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Demystified …

Cell cycle

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The cell cycle is a highly organised and complex process, which ensures that there is complete and accurate replication of the cell before division. Our understanding of the molecules behind these events is both relatively new and as yet incomplete. There are three main proteins that regulate the cell cycle, namely: cyclin dependent kinases (CDKs), the cyclins, and cyclin dependent kinase inhibitors (CKIs). The CDKs, once activated, phosphorylate other proteins, allowing them to carry out their role at a particular stage of the cell cycle. The CDKs are regulated by cyclins, which only activate CDKs when they reach a critical concentration. The CKIs control the activation of the CDK–cyclin complex by inhibiting its formation.

A basic understanding of the interaction between these three main groups of proteins and other regulating molecules during the different phases of the cell cycle should help to clarify this fundamental procedure.

Background

Cell division is a fundamental process that is required throughout the life of all eukaryotes. Although it has been known for many years that cells have the ability to grow and replicate, the actual mechanisms involved have only been discovered relatively recently. In fact, although a number of the mechanisms are beginning to be understood, there is still a great deal that cannot be explained and proteins that have not been identified. Hence, in the past thirty years or so there has been intense study of the cell cycle. This was aimed at trying to find out how the cell controls its own replication in such a way that it can ensure that all stages are carried out in the correct sequence, that one stage is completed before the next one starts, and that a damaged cell is not allowed to continue to proliferate unchecked.

In humans, some cells, such as neurones, do not replicate at all once they become differentiated, and others from organs such as lung, kidney, and liver only replicate in response to part of the organ being damaged or removed. Although some human cells may proliferate relatively rapidly, such as the epithelial cells of the small intestine, even the quickest of these will take at least 24 hours to complete a single cell cycle. Hence, human cells were not considered an ideal model for the study of the cell cycle, not only because of their prolonged cell cycle time but also because the mechanisms were likely to be more complex in a higher eukaryote than in a lower order one. Therefore, investigators turned to lower order organisms to study the cell cycle. Work has primarily been carried out using frog oocytes, the budding yeast (*Saccharomyces cerevisiae*), the fission yeast (*Schizosaccharomyces pombe*), sea urchins, and clam embryos.

These studies resulted in the discovery of the three main components of the cell cycle; namely the activating enzymes, cell division cycle (CDC) kinases or CDKs; their activating cyclins; and CDC/CDK inhibitors, collectively known as CKIs. In 1971, two groups of workers[1 2] independently found that if a frog oocyte that had been arrested after DNA synthesis was injected with the cytoplasm of another oocyte, which had not been arrested, the cell would enter mitosis. The substance present in the cytoplasm that induced this continuation of the cell cycle was called "maturation promoting factor". A term that has continued to be used until recently when it was replaced by the more appropriately named "M phase promoting factor".

In 1973, Hartwell identified mutant proteins in *S cerevisiae* that arrested the cell at certain phases of the cell cycle.[3] He called these CDC mutants and concluded that the wild-type genes found in their wild-type counterparts were responsible for progression through the cell cycle. In the CDC28 mutant, the cell cycle arrest occurred before mitosis, suggesting that normal CDC28 was a component of the maturation promoting factor. At about the same time, using *S pombe*, Nurse and colleagues[4] also identified the protein involved in regulating progression of the cell to mitosis, this was called CDC2. Thus, somewhat confusingly, the same functional protein was given two different CDC names because it was discovered in two different organisms.

Another milestone in our understanding of the control of the cell cycle came in 1983, when Evans *et al* identified two proteins that showed a cyclical pattern of expression during the cell cycle of sea urchin and clam embryos.[5] The concentrations of the proteins, called "cyclin A" and "cyclin B", increased steadily during interphase and declined rapidly during mitosis.

The final major group of proteins associated with regulating the cell cycle are the CKIs, which have been identified only in the last few years.[6] These proteins interact with the complex formed between a cyclin and a CDC/CDK in such a way that they inhibit the kinase

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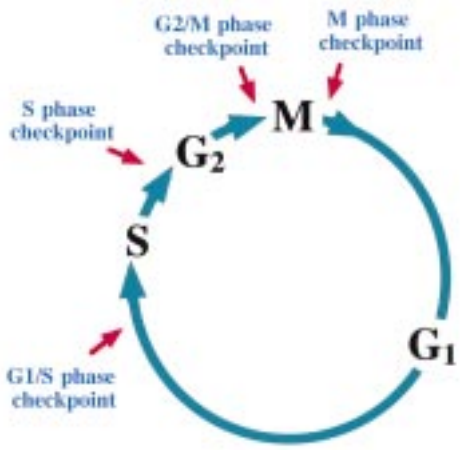

Figure 1 Phases and checkpoints of the active cell cycle.

activity and prevent the cell from progressing through the cell cycle.

The use of different models for studying the cell cycle and the gradual understanding of the role of different cell cycle associated proteins accounts for much of the confusion in terminology. For example, a protein that carries out a similar role in different organisms might be given a different name for each organism. The protein CDC2, referred to above, from *S pombe*, which is known as CDC28 in *S cerevisiae*, is known in human cells as either CDC2 or, more appropriately CDK1. In general, for yeast cells, sea urchins, clams, and frog oocytes CDC is used, but for mammalian cells CDK is used; however, this is not always the case.

Yeasts and other eukaryotes continue to be used as models for studying the cell cycle. However, although many of the proteins discovered in yeasts have their human counterparts, it is not appropriate to assume that all of the new proteins identified in lower eukaryotes have a human equivalent.

The following overview describes the proteins involved and their interaction in the human cell cycle.

Mechanisms of initiating and maintaining the cell cycle

The proteins involved in the cell cycle are closely linked with the common purpose of ensuring that, after appropriate stimulation, the cell accurately and completely replicates its DNA before cell division. Hence, these proteins have a particular role during specific parts of the cycle, so that events occur in the correct sequence. The active cell cycle has been divided into four phases (fig 1). Mitosis (M phase) was the first to be identified because it has distinctive morphological stages, unlike other phases of the cycle, which are known collectively as interphase. Subsequently, increased knowledge of the structure and function of DNA gave rise to the introduction of the term S (synthesis) phase, which related to DNA synthesis. It was apparent that these two phases could not just run from one to the other (although this is the case in some rapidly proliferating embryonic cells) and that there must be a break between the two. These breaks or gaps occur between rounds of mitosis and DNA synthesis. The first break immediately after mitosis was called gap 1 (G1) and the second break after S phase, gap 2 (G2). A fifth phase has also been identified, which occurs when a viable cell is not involved in the active cell cycle. Such quiescent cells enter the gap 0 (G0) phase from which they can re-enter the cell cycle upon stimulation. The phases have no definite start and finish point, but each one tends to be a stage at which one function is coming to an end and another is beginning. However, when discussing the cell cycle, these phases are commonly referred to, because they are a useful way of navigating through the processes, but it should be understood that they do not delineate distinct parts of the cycle, but merely "phases".

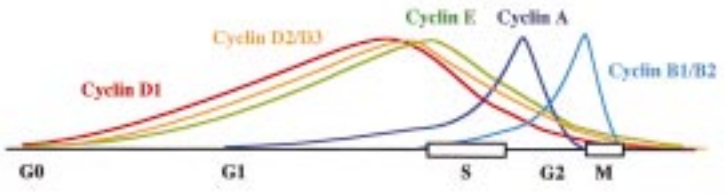

Figure 2 Cyclin expression during the cell cycle.

CDKs and cyclins

The cell cycle is controlled by phosphorylation of various proteins carried out by a family of enzymes (serine/threonine kinases), the different members of which have specific roles during certain parts of the cycle, and are termed cyclin dependent kinases. As the name implies, the kinases are activated by specific cyclins, named because of their cyclical concentrations during the course of the cell cycle (fig. 2). Thus, a CDK can only be activated once its partner cyclin has reached a critical concentration, it carries out its role and then becomes "deactivated" as the cyclin concentration decreases. Some CDKs are activated by more than one cyclin, which sequentially form a complex with the CDK, thus maintaining its activity through a longer period of the cell cycle. These waves of CDK activation continue throughout the cell cycle from early G1 to M phase (fig 3). Eight CDKs have been identified so far (CDK1 to CDK8), although not all of them are involved directly in the cell cycle.[7] They show remarkable structural similarities, with a 75% sequence homology. However, to enable them to bind specifically to their activating cyclin they possess unique binding sites.[7]

Currently, 14 cyclins (cyclin A to cyclin J, some of which have been subdivided—for example, there are three D type cyclins: D1, D2, and D3) have been identified, although the functions of all of them have not been resolved.[6] Common to the structure of all members of this family is the cyclin box, which is a series of 100 amino acids.[8 9] Some cyclins also have similar sequences of amino acids at their N-terminal region, called the destruction

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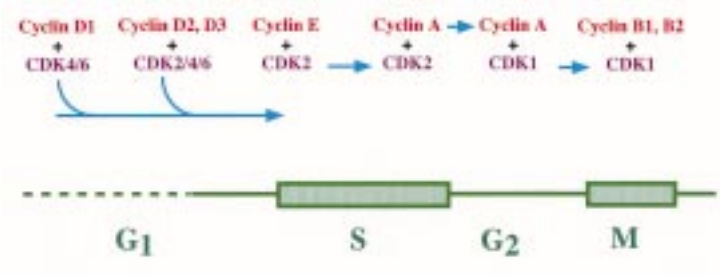

Figure 3 CDK–cyclin associations in the cell cycle.

box, which brings about their own degradation.[10 11] The concentrations of different cyclins oscillate throughout the cell cycle. When concentrations are high, the cyclin can bind CDK, but when the activated CDK–cyclin is no longer required, cyclin is destroyed and concentrations subsequently drop.

Cyclin alone does not fully activate CDK, the latter also requires phosphorylation and dephosphorylation of some specific amino acid residues to become fully functional. This is, in part, carried out by CDK activating kinase (CAK), which is composed of cyclin H and CDK7.[12 13] Thus, only when CDK has been activated by its partner cyclin and also by CAK and other similar functional proteins, can it carry out its role within the cell cycle.

Start of proliferation

A cell can be stimulated to divide by numerous external signals, including growth factors and hormones. These ligands are bound by receptors present on the surface and within the cell which signal the cells in G0 or early G1 to initiate progression through the cell cycle and pass ultimately to cell division. The signal to replicate affects the concentrations of both cyclin D and cyclin E, collectively known as the G1 cyclins.

As a result of stimulation there is an increase in the expression of the cyclin D-type proteins (cyclin D1 to cyclin D3). Cyclin D1 can form a complex with both CDK4 and CDK6, cyclin D2 and cyclin D3 also form a complex with these two kinases and, unlike cyclin D1, with CDK2.[14 15] The functional differences between the three different D-type cyclins have not been clarified. There is evidence, based on cell line work, to suggest that they are each expressed at different times during G1, although this is dependent on cell type.[16] Ultimately, they all contribute to the phosphorylation of the retinoblastoma gene product (pRb) and its associated proteins, p107 and p130.

In early G1, pRb is present in a hypophosphorylated form that binds to and inactivates some of the E2F transcription factors that have a fundamental role in preparing the nucleus for DNA replication. Hence, until pRb is fully phosphorylated, E2F cannot be released and DNA synthesis cannot take place.[17] The activation of CDK4 and CDK6 by all three D-type cyclins and CDK2 by cyclin D2 and cyclin D3, in response to growth stimulatory signals, is the start of the phosphorylation process that removes the inhibitory effects of pRb (fig 3). Having initiated the phosphorylation of pRb, concentrations of D-type cyclins increase throughout G1 until they reach a peak just before S phase (fig 2). At the onset of S phase, their concentrations decline so that they no longer activate CDK4/6. However, at this stage pRb is not yet sufficiently well phosphorylated to release E2F, so the role of phosphorylation is taken over by the next set of cyclin–CDK complexes, cyclin E and CDK2 (fig 3). Towards mid to late G1, levels of cyclin E increase until they reach the stage when they can activate their partner, CDK2 (fig 2). This complex achieves maximum activity during the transition from G1 to S phase and primarily takes over the role of cyclin D–CDK4 and cyclin D–CDK6 until phosphorylation of pRb reaches the stage at which it can no longer bind E2F. DNA synthesis can now proceed. Hence, it is thought that it is the action of cyclin E–CDK2 that triggers the cell to enter S phase.

DNA synthesis

Once the cell enters S phase, cyclin E is degraded rapidly and the activation of CDK2 is taken over by cyclin A (fig 3). The concentrations of cyclin A increase gradually during the latter stages of G1 and some binding with CDK2 is a prerequisite for entry into S phase (fig 2). However, once S phase has begun, the cyclin A–CDK2 complex has an important role in both the initiation and maintenance of DNA synthesis, because it activates the proteins at the DNA replication origins that initiate DNA synthesis at different sites or origins on the chromatids.[18] The mechanisms of DNA replication are complex and beyond the scope of this article, but suffice to say that numerous proteins are involved in this intricate procedure, all of which work together not only to replicate the DNA template but also to provide the structural proteins required for a functional chromosome. There have been a number of review articles published on the mechanics and control of DNA synthesis, but like many aspects of the cell cycle it is a rapidly evolving area of research and discovery.[18–21]

The activation of CDK2 by cyclin A is necessary for the continuation of the S phase, but towards the end of this phase cyclin A starts to activate another kinase, CDK1, in preference to CDK2 (fig 3). This signals the completion of the S phase and the onset of another gap phase, G2. Upon the completion of the S phase, the G2 phase provides a break between the fundamental procedures of DNA synthesis and mitotic division. During this gap the cell can ensure that DNA replication is both complete and accurate. As yet, the control of these processes is less well understood.

Mitosis

For some time it has been known that a specific protein (or group of proteins) is involved in the signal to initiate the onset of mitosis. This protein was initially known as maturation promoting factor but is now called M phase promoting factor (MPF). MPF consists of CDK1 and its activating mitotic cyclins, cyclins A or B. While it appears that cyclin A–CDK1 has a more important role in the completion of S phase

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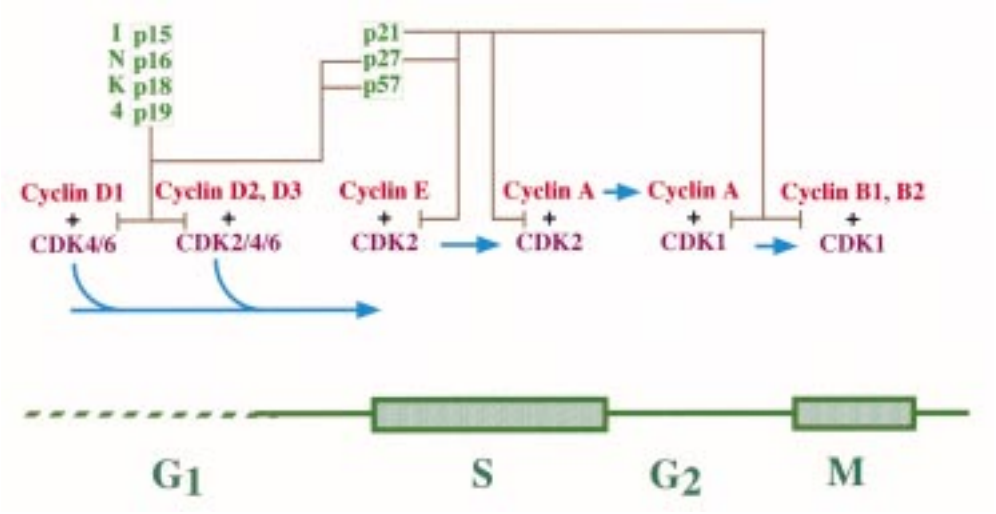

Figure 4 Action of CDK inhibitors in the cell cycle.

and the preparation for mitosis, cyclin B–CDK1 controls the onset, sequence of events, and the completion of mitosis. Currently, two B-type cyclins have been identified, B1 and B2, which are present at very low concentrations in G1. The concentrations increase gradually during the latter stages of the S and G2 phases, until they reach a peak during mitosis (fig 2). The B cyclins take over from cyclin A as the activating partner of CDK1 during mitosis; however, the kinase is not functionally active until a sequence of phosphorylation and dephosphorylation steps have occurred. This provides a safety mechanism to ensure that mitotic division does not occur until DNA synthesis has been completed. Although cyclins B1 and B2 appear to carry out the same functions, they act at different locations. Cyclin B1 co-localises to the microtubules, whereas cyclin B2 co-localises to the Golgi region.[22]

Mitotic division commences only when CDK1, with its bound cyclin B, has been phosphorylated at threonine 14 and tyrosine 15 by WEE1,[23] at threonine 161 by CAK, and dephosphorylated at threonine 14 and tyrosine 15 by CDC25.[24] The activation of CDK1 in this manner leads to the phosphorylation of structural proteins in the nucleus, including nucleolin, nuclear lamins, and vimentin. The nucleus then embarks on the well recognised, although not fully understood, stages of mitotic and cellular division. Prophase is the first recognised step in mitosis and is initiated when CDK1 is fully phosphorylated; metaphase, anaphase, and telophase follow on as the nuclear proteins and chromosomes pass through the sequence of events which ensures that an equal number of chromosomes end up in each daughter cell.[25 26] Upon completion of mitosis, cytokinesis or cell division can then take place. Cyclin B is destroyed, CDK1 is no longer activated, and the cell enters G1 or G0.

G0 phase

Depending upon the type of cell and its state of differentiation, the cell may step out of the cell cycle, while still remaining viable; this is known as entering G0. In general, terminally differentiated cells enter G0 from which they do not return. However, cells in organs with an extremely slow proliferative capacity, such as hepatocytes, are known to re-enter the cell cycle if there has been surgical removal of all or part of the lobes.

The constant supply of CDKs and fluctuating concentrations of cyclin that bring about progress through the cell cycle are a series of positive factors. However, we must consider what stops the cell from embarking on a round of proliferation when either the environmental factors are not suitable to sustain viability or when there are intrinsic errors in the replicated DNA. The latter may have occurred before the onset of cellular replication or might have happened during the process of DNA synthesis. There are a number of regulators or inhibitors that become activated at different stages of the cell cycle and some "checkpoints" that try to overcome these problems with their potentially fatal outcome.

Inhibitors of the cell cycle

The activation of CDK by cyclin has the potential to form a self perpetuating cycle, with little regulation. It has been recognised for some time that there are two main checkpoints in the cell cycle, one controls the mechanisms that initiate the onset of DNA synthesis and the other controls the start of mitosis itself. There are many other "points" in the cell cycle that check the previous stages, for example correct DNA replication, adequate microtubule formation, and attachment during mitosis.

CDK INHIBITORS

Control of the transition from one phase of the cell cycle to the next is brought about by regulating the activation of CDKs by their partner cyclins with CKIs (fig 4). These inhibitors can be upregulated when required, thus blocking the activation of CDK by cyclin. This retains the cell in a particular part of the cell cycle until conditions are such that it can continue towards proliferation or, if necessary, be steered towards cell death (apoptosis).

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There are two main groups of CKIs, the p21 family and the INK4 family, members of which have similar structural features. The p21 family consists of p21 itself, p27, and p57. p21 was the first CKI to be identified; unfortunately, it was described independently by several different workers and, consequently, has been given a number of different names. Thus, p21 is also known as wild-type p53 activated fragment 1 (WAF1), CDK2 interacting protein 1 (CIP1), senescent derived inhibitor 1 (SDI1), and melanoma differentiation associated gene (mda-6). p27 and p57 are also known as kinase inhibiting protein 1 (KIP1) and 2 (KIP2), respectively. These inhibitors tend to have wide ranging roles and can potentially inhibit a number of different CDKs. The INK4 family is far more specific, consists of $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$, and until recently was thought only to have a role in G1, where members of the family inhibited the action of CDK4. However, a second INK4A gene product has been identified recently, $p19^{ARF}$, which binds to and inactivates the p53 regulatory protein, MDM2, thus allowing increased p53 stability and subsequent cell cycle arrest.[27]

Control of progress from G1 to S

Before the onset of the active cell cycle, p27 is present at a high concentration, and this prevents either CDK4 or CDK6 from becoming activated by cyclin D1, cyclin D2, or cyclin D3. Hence, the cell is kept in either G0 or early G1 awaiting mitogenic stimuli. Upon suitable stimulation, p27 concentrations are reduced and this, in conjunction with increasing concentrations of cyclin D, allows the activation of CDK and the subsequent chain of events that allows the phosphorylation of pRb. pRb itself is an inhibitor of the cell cycle, and it has been termed the "gateway" to proliferation because it needs to be phosphorylated to allow the cell to progress.[28] Thus, pRb in its active form prevents progression through the cell cycle and its phosphorylation renders it inactive.

Concentrations of p27 can remain relatively high during G1 so that it maintains some control during this phase of the cell cycle. If mitogenic stimulation is suddenly stopped, the concentration of p27 increases further and the cell is held at that particular stage in G1.[29] Upon the re-introduction of growth sustaining stimuli, these inhibitory mechanisms are released and the cell proceeds through the cycle. It is well recognised that there is a "point of no return" during late G1 at which the cell is committed to proliferation and the removal of growth stimulating factors has no effect. This is called the "restriction point" or "R point" (or "START" in *S cerevisiae*).[30] Although the cell is no longer influenced by external factors, it retains some self control, and intrinsic factors can lead to further inhibitory effects during the course of the cell cycle. The primary function of p27 appears to be during these early stages of the cell cycle (G0 and G1), when it inhibits CDK4, CDK6, and CDK2 (fig 4).[31] The other main inhibitors of CDK4 and CDK6 are the INK4 CKIs (p15, p16, p18, and p19), which interact almost specifically with these kinases. Both p15 and p16 are recognised as tumour suppressers that are regulated by pRb. However, there is no evidence to suggest that p18 and p19 have similar functions. All four proteins prevent the activation of CDK4 and CDK6 by either destabilising the association between CDK and cyclin or displacing the cyclin from the CDK.[32] Although p16 and p27 have the ability to inactivate CDK4 and CDK6, it appears that p16 has the stronger association. It is thought that if the concentration of p16 is increased so that it can inactivate CDK4/6, p27 then becomes the prime inactivator of CDK2.[33]

During the early part of the cell cycle, any damage that might have occurred to the DNA can also be detected and the cell can be delayed in G1 to allow time for repair. For example, in response to damage caused by either ultraviolet or ionising radiation, the tumour suppresser gene product, p53, induces transcription of p21.[34] The increased concentrations of p21 can then inhibit the activation of CDK2 by either cyclin E or cyclin A. This arrests the cell in the late G1 phase or early S phase so that the DNA can either be repaired or, if damage is too extensive, so that the cell can be directed towards apoptosis.

The third member of the p21 family also has a role in the early phases of the cell cycle. p57 can bind to and inactivate CDK2, CDK3, and CDK4; however, its role within the cell cycle is not clear and it is not fully understood what stimulus and which proteins control the expression of p57.[35]

Control of progress from G2 to M

The response to DNA damage can also occur later in the cell cycle, just before the onset of mitosis. Again, p53 mediates the increased expression of p21, which can arrest the cell cycle by preventing the activation of CDK1 by cyclin B (fig 4). The different stages of mitosis are also monitored closely so that one stage cannot start without the previous one being completed. Suffice to say, many of the inhibitors that maintain control of mitosis are still not identified. Although some have been discovered in yeast, their mammalian counterparts have remained elusive. Two proteins found recently to have a role in mitosis are BUB1 (budding uninhibited by benomyl) and MAD2 (mitotic arrest deficient), both of which monitor the assembly of the chromosome on to the mitotic spindle in metaphase.[36 37] Until the chromosomes are attached correctly to the spindle, MAD2 binds and inactivates CDC20. Once activated, CDC20 can destroy the proteins that prevent the separation of the two chromatids.

Value of studying the cell cycle

A considerable amount of time, effort, and expense is being invested worldwide in the study of the mechanisms that control the cell cycle. Obviously, from a purely biological point of view it is interesting to know how the associated proteins interrelate to perform such a complex and important task. However, the real value of this effort is to apply the knowledge of

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events in the normal proliferating cell to increase the understanding of how the cycle can become deregulated. We need to be able to explain why some cells have uncontrolled proliferation (hyperplasia), and how malignant cells can overcome the checkpoints that should prevent proliferation of any cell in which the DNA has been replicated incorrectly.

The measurement of proliferative activity has been used for many years to predict the likely course of disease progression in different cancers.[38] If the cells are proliferating rapidly without a corresponding increase in cell death, the accumulation of these rapidly dividing cells forms a mass or lump. Thus, in malignant tumours, the speed with which the cells are dividing provides a reasonable measure of the aggressiveness of the tumour. Initially, the only way of measuring cellular proliferation was to draw conclusions from the number of mitotic figures in a sample of the tumour.[39] In many ways, this is still the preferred method of estimating the proliferative capacity of a lesion, despite the fact that other "markers" are available for demonstrating events during interphase. These markers include the incorporation of tritiated thymidine into the DNA template during synthesis over a specified period of time, followed by measurement of the amount of uptake by autoradiography.[40]

More recently, antibodies have been produced against cell cycle associated proteins, which are present during particular phases of the cycle. Antibodies against the Ki67 antigen in particular have been used in studies involving cancers of most of the major organs.[41 42] In fact, we are now at a stage when antibodies have been produced against so many of the proteins involved in regulating the cell cycle, including the different CDKs, cyclins, and CKIs, that it is very difficult to ascertain their relative prognostic value. This problem is not helped by the way in which the cell, even a malignant one, appears to be able to compensate in part for the deregulated expression of one of these proteins. Experiments with mice in which a gene encoding a particular protein involved in cell cycle control has been deleted (knockout mice) have shown that many such animals are viable, although they do have some defects. For example, cyclin D1 knockout mice are small, have defective retinas, and do not develop mammary glands[43]; p27 knockout mice grow considerably larger than their litter mates[44]; and p21 knockout mice are no different to their normal counterparts.[45]

Although our understanding of the proteins and mechanisms controlling the cell cycle is improving rapidly, there are still many aspects that cannot be explained fully. These will continue to be resolved as new proteins are identified and as the functions of those that are already known are clarified. Once a basic understanding of the relation between cyclins, CDKs, and their inhibitors has been grasped, new findings can be inserted into this model and the mysteries of the cell cycle resolved.

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