding site. Another (Thr161) has to be phosphorylated to turn on the Cdk–cyclin complex's kinase activity (Fig. 2). Intriguingly, the activating kinase that phosphorylates Thr161 is the Cdk7–cyclin H complex, raising the question of which kinase activates the activator. Finally, specific inhibitors can act at different levels to block the action of Cdk–cyclin complexes. Some inhibitors bind to cyclins and prevent them from complexing with Cdks,

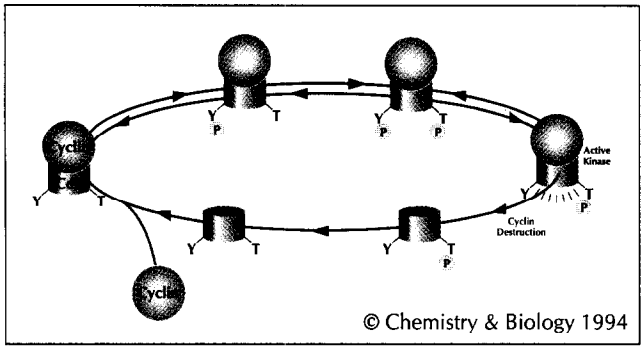

Fig. 2. Control of Cdk activity. Phosphorylation at Tyr15 (Y) inhibits Cdk activity, while phosphorylation at Thr161 (T) enhances activity. Once a cyclin binds to a Cdk, Tyr15 is often phosphorylated first, followed by Thr161. For the complex to become active, Tyr15 must be dephosphorylated. These events are under complex control. Active Cdk–cyclin complexes are then inactivated by cyclin destruction.

whereas others, named cyclin-dependent kinase inhibitors (CKIs), bind tightly and specifically to Cdk–cyclin complexes to inhibit their kinase activity. For a much more detailed treatment of these topics see [6].

The malignant consequences of deranging the various levels of Cdk–cyclin regulation underscores their biological importance. Many of the most important mutations in human cancers affect the control of cyclin activity; these include mutations that increase the transcription of a G1 cyclin (cyclin D), inactivate a cyclin-binding protein (the retinoblastoma protein), or inactivate a CKI (p16) [7]. Thus the uncontrolled activity of Cdk–cyclin complexes can lead to uncontrolled cell proliferation.

Unresolved chemical questions

Despite the speed at which our understanding of Cdk–cyclin complexes has advanced, many questions remain, most of which are applicable to all families of protein kinases. One set concerns the relationship between the structure of proteins and their chemical activities: how does the association of Cdks with cyclins and their post-translational modification regulate their protein kinase activity? What determines the substrate specificity of a particular Cdk–cyclin complex? In the long run, these questions can be answered by determining the three-dimensional structure of Cdks and cyclins, and their active and inactive complexes, with and without bound substrates, and analyzing the structure and activity of mutants in the cyclin and Cdk subunits.

Can chemistry assist with this daunting task? Comparing the structure of inactive monomeric Cdk2 with active cAMP-dependent protein kinase suggests two major changes that have to occur in Cdk2 before it can become active: a realignment of the β–γ phosphate bond in ATP that will produce a favorable geometry for the S_N2 attack by the protein substrate's hydroxyl group, and a major displacement of a peptide loop that blocks access to the putative binding site for the protein substrate [8]. Which of these changes depend on cyclin binding and the activating phosphorylation, and which are inhibited by the inhibitory phosphorylation? One way of addressing this question would be to engineer fluorescence, NMR, or other probes that could report on the conformation of the Cdk and cyclin subunits and the binding and conformation of the ATP and protein substrate. Two techniques offer promise for creating such probes. One is combinatorial chemistry (reviewed in [9]). By preparing libraries of compounds containing suitable fluorescent reporter moieties and selecting those members that bind to an active Cdk–cyclin complex, but not an inactive one, it should be possible to isolate probes that will report on the conformational changes that occur on activation. Those compounds that can compete for protein substrate binding to the active complex will act as reporters on the conformational changes that open the protein substrate binding site. Combining bead-based chemistry and fluorescent reporters should allow direct selection of probes whose fluorescence changes on protein binding. The beads could be passed through a fluorescence-activated cell sorter (FACS) machine once and pooled according to either the amount of light emitted, or the ratio of emission at different exciting or detection wavelengths. Each pool would then be incubated with the Cdk–cyclin complex and resorted, selecting for those beads whose fluorescence properties differ from those shown on initial protein-free sorting (Fig. 3). Compounds identified by such methods could be further subdivided on the basis of their binding to active and inactive complexes, and their ability to compete with different ligands for binding to the complexes.

Phage display offers a possible alternative to combinatorial chemistry. This technique selects those members of a population of bacteriophages that express a peptide sequence that binds a particular compound [10]. Since the peptide sequence is specified by the genetic material of the phage, randomization of the peptide coding sequence produces a population of up to 10^{10} members in which each phage expresses a different peptide. After selection, any member of the library can be amplified to produce a genetically and chemically homogenous population for further characterization. Such libraries have

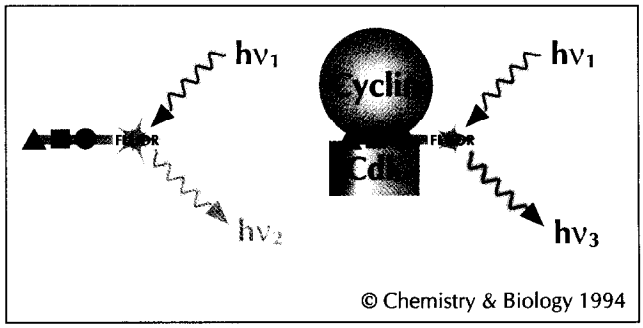

Fig. 3. Compounds that bind specifically to an active Cdk–cyclin complex could be selected to give a different fluorescent signal when the complex binds to them, or if it changes conformation while bound.

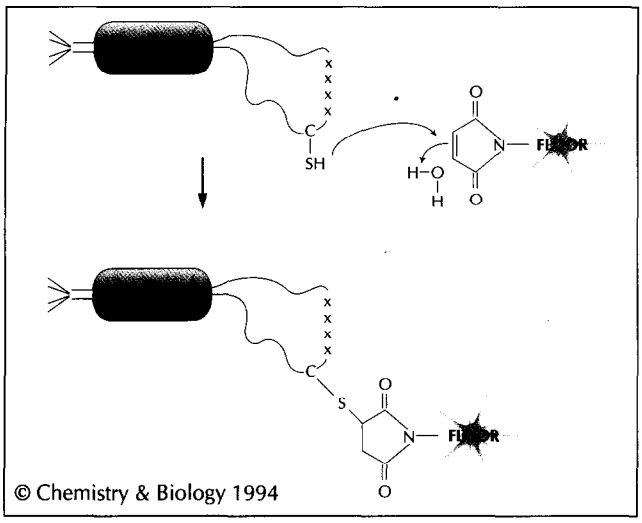

Fig. 4. Making a fluorescently labeled peptide library. Random peptides, displayed on phage, could be engineered to contain an invariant cysteine residue. All of the peptides in the phage library could then be linked to a fluorescent moiety via N-ethyl maleimide, or a similar linking agent, before screening.

been used to select short peptides that bind to both small chemical ligands and intact proteins, and the technique has recently been expanded to allow selection of peptides produced by direct *in vitro* translation of large libraries of RNA molecules. By making libraries in which each peptide contained an invariant cysteine, a chemical reporter group could be added to each member of the library by coupling it to a sulfhydryl reactive compound such as an N-ethylmaleimide derivative carrying a fluorescent moiety (Fig. 4). Screening those members of the derivatized population that bind to immobilized Cdk complexes should then yield peptide epitopes that would bind in their derivatized forms.

By using derivatized peptide libraries based on the consensus sequence for phosphorylation by a given Cdk–cyclin complex, it should be possible to isolate peptides whose fluorescence properties change when they are phosphorylated. Such molecules would bring rapid spectroscopic techniques to bear on the formation of the protein hydroxyl-phosphate bond, as well producing reporters that could be introduced into living cells to report on where and when Cdk–cyclin complexes are active in individual cells.

Unresolved biological questions

How do Cdk–cyclin complexes produce their biological effects? Is the specificity of Cdk–cyclin complexes the same in a test tube and in a cell? If not, why not? Can the same complex in different parts of the cell be regulated in different ways? How do we determine the role of a single Cdk–cyclin complex in a cell that has many different complexes? Because they apply to all protein kinases, and because protein kinases are involved in regulating every aspect of cell behavior, these questions are critical to all biologists as well as to the pharmacologists who want to interfere with protein kinases that are important in disease processes.

Even the simplest questions, like monitoring which proteins are phosphorylated in a given cell, are often difficult to answer. The commonest method, radioactive labeling, is impossible in clinical situations or in non-laboratory animals. Furthermore, the extent of labeling depends on the turnover of individual phosphate groups, and cannot be quantified without measuring the specific activity of intracellular ATP pools. One solution to this problem would be to devise methods that would convert unlabeled phosphate groups on proteins in cell extracts into some other chemically (or radioactively) labeled amino acid modification. In principle this task could be accomplished in three steps. First, reaction with one reagent would modify all the free hydroxyls, sulfhydryls and amino groups on the amino-acid side chains. Second, chemical or enzymatic hydrolysis of the phosphate groups would unmask one side chain hydroxyl for each phosphate group removed. Finally, these unmasked hydroxyls would be derivatized with a labeled reactive compound that was different from that used in the initial blocking reaction. The modifying groups used in the final

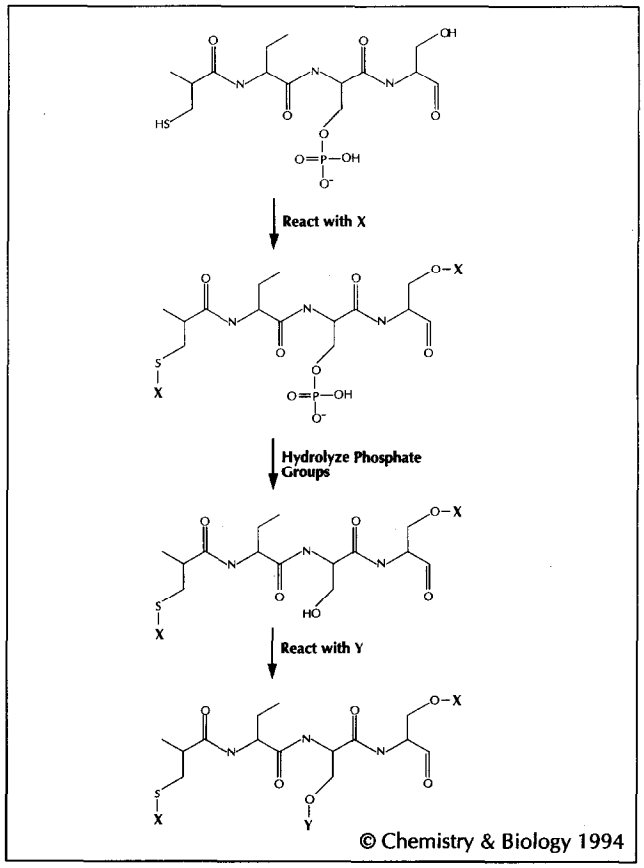

Fig. 5. Schematic method to detect phosphorylation state of a protein. Reaction with X blocks all of the free hydroxyls, sulfhydryls and amino groups on the protein; hydrolysis of phosphates would then unmask a side chain hydroxyl, which could then be 'tagged' with a different reagent, Y. Determination of the number and position of Y-modified residues would then provide information on the phosphorylation status of the native protein.

step could be designed to permit either affinity-matrix or antibody-based methods to separate unphosphorylated from phosphorylated peptides (Fig. 5). Since phosphorylation stoichiometries as low as 0.01 mol of phosphate per phosphorylation site can induce biological effects, designing a set of reactions that can accurately convert phosphorylation status into some other chemical signature will represent a formidable chemical challenge. Recent progress in this area is reviewed in [11].

Which substrates does a particular kinase phosphorylate *in vivo*, and which of these actually produce the biological response that follows activation of the kinase? Three problems hinder approaches to this central question in modern biology. The first is that protein kinases often act in cascades so that the biological effector may not be a direct substrate of the kinase that was initially activated. Thus a protein phosphorylated in response to treatments that activate protein kinase A, may be a substrate not of A, but of protein kinase B, which is activated when it is phosphorylated by A. The second is the need to inhibit one protein kinase without inhibiting any of the hundreds of others in a cell. The third is that many conditions or agents claimed to activate a single protein kinase fail to deliver the advertised level of specificity. All of these problems are more acute when we want to determine the function of one member of a family of protein kinases, such as the Cdk–cyclin complexes.

So far, the best way of controlling the specificity of kinase inhibition has been genetics. By mutagenizing the gene for a protein kinase and isolating mutants that are inactive at a particular temperature we can determine the consequences of inactivating a particular protein kinase. Alternatively, by isolating mutants that make the activity of a protein kinase constant and unregulated, and then placing the production of the kinase under the control of a gene promoter that can be experimentally regulated, we can determine what happens when this kinase is specifically activated. These considerations explain why two genetically tractable organisms, budding yeast and fission yeast, have made such major contributions to our understanding of the cell cycle and many other areas of cell biology.

The evolutionary conservation of the cell cycle means that many of the lessons learnt from genetic studies on yeast are directly applicable to mammalian cells. Nevertheless, we would still like to know what happens when we inactivate a particular protein kinase in a specific human cell type. A simple solution to this problem is chemical genetics: the production of kinase inhibitors and activators whose specificity equals that of kinase mutants. The advent of combinatorial chemistry, peptide selection techniques, and *in vitro* evolution suggests that it should soon be possible to produce such inhibitors. One limitation that synthetic compounds might be able to overcome is the fact that the time required to turn mutant proteins on or off is long, ranging from minutes to hours. On the time scale of the cell cycle, these delays are a minor annoyance, but in faster processes, such as chemical communication at nerve terminals, they are crippling. By making bioactive compounds whose functional groups are masked by photoreactive groups it would be possible to create compounds that, like caged ATP and caged fluorophores [12], could be activated in milliseconds, allowing us to study the role of protein kinases in fast biological processes.

The application of sophisticated chemical techniques to the study of protein kinases could vastly improve our understanding of the enzymology of protein kinases, and the reactions that regulate protein kinase activity. Most appealingly, the advent of chemical reporters of kinase activity, and bioactive compounds that allow the rapid and specific modulation of kinase activity, would begin to unravel how these enzymes can produce such specific effects in reaction systems that are as complex as a living cell.

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