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David Morgan's Lecture Part 2:
Cyclin-Dependent Kinases and the Cell Cycle

Teaching Tools were prepared by David Morgan.

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1. Review Questions

1. Review the molecular mechanisms that generate waves of Cdk activity during the cell cycle.
2. Review how protein kinases work, and explain how the analog-sensitive kinase mutant system allows identification of kinase substrates in a crude cell extract containing many different kinases.
3. Explain how the analog-sensitive kinase mutant system allows the construction of a cell line in which a single protein kinase can be specifically inhibited by addition of a chemical to the medium.
4. Describe some of the cellular processes that Cdks regulate as the cell proceeds through the various stages of the cell cycle.
5. Explain how cyclins might influence the physiological function of the associated Cdk.

6. Discuss some of the mechanisms by which phosphorylation changes protein function.

2. Answers to Review Questions

1. Oscillations in cyclin levels result in a series of distinct cyclin-Cdk complexes at different cell-cycle stages. Cyclin levels are controlled by changes in the expression of cyclin genes and by changes in the rate of cyclin destruction. The activities of different cyclin-Cdk complexes are also regulated by Cdk phosphorylation and association with various Cdk inhibitor proteins.

2. Protein kinases catalyze the transfer of the gamma-phosphate of adenosine triphosphate (ATP) to hydroxyl groups on specific amino acids in a protein substrate. The most common experimental method for labeling the substrates of a protein kinase is to use a version of ATP in which the gamma-phosphate is radioactively labeled with 32P, so that transfer of this phosphate places a convenient radioactive tag on the substrate. This method is not useful when searching for kinase targets in a crude cell extract, however, because the extract contains dozens of different protein kinases that can use 32P-labeled ATP to label their targets. The 'analog-sensitive' mutant approach provides a way around this problem. It is based on the fact that the adenine base of ATP binds in a deep hydrophobic pocket in the kinase active site. In most kinases, part of the adenine-binding site is formed by a large, hydrophobic amino acid at a specific position in the amino acid sequence. Kevan Shokat reasoned that changing this large amino acid to something smaller (alanine or glycine) would result in a 'hole' in the side of the adenine-binding site. This mutant kinase would then be able to bind a modified version of ATP (e.g. N6-benzyl ATP) in which the adenine base carries an extra benzyl ring to fit in the new hole provided by the mutation. Wild-type kinases cannot bind this bulky ATP derivative. The analog-sensitive mutant kinase could then be added to a crude cell extract with 32P-labeled N6-benzyl ATP, and only the mutant kinase would be able to use the radiolabeled ATP to label its targets.

3. Analog-sensitive kinase mutants contain an extra 'hole' in the side of their adenine-binding site. It is therefore possible to develop small adenine-related chemical compounds that fit tightly into the expanded adenine-binding site of a mutant kinase but do not fit into the smaller adenine-binding site of a wild-type kinase. In the case of analog-sensitive yeast Cdk1, as discussed in this lecture, a

small chemical called 1-NM-PP1 binds very tightly to analog-sensitive Cdk1 but has little affinity for wild-type Cdk1 or other kinases. It was therefore possible to construct a yeast strain (called the cdk1-as strain) in which the CDK1 gene is replaced with a gene encoding the analog-sensitive mutant. When 1-NM-PP1 is added to the culture medium, Cdk1 in this strain is rapidly and specifically inhibited, resulting in a cell-cycle arrest.

4. Cdks act on numerous DNA replication proteins, as well as chromatin proteins, to trigger chromosome duplication in S phase. They also act on large numbers of proteins to initiate the various events of early mitosis (particularly mitotic spindle assembly). Mitosis in particular is a major upheaval for the cell, and essentially all major cellular processes are adjusted in some way during mitosis. The substrate lists described in this lecture revealed that Cdks are also likely to control cellular processes that were not previously suspected to be important in the cell cycle, such as protein translation and protein secretion, and studies of these processes during the cell cycle might lead to new insights into Cdk function.

5. The primary function of a cyclin is to bind and thereby activate a Cdk catalytic subunit. In addition, cyclins often carry signals that take them to a specific location in the cell (e.g. the Golgi apparatus, in the case of human cyclin B2; or the nucleus, in the case of many other cyclins), and this subcellular localization clearly influences which substrates are phosphorylated by the associated Cdk. As discussed in this lecture, some cyclins also contain a substrate-docking site that increases their affinity for a specific subset of Cdk targets. In the case of Clb5, increased affinity for proteins involved in DNA replication allows Clb5 to be a particularly effective regulator of chromosome duplication in S phase.

6. Adding a large, negatively-charged phosphate to a protein can have a considerable impact on protein function. As discussed in the lecture, we can divide the effects of phosphorylation into two general categories. In some cases, the precise placement of a phosphate in a globular protein domain can result in a change in the conformation of that domain, which can influence its enzymatic activity or indirectly change its interaction with other proteins. In other cases, addition of clusters of phosphates to a protein surface can directly interfere with or promote interactions with other proteins.

3. Discussion Questions

1. Discuss the basic features of a mass spectrometer, with an emphasis on the methods used to identify Cdk1 substrates in the experiments described in this lecture.

2. Most phosphorylation events in the cell are not permanent: protein kinases are generally opposed by protein phosphatases that remove phosphates. Why are phosphatases critical for the success of the mass spectrometry approach to finding Cdk targets?

3. Imagine that you have discovered a protein kinase that you suspect helps regulate an essential cellular process such as mitotic spindle assembly. (a) Discuss the methods you might use to assess the importance of your protein kinase in mitotic spindle assembly. (b) Discuss some of the approaches you might use to identify the relevant targets of this kinase.

4. Imagine that you have identified a candidate target for your favorite protein kinase. How do you prove that this candidate is a physiologically relevant substrate of your protein kinase in the cell?

5. Imagine that your studies reveal that your favorite protein kinase has hundreds of potential substrates, as in the case for the Cdks described in this lecture. How do you assess the importance of phosphorylation of so many candidates?

6. The experiments described in this lecture led to the identification of several Cdk substrates that are phosphorylated more rapidly by one cyclin-Cdk complex (Clb5-Cdk1) than another (Clb2-Cdk1). Describe some experiments that would allow you to test if Clb5 specificity for these targets is important in the cell.

7. Fission yeast cells are able to survive reasonably well when they contain just a single cyclin that drives both S phase and M phase. It has been proposed that early eukaryotes also controlled their cell cycle with a single cyclin-Cdk complex. What are the potential problems that might arise when the cell cycle is controlled by a single cyclin-Cdk complex, and how did the evolution of multiple cyclins help solve these problems?

8. Cyclins are destroyed in mitosis (as discussed in Lecture 1), leading to Cdk inactivation; this allows phosphatases to dephosphorylate Cdk substrates. Dephosphorylation of Cdk substrates is essential for the completion of many late mitotic events. Interestingly, all cyclins are not destroyed at the same time in mitosis:

Clb5 is destroyed earlier (in metaphase) than most Clb2 (in late anaphase). How might the timing of destruction of different cyclins influence the time at which different Cdk substrates are dephosphorylated?

9. Several Cdk substrates contain clusters of phosphorylation sites that seem randomly scattered in poorly conserved regions. What experiment might you do to test whether the positioning of phosphates in these regions is important for their regulatory function?

4. Answers to Discussion Questions

1. Mass spectrometers are powerful and complex analytical instruments for determining the mass, and thus the structure, of chemical compounds. The mass spectrometers used in protein analysis typically measure the mass of whole proteins or peptides derived from proteins. The protein or peptide sample is vaporized and ionized, and the volatile ions are accelerated by magnetic fields through a mass analyzer. The behavior of the ions in the analyzer is then used to calculate their mass with extreme accuracy. In tandem mass spectrometry, peptide ions are fragmented by collision with inert gas, and the masses of the fragments are analyzed to determine the amino acid sequence of the peptide (and position of phosphate in the sequence).

   In the experiments described in this lecture, complex phosphopeptide mixtures from two parallel cultures were analyzed. One culture (the one treated with Cdk inhibitor) was labeled with heavy isotopes of nitrogen and carbon, so that the peptides from this culture could be distinguished in the mass spectrometer from the peptides in the untreated culture. The relative amounts of each phosphopeptide in the two cultures were determined to assess whether the abundance of that phosphorylation site declined after Cdk1 inhibition. Each phosphopeptide was also fragmented in the instrument to determine amino acid sequences and phosphorylation sites on each peptide. Powerful computer analysis methods were then used to analyze and organize the enormous numbers of phosphopeptide sequences generated in these experiments.

2. The amount of phosphate at any site in the cell is governed by the relative activities of protein kinases and phosphatases acting at that site. As the cell progresses through S phase and early mitosis, the amount of Cdk activity rises dramatically and overwhelms the opposing phosphatases, so that Cdk substrates are highly

phosphorylated. The quantitative mass spectrometry approach requires that chemical inhibition of Cdk1 reduces kinase activity to a level where the phosphatases win the battle, resulting in dephosphorylation of Cdk1 substrates.

In budding yeast, the dephosphorylation of many Cdk1 substrates is carried out by a phosphatase called Cdc14, which is activated transiently in late mitosis. Cdc14 activity is low at most other cell cycle stages; thus, many Cdk1 substrates are not extensively dephosphorylated when Cdk1 is inhibited at these stages. To enhance the number of Cdk1 substrates that are dephosphorylated after Cdk1 inhibition, some of the experiments described in these studies were carried out in cells synchronized in late mitosis, when Cdc14 is most active.

3.

(a) Your first goal is to demonstrate that the protein kinase is involved in spindle assembly. The most straightforward approach to this problem is to develop a method for inhibiting the kinase in the cell and then assessing the effect of that inhibition on spindle assembly. Because mitotic spindle assembly is essential for life, you cannot simply knock out the kinase gene; instead, you need to make a 'conditional' kinase mutant that can be turned off under specific conditions. If you are working in yeast, then one approach is to develop the analog-sensitive strategy used in this lecture. Alternatively, you can construct a temperature-sensitive mutant kinase that is active at low temperature and inactive at high temperature, as in many classical yeast genetic studies. You can place the kinase gene under the control of a gene promoter that can be turned off by adding specific sugars or other chemicals to the culture medium. If you are working in cultured animal cells, it might be possible to turn off synthesis of your kinase with RNA interference methods.

(b) Identification of kinase targets is difficult, and the ideal approach clearly varies in different cases. If your protein kinase has limited biological effects in a specific process, then one approach is to test the ability of purified kinase to phosphorylate candidate substrates that are known to be involved in that process. For kinases with broad functions (like Cdk1), the analog-sensitive method discussed in this lecture provides a relatively unbiased approach but is technically challenging and does not work for all protein kinases. A related strategy involves the use of 'proteomic libraries', which are large collections of yeast strains in which each strain carries a single known protein that is tagged with a purification tag so that it can be isolated from cell extracts. Using these libraries, it is possible to purify large numbers of specific proteins and test them as substrates for a purified kinase in vitro. Alternatively, arrays of different proteins can be 'printed' onto glass slides and tested

as substrates for a purified kinase. In the end, it is essential to carry out further experiments to demonstrate that the candidate substrate is phosphorylated by the kinase in the cell, as discussed in the next question.

4. There is no simple way to prove the importance of a specific phosphorylation site in the cell. Ideally, one should begin by pursuing the following lines of evidence.

   (a) The protein kinase should be able to phosphorylate the candidate substrate at a high rate in a test tube. The phosphorylated sites should conform to the known sequence specificity of the kinase, if any is known (Cdks, for example, almost always phosphorylate serines or threonines followed by a proline).

   (b) The candidate substrate should be phosphorylated in the intact cell at the same sites phosphorylated in vitro. The best approach to identification of phosphorylation sites in vivo is mass spectrometry, as discussed in this lecture.

   (c) Phosphorylation in vivo should occur at a time (i.e. cell cycle stage) when the kinase is known to be active, and at a subcellular location where the kinase is known to be active.

   (d) You should be able to formulate and test a good hypothesis to explain the physiological function of the phosphorylation; that is, why does the kinase phosphorylate that particular protein? Is the protein involved in a process known to be regulated by that kinase? If so, then mutation of the phosphorylation site to a nonphosphorylatable amino acid (e.g. alanine) should have the predicted effects on that process. In addition, replacement of the phosphorylation site with glutamate might mimic the effects of phosphorylation, although this approach works only rarely.

5. This is a question for which there is currently no good answer. Given the amount of labor required to prove the relevance of a single substrate (see previous question), it is hard to imagine rapid methods for assessing the importance of dozens or hundreds of substrates. One approach is to use various subjective criteria to choose the most interesting candidate from among the large list of possibilities, and then study that candidate in detail. An alternative might be to choose several interesting candidates and develop methods for rapidly constructing phosphosite mutants and analyzing their phenotype in the cell.

6. The general importance of Clb5 specificity is nicely illustrated by studies of yeast cells in which Clb5 is deleted and the mitotic cyclin Clb2 is expressed at the beginning of S phase instead of Clb5 (Cross, F.R. et al. (1999). Specialization and targeting of B-type cyclins. Mol. Cell 4, 11-19). The initiation of S phase is greatly delayed in these cells, indicating that Clb2 does not have the same S-phase

promoting activity as Clb5. Later studies argued that this activity depends on the substrate docking site of Clb5.

To assess the importance of individual Clb5-specific substrates, you could mutate the substrate binding site that interacts with the Clb5 docking site. The cyclin docking site typically interacts with a substrate motif called an 'RXL' motif, and mutation of this RXL motif abolishes the interaction between Clb5 and that particular substrate. Analysis of the phenotype of this RXL mutant in vivo would provide insights into the importance of Clb5 specificity for that protein in the cell.

7. If S and M phases are both triggered by the same cyclin-Cdk complex, then additional mechanisms must somehow ensure that S phase is initiated and completed before M phase. One possibility, for example, is that low levels of Cdk activity trigger S phase, and M phase is triggered later when cyclin-Cdk activity rises to some higher threshold. This mechanism is not expected to provide robust, switch-like initiation of separate S and M phases; indeed, cells operating on a single cyclin tend to have overlapping S and M phases, increasing the risk that mitosis begins with incompletely replicated chromosomes. This problem is solved by evolving a distinct cyclin (e.g. Clb5) that has high specificity for DNA replication proteins and is expressed earlier than a mitotic cyclin. In addition, cells have evolved mechanisms, called checkpoint mechanisms, that block the onset of mitosis until DNA replication is completed.

8. Because Clb5 is destroyed earlier than Clb2, Clb5-Cdk1 activity drops earlier and Clb5-specific substrates are likely to be dephosphorylated earlier, depending on the phosphatases that are present. In fact, at least two of the Clb5-specific substrates identified in the studies in this lecture (Fin1 and Ase1) are involved in mitotic spindle regulation, and it appears that dephosphorylation of these proteins in early anaphase is important for controlling the behavior of the mitotic spindle in anaphase. For a full discussion of these issues, see the following review: Sullivan, M., and Morgan, D.O. (2007) Finishing mitosis, one step at a time. Nat. Rev. Mol. Cell Biol. 8, 894-903.

9. In principle, the regulatory function of phosphorylation should be preserved even if the phosphorylation sites are moved around in the amino acid sequence.

5. Explain or Teach These Concepts to a Friend

1. Explain how the analog-sensitive mutant system allows identification of kinase substrates in a crude cell lysate containing many different kinases.
2. Explain the mechanism underlying the high activity of certain cyclins for specific targets.
3. Explain the mechanisms by which phosphorylation can change the function of a protein.

6. Research the Literature on Your Own

1. Learn about the basic principles underlying mass spectrometry.
2. Several Clb5-specific substrates (Orc2, Orc6, Cdc6, Mcm3) form a large protein complex (called the pre-replicative complex or pre-RC) at origins of replication. Why is it important for these proteins to be phosphorylated by Clb5-Cdk complexes at the onset of S phase?
3. One Clb5-specific substrate (Sld2) is a component of a large ‘pre-initiation’ complex of proteins that forms at origins of replication. Learn about how phosphorylation of this protein (and a related protein called Sld3) is important for the initiation of chromosome duplication.
4. One of the Clb5-specific proteins is called Fin1. What is the function of Fin1, and why is Fin1 a better substrate for Clb5 than it is for Clb2?
5. Phosphatases reverse the effects of Cdks in the cell. In the budding yeast, the phosphatase Cdc14 is the key regulator of Cdk substrates in late mitosis. Learn how Cdc14 is regulated in late mitosis, and how it is thought to promote various late mitotic events.
6. The proteins Sic1 and Cdh1 are excellent examples of proteins that are regulated by phosphorylation by Cdk1 at multiple sites. Learn how the multi-site phosphorylation of these proteins regulates their function.

7. Papers for Journal Club

The following papers illustrate the usefulness of the analog-sensitive kinase method. The first paper documents the use of analog-sensitive yeast strains to allow specific inhibition of protein kinases in the cell; the second paper illustrates the use of these mutants to identify Cdk1 substrates, as described in the lecture.

- Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., Wood, J.L., Morgan, D.O., and Shokat, K.M. (2000) A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature 407, 395-401.
- Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. (2003) Targets of the cyclin-dependent kinase Cdk1. Nature 425, 859-864.

The following papers illustrate the importance of cyclin specificity in Cdk function. The first paper provides genetic evidence in yeast that the S-phase cyclin Clb5 has greater S-phase promoting activity than the mitotic cyclin Clb2. The second paper includes the identification of Clb5-specific Cdk1 substrates as described in the lecture.

- Cross, F.R., Yuste-Rojas, M., Gray, S., and Jacobson, M.D. (1999). Specialization and targeting of B-type cyclins. Mol. Cell 4, 11-19.
- Loog, M., and Morgan, D.O. (2005) Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. Nature 434, 104-108.

The following paper describes the mass spectrometric approach to the identification of Cdk1 substrates, plus discussions of general issues in the function and evolution of phosphorylation sites.

- Holt, L.J., Tuch, B.B., Villén, J., Johnson, A.D., Gygi, S.P., and Morgan, D.O. (2009) Global analysis of Cdk1 phosphorylation sites provides insights into evolution. Science 325, 1682-1686.