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Recycling the Cell Cycle: Cyclins Revisited

Review

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I discuss advances in the cell cycle in the 21 years since cyclin was discovered. The surprising redundancy amongst the classical cyclins (A, B, and E) and cyclin-dependent kinases (Cdk1 and Cdk2) show that the important differences between these proteins are when and where they are expressed rather than the proteins they phosphorylate. Although the broad principles of the cell cycle oscillator are widely accepted, we are surprisingly ignorant of its detailed mechanism. This is especially true of the anaphase promoting complex (APC), the machine that triggers chromosome segregation and the exit of mitosis by targeting securin and mitotic cyclins for destruction. I discuss how a cyclin/Cdk-based engine could have evolved to assume control of the cell cycle from other, older protein kinases.

Introduction

Last summer marked the 21st anniversary of the discovery of cyclin by Tim Hunt and his colleagues during the Physiology course at the Marine Biology Laboratory at Woods Hole (Evans et al., 1983). Like many important discoveries, cyclin reflected the intersection of fortune, a prepared mind, and the courage to build a hypothesis from one crucial observation. Observing the rise and fall of this novel protein, Hunt stated that "It is difficult to believe that the behavior of cyclins is not connected with processes involved in cell division" and went on to suggest that the synthesis of this protein drove cells into mitosis and its destruction allowed cells to finish one cell cycle and begin the next.

Time has proved him right. Cyclin's discovery led to a model of the autonomous oscillator that drove the cell cycle of early embryonic cells (Figure 1), while the discovery of cell cycle checkpoints (Painter and Young, 1980; Weinert and Hartwell, 1988) revealed that this oscillator could be entrained by events in the chromosome replication and segregation cycle (Murray and Kirschner, 1989). Cyclin provided *the* crucial hint about the chemical mechanism of the cell cycle oscillator and the first important role for regulated proteolysis in eukaryotes.

The discovery of cyclin was one of three strands of work that came together to produce the first working model of the cell cycle oscillator. The others were genetic investigations of the cell cycle in yeasts and studies on the large eggs and oocytes of frogs and marine invertebrates. Nurse and his colleagues had identified a network of genes that controlled entry into mitosis (Nurse, 1975) whose key component was Cdc2 (now renamed Cdk1), while Masui (Masui and Markert, 1971) and Smith (Smith and Ecker, 1971) had identified maturation-promoting factor, a biochemical activity that induced meiosis and mitosis. The key event in unifying these two approaches was Lohka's heroic purification of maturation-promoting factor (Lohka et al., 1988), whose two subunits turned out to be Cdk1 (Gautier et al., 1988) and cyclin B (Gautier et al., 1990), a conclusion supported by a variety of experiments on other creatures (Arion et al., 1988; Draetta et al., 1989; Dunphy et al., 1988; Labbé et al., 1989, 1988; Swenson et al., 1986).

Later work showed that different cyclin-Cdk complexes are activated at different points in the cell cycle (Cross, 1988; Hadwiger et al., 1989; Nash et al., 1988), that cyclins must be destroyed before cells can escape from mitosis (Murray et al., 1989), and that mitotic cyclins were destroyed by ubiquitin-mediated proteolysis (Glotzer et al., 1991; Hershko et al., 1991). We can thus distinguish three parts of the cell cycle: the engine, which produces regular fluctuations in the levels of cyclin and the activity of their associated Cdks; the downstream events the engine drives, such as DNA replication and mitosis; and signaling pathways that regulate the engine in response to events outside and inside cells.

Here, I focus on the structure and evolution of the cell cycle engine, arguing that its apparent complexity reflects its role in integrating the events of the cell cycle with each other and a wide range of external stimuli. In addition, I use mitosis to discuss what we know about the mechanism of the engine, the cellular processes it controls, and the signals that regulate it. I contend that an excessive emphasis on the role of individual proteins (one gene, one PhD) has crimped our knowledge of the cell cycle. This philosophy directs us away from thinking of the cell cycle as an integrated whole, and the ease of genetic manipulation in yeasts (and more recently RNAi in animals) compared to the difficulty of biochemistry has kept all but an honorable minority of labs from trying to fractionate and reconstitute either the cell cycle engine or the processes it controls.

This failure stems from an earlier victory. During the 1970s, two schools fought about how to tackle the cell cycle. The Kornbergian one argued that there was no point in trying to understand the control of the cell cycle until the biochemical details of processes like DNA replication had been understood. They were opposed by geneticists and embryologists who claimed that the logic of the cell cycle could be revealed by treating DNA replication and mitosis as black boxes that received mysterious signals from a cell cycle controller and sent other equally mysterious ones back again (Hartwell et al., 1974). Although the twenty years from 1970 to 1990 proved the second school spectacularly right, we seem to have forgotten that understanding the logic was supposed to be a prelude to uncovering the biochemical details that lie behind it. This is a serious lapse. Until we can make and test biochemically detailed models, we could easily be wrong about the broad pictures that have now become accepted dogmas.

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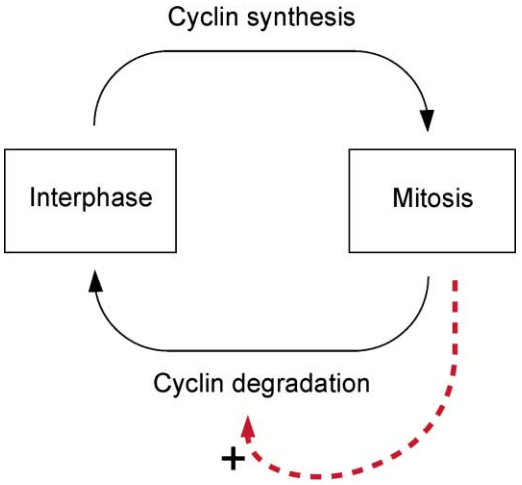

Figure 1. The Original Cyclin-Based Model for the Cell Cycle

The simplest possible model for the cell cycle based on the discovery of cyclin. See text for details.

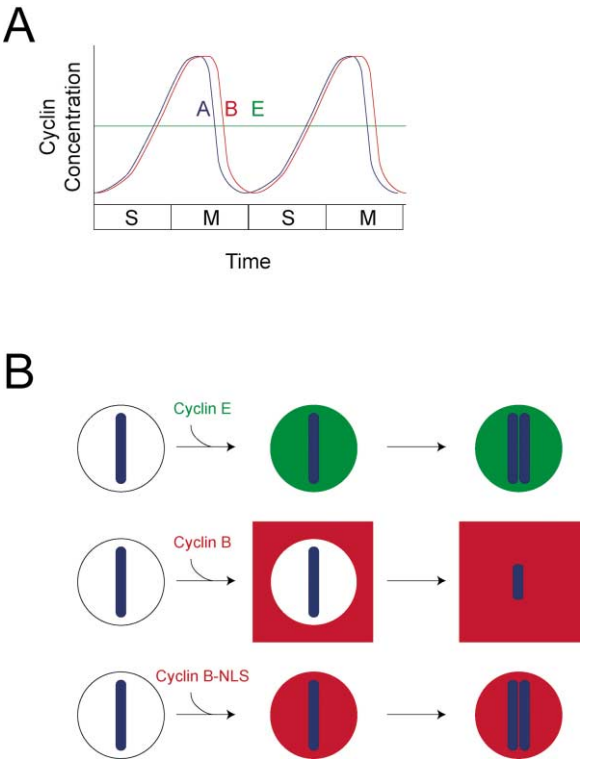

Figure 2. The Activities of Cyclins Are Determined by Their Location

(A) A cartoon of the abundance of cyclins A, B, and E during the early embryonic cell cycles of frogs.

(B) The consequences of adding different cyclins to a cell cycle extract depleted of cyclins A, B, and E. The nucleus is shown as a circle containing a single chromosome. Cyclin E enters the nucleus and induces DNA replication, but wild-type cyclin B fails to do so. Adding a nuclear localization sequence to cyclin B allows it to enter the nucleus and induce DNA replication.

A Single Cyclin Engine

Cyclins were the first example of accessory subunits that activated rather than inhibited protein kinases. Initially discovered as a pair of cyclins, A and B (Evans et al., 1983), which associated with a single kinase subunit, Cdk1 (also known as Cdc2 and Cdc28), the family has expanded to contain multiple cyclins and cyclin-dependent kinases involved in processes that include the cell cycle, transcription, and differentiation. Each cyclin associates with one or two cyclin-dependent kinases, and most cyclin-dependent kinases associate with one or two cyclins, although some, such as Cdk1 in budding yeast, associate with as many as nine distinct cyclins (reviewed in Andrews and Measday, 1998). In the cell cycle, there are cyclins associated with G1 (cyclin D), S phase (cyclins E and A), and mitosis (cyclins B and A).

There are two ways of explaining the plethora of cyclins: each cyclin is biochemically distinct and catalyzes the phosphorylation of a distinct set of proteins or the different cyclins and Cdks are fundamentally the same, but exist in several flavors to allow exquisite regulation of where and when they appear. I argue the latter is the correct view and that it reflects the evolution of cyclins and Cdks as an integrating circuit that was used to refine the control of a preexisting cell cycle.

In the early embryonic cell cycles, where cyclin was first discovered, there are three cyclins and two Cdks. Two cyclins, A and B, rise during interphase and fall during mitosis, whereas the third, cyclin E, remains constant. Cdk1 binds cyclins A and B and Cdk2 binds cyclins A and E. The cyclin/Cdk complexes induce two processes, duplication of centrosomes and DNA during interphase, and mitosis. The roles of individual cyclins were tested by adding recombinant proteins to cyclin-depleted extracts. Cyclin E supports DNA replication and centrosome duplication, cyclin A supports both of these processes and mitosis, and cyclin B supports mitosis alone (Strausfeld et al., 1996). Cyclin B's inability to induce replication could reflect a biochemical inability to catalyze phosphorylate proteins required for DNA replication or a physical inability to reach the compartment those proteins inhabit, since cyclin A is nuclear and cyclin B is cytoplasmic in interphase cells (Pines and Hunter, 1991).

To test the location hypothesis, Moore et al. (Moore et al., 2003) depleted frog egg extracts of cyclins and added back two versions of cyclin B. As reported (Strausfeld et al., 1996), the wild-type protein was a potent inducer of mitosis but never induced DNA replication. But if the nuclear localization sequence from cyclin E was grafted onto cyclin B, the chimeric protein entered the nucleus and induced substantial DNA replication (Figure 2). Thus the principal reason that cyclin B induces mitosis rather than DNA replication is that it and the proteins it *could* phosphorylate to induce DNA replication are separated from each other by the nuclear envelope. The multiplicity of cyclins reflects duplication and divergence that sends different family members to different places at different times, thus allowing biological functions that are located in different parts of the cell to be separately regulated.

This conclusion is supported by genetics. A single mitotic cyclin produces alternating rounds of DNA replication and chromosome segregation in fission yeast, an observation that produced the original suggestion that

low levels of Cdk1 activity induced DNA replication and higher levels led to mitosis (Fisher and Nurse, 1996). In budding yeast, the four mitotic and two phase cyclins can be pared down to a single mitotic cyclin (Haase and Reed, 1999). Mice can develop normally and survive in the absence of Cdk2 (Ortega et al., 2003), and embryonic development proceeds normally in the absence of cyclin E (Geng et al., 2003). Within a particular cyclin class, the presence of multiple members likely reflects temporal and spatial variations in expression patterns; the phenotypes of mice that express only a single cyclin D appear to result from the failure to transcribe that gene in certain tissues rather than differences between the properties of the different cyclin D proteins (Ciemerych et al., 2002).

To sum up, much of the difference between the behaviors of different cyclins reflects where and when they are found rather than their direct effects on substrate specificity. There may also be direct, biochemical differences between cyclins that account for things like the inability of cyclin E to induce mitosis and the apparently restricted specificity of G1 cyclins and Cdks, but we need careful experiments to distinguish the differences in substrate specificity from those in space and time. I return to this point below.

Cyclins Overcome G1 Inhibitors

Budding yeast and animal cells have long G1 phases. It is here that nutrients and growth factors regulate whether cells progress or leave the cell cycle to enter the specialized resting phase known as G0, and as a result, defects in the control of G1 are universal among tumors. The importance of G1 is reflected by the existence of G1 cyclins, which are needed for cell cycle progression.

How do G1 cyclins regulate cell cycle progress? In early embryos, where cyclin E levels are always high, DNA replication begins the moment mitosis ends. One idea was that post-embryonic cells had invented a way of blocking this rapid progression and that the role of the G1 cyclins was to override this inhibition (Figure 3A; Murray, 1991). The discovery of Cdk inhibitors supported this notion, but the strongest evidence was that removal of these inhibitors allows cells to survive without G1 cyclins. In budding yeast, three G1 cyclins phosphorylate and mark Sic1, an inhibitor of the S phase and mitotic cyclins, for destruction (Verma et al., 1997). Removing Sic1 allows cells to proliferate without the G1 cyclins (Schneider et al., 1996; Tyers, 1996), and removing the retinoblastoma protein allows mammalian cells to proliferate in conditions that inhibit the activity of cyclin D (Guan et al., 1994; Lukas et al., 1995).

Animal cells make it even harder to escape G1. Cyclin D is a G1 cyclin needed for the subsequent expression of cyclins E and A (Figure 3B). Cyclin D expression plays a crucial role in regulating cell growth and proliferation since many tumor cells either overexpress D type cyclins, have inactivated proteins that inhibit it, or have removed other inhibitory proteins, such as the retinoblastoma protein, which cyclin D-containing complexes must phosphorylate to induce progress through the cell cycle (reviewed in Sherr and Roberts, 1999). An important role of the Rb protein is to repress the transcription

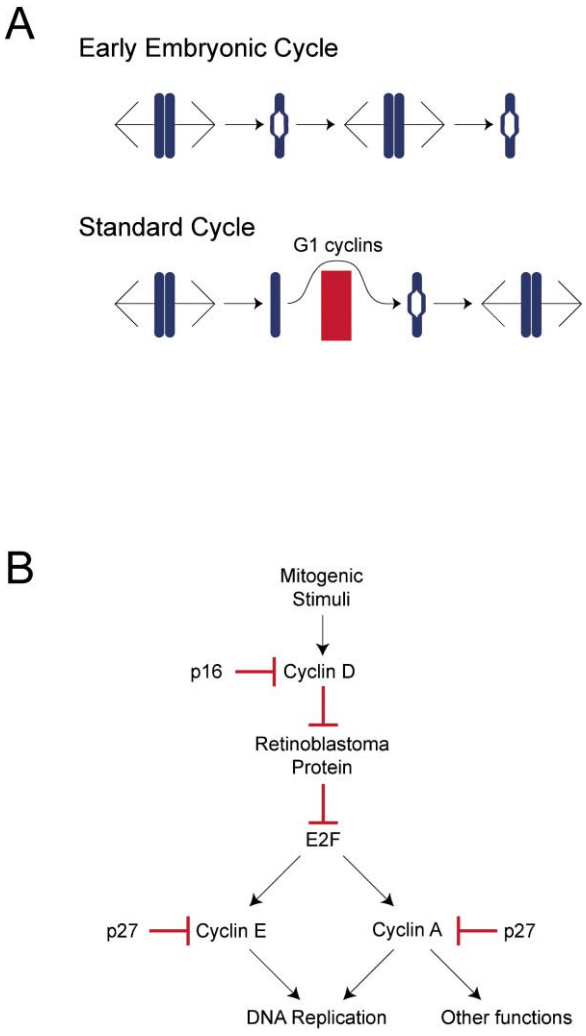

Figure 3. G1 Cyclins Overcome Inhibitors of Cell Cycle Progression

(A) In early embryonic cell cycles, DNA replication begins as soon as cells leave mitosis. In most cell cycles, however, the combination of anaphase promoting factor activity and Cdk inhibitors ensures that cells spend appreciable time in G1 and require the synthesis of G1 cyclins that overcome these inhibitory factors.

(B) The relationship between G1 (cyclin D) and S phase cyclins (A and E), growth factors, and Cdk inhibitors in animal cells. See text for details.

of cyclin E, suggesting that cyclin D's essential task is inducing cyclin E expression. Forced expression of cyclin E induces cell proliferation under conditions that inhibit the activity of Cdk-cyclin D complexes (Lukas et al., 1997), and expressing cyclin E from the cyclin D1 promoter restores some of the defects of cyclin D1-deficient mice (Geng et al., 1999). In *Drosophila* embryos, eliminating Cdk4, the partner of cyclin D, has little effect on late cell proliferation, and overexpressing cyclin D and Cdk4 does not prevent the developmentally programmed halt of cell proliferation, suggesting that in this organism, cyclin D may play an accessory rather than an essential role in controlling cell proliferation, although it does appear to play a role in controlling cell growth (Meyer et al., 2000, 2002). These findings highlight the important question of how involved cyclins

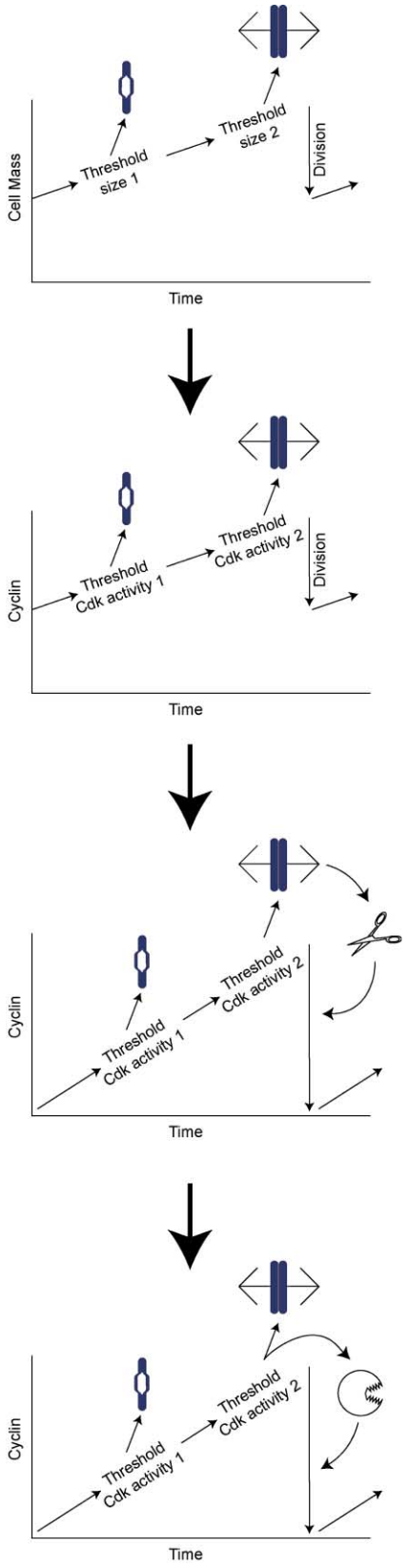

Figure 4. Evolution of the Cell Cycle Engine

The figure shows an hypothesis for the evolution of cell cycle control. In an ancestral proto-eukaryote, DNA replication and mitosis were regulated by cell size, with cells needing to reach a smaller threshold size to initiate DNA replication and a larger one to enter mitosis. In the first step, the size thresholds are replaced by thresholds of Cdk activity, which increases throughout the cell cycle as a result of cyclin synthesis. Even in the absence of cyclin destruction, the amount of cyclin is halved every time a cell divides. Next, cyclin destruction evolves to be coupled to the end of mitosis, resulting in a much larger range in the cyclin concentration as cells pass through the cell cycle and allowing correspondingly more precise regulation. Finally, cyclin destruction comes under the control of Cdk activity, possibly by co-opting a relative of the ancestral SCF complex, creating an autonomous Cdk oscillator similar to those used today. See text for further details.

D and E are in controlling cell growth (the rate at which cells increase in mass) since it should be impossible to increase cell proliferation without increasing cell growth (reviewed in Prober and Edgar, 2001).

The Cell Cycle and Evolution

The importance of cyclins depends on your point of view. If you're a eukaryote they are indispensable, but if you're a prokaryote they do not exist. Remarkably, sequence gazing suggests that Cdks appeared after other protein kinases that regulate aspects of the cell cycle (Krylov et al., 2003), even though these other kinases are now regulated by Cdks and Cdk1 directly phosphorylates and activates many of the proteins responsible for DNA replication and mitosis. If the evolutionary analysis is correct, how did such a late arrival end up in such overwhelming control?

I speculate that cyclins and Cdks began life as integrating functions rather than components of a central oscillator. In bacteria, a round of DNA replication can take twice as long as a cell cycle, meaning that the coupling between DNA replication and cell division is flexible: as growth accelerates, the single replication origin can fire more than once in a cell cycle, and when it slows down, there are cycles in which it doesn't fire at all. In most of today's eukaryotes (budding yeast being a notable exception), entering mitosis with partially replicated DNA leads to irreparable chromosome damage. Before such a feature could appear, a tighter coupling between replication and segregation was necessary. One form of coupling would be a clock that told previously independent processes when to begin, coupled with long intervals between the striking of the hours so that one event would finish before the next began (Figure 4).

Initially, the clock could be provided by a measurement of cell size since most cells take longer to double in size than they do to replicate their DNA and segregate their chromosomes. Cyclins might have first appeared as molecules that accumulate continuously through the cell cycle and thus serve as a proxy for cell size, a role that one of the G1 cyclins in budding yeast still seems to play (Cross, 1988; Nash et al., 1988). If the ancestral cyclins were stable throughout the cell cycle, the amount of cyclin would fall 2-fold when cells divided, giving a narrow range for the thresholds associated with DNA replication and mitosis. This problem would be overcome if cyclin evolved to be destroyed by the proteolytic machinery that separated the sister chromatids. After this improvement, the cell cycle would start with no

cyclin, allowing cells to set well-separated thresholds of Cdk1 activity for replication and segregation. Finally, as the segregation module sent the signal to separate the sister chromatids, it would also reset the clock by initiating cyclin degradation.

This early stage would differ in three respects from the eukaryotic cell cycle of today. First, reactions within the replication and segregation machinery would still be driven by their ancestral, non-Cdk controllers, but these would themselves be activated by a cyclin-Cdk complex. Second, the resetting of the clock would be accomplished by reactions within the segregation machinery, rather than as part of an autonomous oscillator. For example, cyclin destruction could be turned on by the protease separase, which now triggers sister chromatid separation. Finally, the events of the cell cycle would be separated from each other by timing rather than by checkpoints that rely on signals from one event in the cell cycle to regulate the next.

As time went by, these features disappeared from the majority of cell cycles. Checkpoints evolved to control Cdk1/cyclin activity in response to events in the chromosome cycle, a task that may have been easier to accomplish for an enzyme that initially had a limited number of substrates and other interacting proteins. A new form of cyclin proteolysis machinery fell under control of Cdk1 to create an autonomous regulator, most likely by duplicating an activity that was originally constitutive and then placing one of the two copies under Cdk control (see below). Finally, the Cdks would gradually take over the phosphorylation events that drove chromosome replication and segregation. As they did so, they would need a close physical association with the substrates that make chromosomes replicate and segregate, creating pressure for the cyclins and Cdks to duplicate and diverge to allow finer control of cell cycle events in space and time.

If this idea is right, we ought to see echoes of the precyclin era, just as we see echoes of the RNA world. The most obviously added on features are checkpoints; early frog embryos do fine without them (Hara et al., 1980), and budding yeast cells whose checkpoints have been genetically inactivated are almost indistinguishable from wild-type cells as long as no one damages their DNA or spindles. What might be the defeated remains of ancestrally dominant kinases can be glimpsed in both DNA replication (the Cdc7/Dbf4 kinase/activator couple) and in mitosis (Polo and others). Finally, the complicated and disputed influences of Polo and Cdk1 in activating the APC leave open the possibility that it was Polo who initially activated the APC to reset the cell cycle clock, a process that Cdk1 is still in the middle of taking over. One exciting possibility is that whole genome sequences and experimental analysis of the most primitive eukaryotes may either support or refute this scenario for how cell cycle control evolved.

Gene duplication and promoter divergence appear to be an evolutionary cheaper way of fine-tuning the control of gene expression than evolving the enormously complex and sophisticated promoters that specify embryonic patterning (e.g., Lehman et al., 1999). At the level of the protein, duplication and divergence allows different versions of a protein to be sent to different parts of the cell and to fall under different modes of posttranslational modification. Thus, cyclin B is excluded from the nucleus while cyclin A is allowed entry (Pines and Hunter, 1991) and complexes of cyclin B and Cdk1 are much more susceptible to phosphorylation and inhibition by the protein kinase Wee1 than are cyclin A/Cdk1 complexes.

How Do Cyclins Die?

During the 1970s, it had been widely argued that periodic protein synthesis would play a role in controlling the cell cycle. The idea that protein destruction could drive cell cycle transitions had few, if any, advocates. Thus the discovery of cyclin transformed our understanding of the cell cycle. That it did so shows the difficulty in making genuine connections between fields since by 1975, the activation of bacteriophage lambda was known to be accompanied by the RecA-dependent proteolytic cleavage of its repressor (Roberts and Roberts, 1975).

The obvious questions for cyclin were how is it degraded, by whom, and how is its degradation regulated? All known cyclins are targeted to the proteasome by the addition of a chain of ubiquitins, but the details of this conjugation differ for the different cyclins. G1 cyclins are ubiquitinated by the SCF complex, whereas mitotic cyclins are ubiquitinated by the anaphase-promoting complex (APC). Both complexes (reviewed in Jackson et al., 2000) also degrade other proteins, and they share a core organization (the association of a cullin-like protein [Cdc53 in SCF and APC2 in APC] with a protein containing a particular zinc finger domain [Rbx1 in SCF and APC11 in APC]) and possibly a common origin. Despite these similarities, they are regulated in different ways. The SCF complex is active throughout the cell cycle and the destruction of its substrates depends on their phosphorylation, with different phosphate binding proteins (F box proteins) guiding different sets of substrates to destruction. The APC is activated at the onset of anaphase and degrades its substrates as cells exit mitosis, suggesting it might first have appeared as a specialized version of SCF to take over the role of cyclin destruction from some other proteolytic system (Figure 5).

As cells proceed through mitosis, the APC is activated in two ways: its subunits are phosphorylated and its interactions with an activating protein variously known as Cdc20 or Fizzy increase (reviewed in Peters, 2002). Both changes are stimulated by the activation of Cdk1, but there has been considerable disagreement about whether this role is direct or involves intermediary protein kinases (Golan et al., 2002; Rudner and Murray, 2000). As cells leave mitosis, the APC remains active but switches from depending on Cdc20 to depending on a related protein (Hct1/Cdh1/Fizzy related) for its activity. Unlike Cdc20, Hct1 is inhibited by Cdk1 (Jaspersen et al., 1999; Zachariae et al., 1998), and it is unclear whether the APC must remain partially phosphorylated in order to remain active, and if so, which kinase modifies it. Finally, in late G1, the reactivation of Cdks leads to the phosphorylation and inactivation of Hct1, terminating the activity of the APC until cells reach mitosis. We would like to understand how phosphorylation of the APC controls its activity, which kinase modifies it, how the accessory subunits (Cdc20 and Hct1) activate the

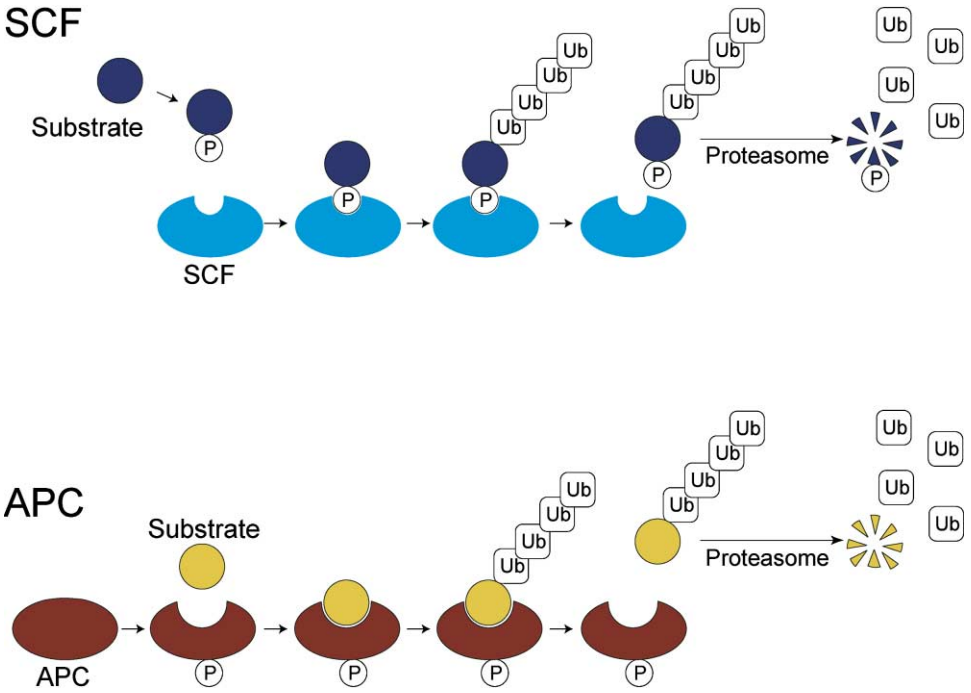

Figure 5. Regulating Protein Destruction in the Cell Cycle

Cartoons of the two major proteolytic systems that regulate the cell cycle. Both are E3 complexes that facilitate the transfer of ubiquitin from the active site of an E2 enzyme to a substrate that is thus marked for destruction by the proteasome. SCF binds phosphorylated substrates through a variety of accessory proteins, which present them to the complex for ubiquitination. SCF is active throughout the cell cycle, and the stability of individual substrates is regulated by their phosphorylation and the level of the accessory proteins that recognize them. By contrast, the APC appears to recognize substrates directly and its activity is regulated during the cell cycle as a result of phosphorylation by Cdk1 and other kinases.

it, how its activation is regulated by the cell cycle oscillator and the spindle checkpoint, and how the substrate specificity of the APC is controlled during the cell cycle.

Although the broad outlines of APC regulation are understood, the details are both confusing and important. The details matter because activating the APC triggers the key biochemical events that lead cells out of mitosis, inactivation of Cdk1 and the activation of separase, the enzyme that triggers chromosome segregation (reviewed in Nasmyth, 2002). As Hunt originally proposed, the mitotic cyclins trigger their own destruction by activating the APC, but pulling this trigger before the cell's chromosomes are properly aligned on the spindle would be disastrous. Two mechanisms slow the activation of the APC, a built in delay within the cell cycle engine that produces a lag between Cdk1 activation and that of the APC and the spindle checkpoint, which monitors interactions between chromosomes and the spindle and inhibits APC activation in cells with misaligned chromosomes. In some cell cycles, such as the early divisions of frog eggs, the spindle checkpoint is too weak to restrain the cell cycle engine, meaning that the built in delay must give a long enough interval between Cdk1 and APC activation to allow spindle assembly and chromosome alignment (Clute and Masui, 1995; Hara et al., 1980; Minshull et al., 1994).

Two general mechanisms that could account for the delay between activating Cdk1 and the APC are making Cdk1 inactivate an inhibitor of the APC or having the activation of the APC depend on multiple events, which occur either in parallel (multiple phosphorylation sites on the APC) or as a series (a cascade of kinases connecting Cdk1 activation to APC phosphorylation). There is evidence to support both mechanisms, although their relative importance is unclear. In vertebrates, a protein called Emi1 binds to and inhibits Cdc20 and cannot be degraded until it has been phosphorylated, possibly by Cdk1 (Reimann et al., 2001), and a fly homolog, Rca1, has similar properties (Dong et al., 1997; Grosskortenhaus and Sprenger, 2002). Emi1 is destroyed by SCF, linking the two different proteolytic systems that control the cell cycle (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). Emi1 has only been found in animals, suggesting that this protein is either poorly conserved or an animal-specific invention. Multiple events seem to be required to activate the APC, including phosphorylation on several subunits (Rudner and Murray, 2000), multiple phosphorylations on individual subunits, and the involvement of other protein kinases, such as polo, in the phosphorylation of the APC (reviewed in Zachariae and Nasmyth, 1999).

The details of these reactions have not been worked out in any organism and highlight the gap between understanding general principles and the details of molecular mechanism that bedevil cell cycle research. Lifting the veil of ignorance will take a concerted effort to follow the kinetics of APC modification and activity in vivo, decipher the details of who phosphorylates which site, understand the molecular mechanism of the APC, reconstitute its activation in vitro, and use comparative

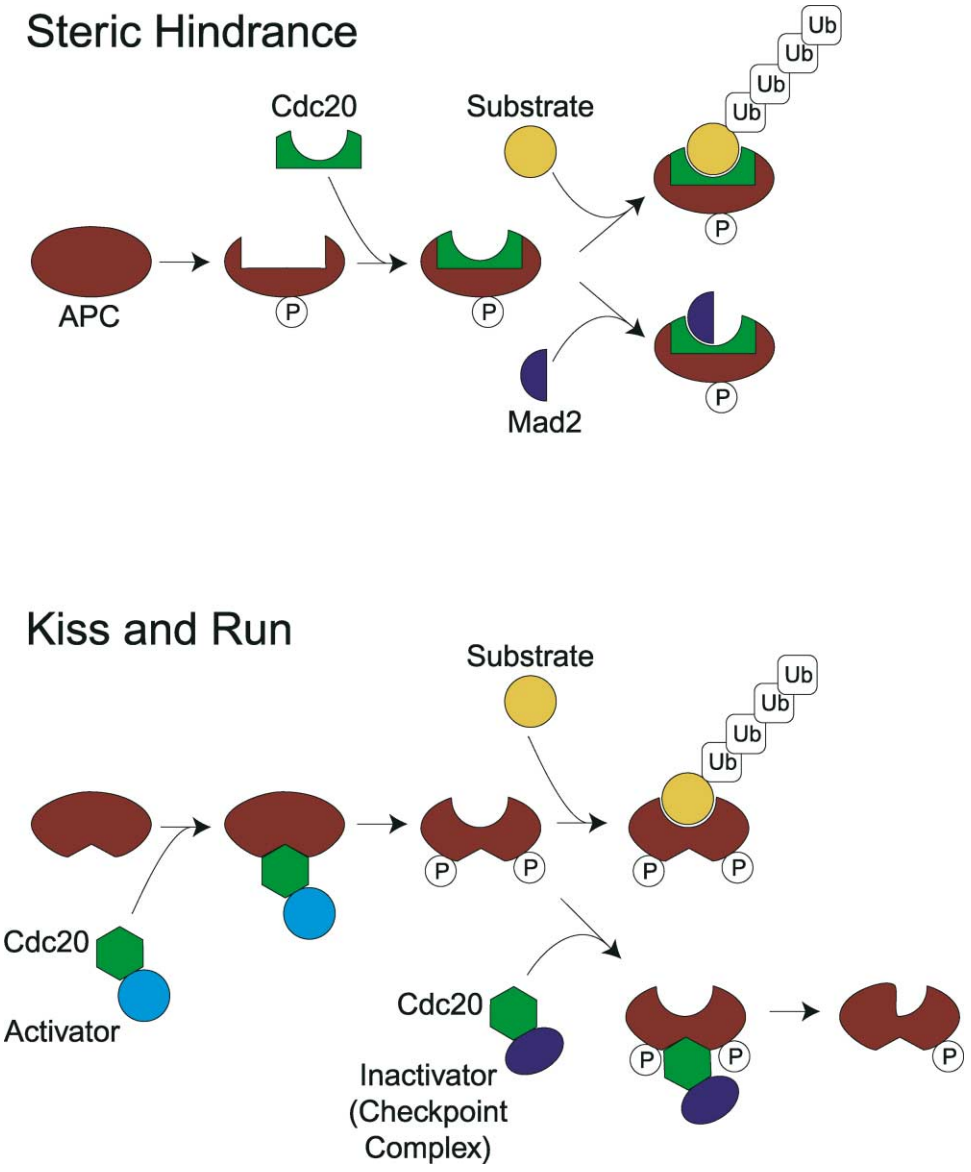

Figure 6. Possible Mechanisms of APC Regulation

Cartoons of two different models for controlling APC activity. In the first (Steric Hindrance), the phosphorylation of the APC leads to the binding of Cdc20, which forms the substrate recognition site in the APC. Binding of checkpoint components to Cdc20 fills most of the substrate binding site, thus preventing most substrates from binding to the APC. In the second, (Kiss and Run) Cdc20 delivers complexes to the APC that catalytically modify it, thus altering its activity. These Cdc20-delivered functions can either activate the APC by phosphorylating it or partially inactivate it by removing some phosphate groups.

studies to reveal how strongly the these details have been conserved in evolution.

Given the importance of Cdc20 and Hct1 in regulating the APC, we are surprisingly ignorant about them. The key question is whether they serve as essential and stoichiometric components of the APC or as kiss-and-run warheads that deliver modifying enzymes that either activate or inhibit the APC (Figure 6). The accepted wisdom is that Cdc20 and Hct1 present substrates to the APC by binding to both simultaneously (Fang et al., 1998b), and that Cdc20 and Hct1 preferentially recognize different sets of substrates (Schwab et al., 1997; Visintin et al., 1997).

Appealing as this model is, it is not a perfect fit with the evidence. The clearest experiments have been done in frog egg extracts. When mitotic extracts are passed over an affinity column containing the N terminus of cyclin, the APC is quantitatively retained, even though very little Cdc20 binds to the column (T. Hunt, personal communication). When the extracts are depleted with anti-Cdc20 antibodies, most of the APC remains in the extract and remains capable of binding to the cyclin column, leading to the conclusion that most APCs are not bound to Cdc20, and those that are not are still capable of interacting with their substrates. Although rigorously showing these Cdc20-free complexes are ac-

tive will be a challenge, these experiments support the idea that Cdc20 is needed to activate the APC but not to maintain that activity.

The spindle checkpoint arrests cells in mitosis by inhibiting the APC. If the checkpoint inhibited Cdc20 and Cdc20 was continually required for APC activity, the APC should be completely inactive in cells with damaged spindles. This is not so since although the spindle checkpoint stabilizes cyclin B, it fails to prevent the destruction of cyclin A (Minshull et al., 1994), which is also a substrate of the APC and also requires Cdc20 for its destruction (Dawson et al., 1995). A model in which Cdc20 is a targeting device (Figure 6) can explain these facts and is appealing since Cdc20 is a member of the WD40 family of proteins that seem to have evolved as assembly platforms for complex protein-protein interactions. In this scenario, Cdc20 can bind to either enzymes that modify and activate the APC or those that modify and inhibit it. Thus, activating kinases would be brought to the APC under the influence of Cdk1, whereas the spindle checkpoint would deliver inhibitory factors that could catalytically inactivate the APC.

How can the activity of the APC toward different substrates be differentially regulated? The simplest explanation is that the APC adds ubiquitins to its substrates processively, the number of ubiquitins added to a substrate reflects how long it was bound to the APC, and a critical number of ubiquitins is required to target a substrate to the proteasome. The number of ubiquitins added in one visit to the APC is important because of the abundance and activity of ubiquitin hydrolases, which rapidly remove ubiquitins and thus save modified substrates from proteolysis. Changing the binding time or the catalytic activity of the APC will alter the ubiquitin chain length and thus the stability of the substrate. Tight binding substrates could still be degraded despite substantial reductions in catalytic activity, whereas weaker binding ones could not. A variety of studies suggest that the N terminus of cyclin B carries all the information needed to regulate its destruction, whereas that of cyclin A depends on a collaboration between signals in the N terminus and elsewhere in the protein (Geley et al., 2001; A. Szidon and A.W.M, unpublished results).

What role do Cdc20 and Hct1 play in controlling the substrate specificity of the APC? Both have been reported to interact physically with APC substrates and differences between them have been proposed to account for different substrates being destroyed at different times as cells leave mitosis (see Peters, 2002 for references). Both recognize the classic destruction boxes first identified in cyclins, and Hct1 also recognizes a shorter sequence called the KEN box (Pfleger and Kirschner, 2000). In budding yeast at least, these differences must be minor since cells can use either protein alone to perform the cell cycle (Rudner et al., 2000; Schwab et al., 1997; Shirayama et al., 1999; Visintin et al., 1997). Like the cyclins, the clearest difference is not in substrate specificity but in how the APC's helpers are regulated in time and space, with Cdc20 requiring Cdk1 for its activity and Hct1 being inhibited by the same enzyme. Again, a real resolution of these questions will require the correlation of careful, quantitative studies performed both in vitro and in vivo.

Monitoring the Spindle

The spindle checkpoint uses information about the interaction between chromosomes and microtubules to regulate the APC. The key step in the spindle checkpoint appears to be the interaction of two complexes at the kinetochore. One, a complex of the Bub1, Bub3, and Mad3 (or BubR1) proteins is thought to reside at the kinetochore, whereas the other, which contains the Mad1 and Mad2 proteins, is soluble. At kinetochores without attached microtubules, the complexes interact and generate a new complex containing Mad2, Mad3/BubR1, Bub3, and Cdc20, which appears to be the checkpoint's warhead (reviewed in Musacchio and Hardwick, 2002). It has been proposed that this complex inhibits the activity of Cdc20 (Fang et al., 1998a). This model cannot easily explain why cyclin A is still degraded in cells whose spindle checkpoint is active, and it requires that most of the Cdc20 in the cell pass through a single kinetochore fast enough to keep it all inactive. This is challenging since the inhibition generated by the checkpoint is short lived; cells start degrading cyclin almost as soon as the last unattached kinetochore binds to microtubules (Clute and Pines, 1999). I propose that the checkpoint complex uses Cdc20 to target enzymes that will posttranslationally modify and inhibit the APC. In budding yeast, Cdc55, a regulatory subunit of protein phosphatase 2A, is part of the spindle checkpoint (Minshull et al., 1996), suggesting that Cdc20 could bring PP2A to the APC to remove the activating phosphorylations that were added as cells entered mitosis.

After Mitosis Is Over

The end of mitosis marks the boundary between the end of one cell cycle and the start of the next. In early embryonic cells, DNA replication begins very shortly after a nuclear envelope has reformed. In these rapid cycles, there is no sequence specificity to where DNA replication begins and origins are very closely spaced, allowing replication to finish in as little as four minutes. To ensure that each part of the chromosome is replicated exactly once per cell cycle, replication origins must be evenly distributed and none can fire after they have already been replicated once. Multiple firing of one origin is prevented by separating the binding and activation of the proteins that initiate DNA replication (reviewed in Bell and Dutta, 2002; Kelly and Brown, 2000). In vivo, initiating proteins like Cdc6 and the Mcm complex can only associate with DNA in the absence of Cdk1 and Cdk2 activity, but they can only trigger replication in its presence. This scheme guarantees that no new initiation complexes can form after replication has begun.

In most cell cycles, there is a pronounced gap (G1) between mitosis and the beginning of DNA replication. The Hct1-dependent activity of the APC degrades mitotic and S phase cyclins throughout G1 (Amon et al., 1994). Why do cells destroy cyclins and other APC substrates in G1? The simple answer is that G1 gives cells time to integrate information from their environment before committing themselves to cell growth and DNA replication, an intrinsically risky business. A second possibility relates to the much greater separation of origins in postembryonic cell cycles. The further the separation,

the more important it is for each origin to fire, and a long period without Cdk1 activity could ensure that each origin bound the replication factors it will need to fire later on. If this logic is correct, the mitotic and S phase cyclins must be banished from G1 cells, and we might expect that their expression there would lead to problems in DNA replication that could cause genetic instability. In budding yeast, shortening G1 by overexpressing cyclins leads to reduced efficiency of protein assembly at replication origins and genetic instability (Tanaka and Diffley, 2002).

How Do Cyclins Find Their Substrates?

I have argued that the defining features of different cyclins is when and where they appear, rather than which substrates they can induce Cdks to phosphorylate. This conclusion comes from the observation that Cdk1 and a single B type cyclin can suffice for DNA replication, centrosome duplication, mitosis, and cell division. Such mutants are, however, clearly compromised, even when the expression of the default cyclin has been adjusted to occur at the right time (Cross et al., 1999), suggesting that there must be some differences in the substrate specificity that different cyclins confer on the same Cdk. A similar conclusion comes from the observation that even though cyclin A can induce mitosis in embryonic cell cycles (Knoblich and Lehner, 1993; Minshull et al., 1989; Strausfeld et al., 1996), cyclin A can exist and be active in the nucleus of tissue culture cells without inducing nuclear envelope breakdown.

Do these differences reflect differences in the enzymatic preferences of different cyclin-Cdk complexes or their different distributions in cells. There have been two approaches to this problem, carefully investigating kinase substrate interactions in vitro and devising new methods to find out exactly which kinase is phosphorylating a given substrate in vivo. In vitro experiments have examined the idea that cyclins can bind to Cdk substrates, thus vastly increasing their local concentration to allow efficient phosphorylation of sites that would be extremely poor substrates if they were presented as free peptides. This has been clearly demonstrated in the case of Pho80/Pho85, a cyclin/Cdk complex involved in regulating yeast genes for phosphate uptake. The cyclin Pho80 binds to Pho4, a transcription factor, and holds it long enough to allow Pho85 to phosphorylate an average of two sites on Pho4 for each encounter with the cyclin/Cdk pair (Jeffery et al., 2001). There has been less success in showing that differences between closely related cyclins lead to differences in kinase specificity. An "hydrophobic patch" has been reported to account for the different abilities of cyclin A- and cyclin B-containing complexes to phosphorylate substrates like the retinoblastoma protein (Schulman et al., 1998), but the residues that were mutated to reach this conclusion are conserved between cyclins A and B.

Examining the phosphorylation of peptides by Cdk/cyclin complexes supports the notion that both Cdk and cyclin can play a role in recognizing substrates. Peptides that match the consensus sequence, (S/T)PX(R/K), are recognized with a Km of about 100 μM, the absence of the final charged residue dramatically weakens binding, and there is little difference between Cdk1 and Cdk2 and amongst cyclins A, B, and E in the effects of amino acid substitutions in the substrate (Holmes and Solomon, 1996). Coupling a consensus peptide to sequences that interact with the hydrophobic patch on cyclins makes binding a hundred times stronger, showing that interaction with cyclin can dramatically improve substrate binding and explaining why many known phosphorylation sites match the much less restrictive consensus (S/T)P (Takeda et al., 2001). The most extreme case is the phosphorylation of the Cdks themselves. All Cdks require phosphorylation in a region called the T loop as well as cyclin binding to become active protein kinases. This reaction is carried out by cyclin-activating kinase (CAK, a complex of Cdk7 and cyclin H), and the phosphorylation sites in the T loop of both Cdk7 and Cdk2 lack any of the features of the Cdk consensus site. Making chimeras between Cdk2 and Cdk7 reveals that sequences outside the T loop control which Cdk can recognize this critical phosphorylation site (Garrett et al., 2001).

The second approach has been to devise methods that can reveal which kinase phosphorylates a substrate in vivo, even when several different kinases have overlapping substrate specificities. Shokat and his colleagues rose to this challenge by genetically engineering kinases so they are uniquely able to utilize analogs of ATP that have been made too bulky to fit any wild-type kinase (Bishop et al., 2000; Shah et al., 1997). If a substrate receives radioactive phosphate derived from the modified ATP, it must have been directly phosphorylated by the altered kinase. Because the ATP analog is charged, it can only be used in cell extracts (Ubersax et al., 2003), raising the question of how different the extracts are from the cell they were made from. The modified kinases can be uniquely inhibited in vivo by using cell-permeable inhibitors, but the effects seen here have the same caveat associated with genetically manipulating kinases; when cells alter their behavior and a target protein's phosphorylation falls, the changes could be indirect effects caused by reduced phosphorylation of a downstream kinase that actually phosphorylates the substrate. An equally serious problem is the enormous number of substrates such experiments reveal and the fact that for many of them, mutating the putative phosphorylation sites has little effect on cells. This frustrating result may reflect the overlapping effects of phosphorylation of several different proteins in the same pathway, making it impossible to see strong effects by abolishing the phosphorylation of a single protein. In addition, there may be little or no selection against consensus phosphorylation sites (for Cdk1 or other kinases) that are located outside functionally important parts of proteins.

A final approach is identifying cDNA clones that encode protein kinase substrates by looking for translation products whose electrophoretic mobility changes on protein phosphorylation (Stukenberg et al., 1997). This approach has been applied to look for proteins that are phosphorylated by cyclin A/Cdk2 and cyclin B/Cdk1 complexes. Of the substrates for cyclinB/Cdk1, roughly a quarter show some preference toward one kinase or the other, but in none of these cases is the specificity absolute (T. Hunt, personal communication).

Taken together, the evidence reveals two classes of

substrates for Cdk1. The first are high turn over substrates, like histone H1, that have a close match to the extended consensus phosphorylation site, are recognized only by the substrate binding pocket on Cdk1, and bind relatively weakly to the cyclin/Cdk complex. The second are low turn over substrates that bind tightly to a substrate recognition site on the cyclins and can be phosphorylated at sites that can lack all features of the consensus sequence. The low turn over substrates raise two problems. The more strongly they bind, the more they will act as competitive inhibitors that sequester Cdk molecules that could otherwise be phosphorylating substrates, and the ability to see processive phosphorylation of some low turnover substrates (such as Pho4 [Jeffery et al., 2001]) suggests that their must be considerable flexibility in the substrate, the kinase complex, or both. When careful measurements have been made, there are subtle differences (up to 5-fold) in substrate recognition between different cyclins complexed to the same Cdk or between Cdk1 and Cdk2. The size of the differences is comforting. They are small enough to help explain cells' otherwise astonishing ability to survive even after scientists have abolished much of the variety in their cyclins and Cdks. They are also large enough, especially if they are multiplied by differences in the local concentrations of cyclin/Cdk complexes, to explain why such genetically manipulated cells often limp through the cell cycle.

What Does Phosphorylation Do?

The eukaryotic cell cycle engine starts and stops the morphological and biochemical events that make up the cell cycle. To understand the cell cycle, we need to know how the engine induces individual events and how these events are coordinated with each other.

Mitosis illustrates some of these questions. The events of mitosis have been known for more than 100 years (Wilson, 1928). As cells approach this stage, their chromosomes start to condense within the nucleus, the centrosomes divide and migrate to opposite sides of the nucleus. Mitosis proper begins when the nucleus breaks down and the chromosomes align on the mitotic spindle. After a brief pause in this state, the sister chromosomes separate from each other and segregate to opposite poles of the spindle, the daughter nuclei form, and the cell divides in two. In the fastest embryonic cell cycles, this complicated dance can occur in less than four minutes. How does Cdk1 induce nuclear envelope breakdown, chromosome condensation, and the changes in microtubule dynamics that lead to assembly of the mitotic spindle, and how are these processes integrated to align all the chromosomes correctly on the spindle?

Nuclear envelope breakdown is the best understood of these processes, and the gaps in our knowledge indicate how far we have to go before we can reach full knowledge of how Cdk/cyclin complexes induce mitosis. The envelope consists of a double membrane that is contiguous with the endoplasmic reticulum and lies over the lamina, a shell made from polymers of the nuclear lamin proteins. As cells enter mitosis, the lamins are phosphorylated and depolymerize. This phosphorylation can be performed by Cdk1/cyclin B complexes (Peter et al., 1990; Ward and Kirschner, 1990), these complexes can disassemble lamin polymers in vitro (Peter et al., 1991), and mutating the phosphorylation sites blocks disassembly of the nuclear lamina as cells enter mitosis (Heald and McKeon, 1990). So far so good, but there are a number of complications, including phosphorylation sites for other kinases, such as protein kinase C (Hennekes et al., 1993) and S6 kinase II/p90^{rsk} (Ward and Kirschner, 1990), and the demonstration that MAP kinases can phosphorylate at least some of the sites modified by Cdk1 (Peter et al., 1992).

Although the ability of Cdk1 to phosphorylate and depolymerize the nuclear lamina has entered the cell biology textbooks, we are still not sure whether other protein kinases are needed in addition to Cdk1 (Collas, 1999). As the nucleus breaks down, nuclear pores disassemble and the nuclear membranes vesiculate. Both of these processes are substantially less well understood, and their dependence on Cdk1-mediated phosphorylation is not yet clear. Nuclear envelope breakdown also emphasizes the importance of controlling the location of cyclin/Cdk complexes (reviewed in Takizawa and Morgan, 2000). During interphase, cyclin B is actively exported from the nucleus blocking its access to the lamins. As cells enter mitosis, cyclin B is phosphorylated, inactivating its export signal and accelerating its import. The nature of the phosphorylating kinase remains controversial, as do the quantitative aspects of the feedback loop that leads to a very rapid rise in the nuclear concentration of cyclin B before the permeability of the nuclear envelope has fallen (Terasaki et al., 2003). This observation suggests that cyclin B/Cdk1 complexes enter the nucleus and then disassemble the nuclear pores from the inside of the nucleus, leading to large holes in the nuclear envelope that abolish the barrier between nucleus and cytoplasm (Lenart et al., 2003; Terasaki et al., 2001).

Other aspects of mitosis are no better understood than nuclear envelope breakdown. There are two principal problems. We lack a complete catalog of the proteins involved in any of these complicated cell biological events, let alone a description of protein phosphorylation, which would ideally include the sites of phosphorylation, the identity of the phosphorylating kinases, and a kinetic description of how the modification of different molecules changed as cell passed through mitosis. Even if we could obtain all this information, we would need to understand how the overall features of processes like chromosome condensation or spindle assembly arise from the molecular interactions of their components before we could understand how changes in protein modification produce these large architectural changes in the cell.

A Call to Arms

I have emphasized how little we understand about the cell cycle, rather than how much we have learned over the last 25 years. This choice reflects the belief that understanding the cell cycle is crucial to understanding fundamental problems in biology and medicine and that our current knowledge fails to make accurate predictions or reliably satisfy our curiosity about how the cell cycle works.

The history of studies on bacterial chemotaxis is a

useful analogy to the cell cycle. In the mid 1970s, receptor methylation was the only known modification in the bacterial signaling pathway (Koshland, 1977). During the 1980s, it was discovered that protein kinase rapid phosphorylation was responsible for rapid responses, while methylation produces the slower adaptation that couples the cell's responsiveness to the recent history of the attractant concentration (Bourret et al., 1991). In the 1990s, the structures and quantitative biochemical parameters of most of the components were determined making quantitative models of chemotaxis possible, a point well beyond our current understanding of the cell cycle. In the last few years, the remarkable amplification that allows cells to respond to very shallow concentration gradients (Cluzel et al., 2000; Duke and Bray, 1999; Sourjik and Berg, 2002) and the robustness of chemotaxis to environmental and genetic insults has begun to be understood (Alon et al., 1999; Barkai and Leibler, 1997).

The precedent of such major readjustments should be a strong incentive to reach the same sort of detailed understanding of the eukaryotic cell cycle. Strategies that might help us include the following:

(1) Identifying a minimal number of connected problems to concentrate on, for example, the mechanism of the regulation and mechanism of the APC, spindle assembly, and the spindle checkpoint.

(2) Finding one or two organisms that allow the equivalent of genetic manipulation, sophisticated manipulation of the process in vitro, and offering the hope of purifying and reconstituting the full process or partial reactions. For mitosis, the most attractive candidates are budding or fission yeasts and frog egg extracts. The yeasts have extremely sophisticated genetics, but the range of processes that can be closely mimicked in vivo is small. Frog egg extracts (Lohka and Masui, 1983) are attractive because they recapitulate mitosis in its entirety. Removing proteins and replacing them with mutant versions offers an approximation to genetics, and the extracts are accessible to many reagents that cannot easily cross cell membranes, such as the ATP analogs used to directly identify the substrates of a particular protein kinase (Bishop et al., 2000). Intensive work in one or two organisms will refine the comparisons between the workings of the cell cycle in the wide range of eukaryotes that are currently studied.

(3) Developing optical probes that report on the kinetics and localization of protein phosphorylation in living cells and extracts.

(4) Making "toy systems," synthetic assemblies of molecules that are much simpler than those inside cells, yet mimic some of the key features of the process. For example, mixtures of motors and microtubules in small chambers do self-organize (Nedelec et al., 1997), chambers with motors that march in opposite directions can produce spindle-like structures (Surrey et al., 2001), and it would be interesting to explore how the addition of chromosomes and Cdk1 would modify these model systems.

The next ten years will reveal whether we have the commitment for the hard experiments that will be needed to challenge current dogma, overturn it when necessary, and move on to a deeper understanding of the cell cycle.

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