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We are developing methods to identify G1-phase substrates of cyclin-Cdk complexes. In earlier years of this project, we optimized two-dimensional gels for visualization of phosphorylation of Cdc28 substrates, and we developed monoclonal antibodies with reactivity to phospho-Ser-Pro or phospho-Thr-Pro. We identified one important in vivo substrate, Sic1. However, it appears that these methods by themselves are insufficiently powerful to identify many substrates, since most substrates seem to be very rare. We have therefore supplemented these methods with three other approaches: (1) purification of affinity tagged cyclins, to look for co-purifying proteins; (2) complete genome two-hybrid screens with Cdc28 and with G1 cyclins, to look for interacting proteins; and (3) genome-wide analysis of cell cycle regulated transcription, on the basis that some important substrates might be regulated at the transcriptional level as well as at the activity level. All three approaches are going very well, and we have excellent candidates for new substrates, which will be characterized in part using the 2D gel system.

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Bruce Futcher Aug 18, 1998

PI - Signature Date

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5. Introduction.

There is general agreement that in all eukaryotes, phosphorylation by various cyclin-Cdk complexes controls and orchestrates key cell cycle events. In yeast, these events include budding, spindle pole body duplication, and commitment to division in G1 phase; initiation of DNA synthesis in late G1 or early S phase; and spindle elongation in mitosis. However, despite knowing a great deal about the cyclin-Cdk complexes themselves, and despite years of investigation by many laboratories, we know only about half a dozen substrates of the cyclin-Cdk kinases, and none of these explain the control of critical cell cycle events. In particular, we do not know what substrates have to be phosphorylated for commitment to cell division to occur (although in mammalian cells, Rb is almost certainly one of the substrates).

The purpose of the present work is to develop methods for identifying substrates of the cyclin-Cdk complexes. In particular, we are interested in G1 substrates. To begin, experiments will be done in the yeast S. cerevisiae as a model for future work in mammalian cells.

We initially proposed two main approaches. The first approach uses two-dimensional gels to examine phosphoproteins. Various cyclins are expressed from a GAL promoter, and cells with the over-expressed cyclins are labelled with 32P. The pattern of spots on a 2D gel is then compared between cells expressing and not expressing the cyclin. Extra spots in the cyclin-expressing cells may be substrates. The second approach is to develop antibodies against phosphoserine followed by proline, and phosphothreonine followed by proline. Such antibodies would recognize proteins phosphorylated by Cdk complexes. Thus, such proteins could be immunoprecipitated and sequenced. This could also be combined with the 2D gels as an enrichment step.

Both these approaches have been somewhat successful, and we have in fact been able to identify one important substrate, Sic1, as described in publications and previous reports. However, it has been difficult to identify additional novel substrates, probably because the relevant proteins are extremely non-abundant. Therefore, recently, we have focused on finding supplementary methods to identify likely candidate substrates. The supplementary methods we have used are (A) purification of Cdc28-cyclin-(substrate?) complexes; (B) two-hybrid screens; and (C) genome-wide characterization of transcription. The 2D gel approach is still very important in characterization of candidates identified by these methods.

6. Body of the Report.

Parts (A) and (B) below are both based in part on the idea that the interaction of substrate and protein kinase may be weakened after the substrate has been phosphorylated. This would allow unphosphorylated substrate to bind with high affinity, then fall off after phosphorylation, thus allowing the enzyme to bind to a new molecule of unphosphorylated substrate. A change in affinity of this kind is known to occur for protein tyrosine phosphatases (except of course that the higher affinity is for the phosphorylated form) (Flint et al., 1997). To take advantage of this idea, we have built a "kinase negative" mutant of Cdc28, in which the key catalytic residue lys 40 has been mutated to arg (the *cdc28K40R* allele). Our hope is that this mutant protein will bind more tightly to substrates than will wild-type Cdc28, so that we can obtain substrates by purification (part A) and by two-hybrid analysis (B). We have already shown that *GAL-cdc28K40R* is a dominant negative lethal. We are doing reconstruction experiments using the two-hybrid system to see

whether in fact it does bind more tightly to substrates than wild-type Cdc28, but in the meantime we have obtained very promising results with a two-hybrid screen.

A. Purification of Cdc28-cyclin substrates.

This sub-project is based on three ideas. First, that substrates must be bound to some degree to cyclin-CDK complexes. Second, that this binding may be prolonged if the CDK is inactive and cannot phosphorylate the substrate. Third, that tagging proteins of interest with multiple, convenient affinity tags should make purification easy.

To address the third idea, we have developed a triple affinity tag consisting of a calmodulin-binding peptide (CBP), six histidines, and a triplicated hemagglutinin (HA) epitope, all on a NotI cassette. The CBP affinity tag binds calmodulin with high affinity in the presence of calcium, and when calcium is chelated by the addition of EGTA, the CBP is released. The 6x His tag allows binding to immobilized nickel ions, and release in the presence of imidazole. The HA tag allows immunoprecipitation by the monoclonal antibody 12CA5. So far, we have added this affinity cassette to Clb2. This tagged Clb2 was mildly overexpressed in a one litre culture of yeast cells. The Clb2-Cdc28 complex was purified by binding to a calmodulin resin, then eluted with EGTA. (A control experiment was done with cells lacking the affinity tag.) Analysis with anti-HA antibodies, anti-Cdc28 antibodies, and with protein kinase assays showed that a high-yield enrichment of Clb2-Cdc28 had been achieved, though the complex was still not pure. The enrichment was a minimum of 200-fold, but it may have been much more—the amount of total protein in the eluate was below the level of detection of the Bradford assay. As a second purification step, the complex was immunoprecipitated from the eluate using the 12CA5 monoclonal against HA. Protein was released by boiling in SDS, and analyzed after SDS PAGE by both silver staining and Western analysis. Silver staining showed that only two major proteins were present (not including the antibody IgG from the immunoprecipitation); both were specific to the experimental purification. These two bands were identified as Clb2 and Cdc28 by Western blotting. Other weak bands were also seen, and 15 of these were specific to the tagged Clb2 cuclture. One of these was identified as Sic1 (a known substrate) by Western blotting, and another co-migrated with Cks1 at 18 kDA. The 13 remaining specific, unidentified bands were cut out of the silver-stained gel and sent to our collaborators in Seattle (John Yates and colleagues) for analysis by tandem mass spectrometry. We expect results shortly. However, becaue only 1 litre of culture was used and the quantitiy of protein small, identification may not be possible for all proteins. We can easily scale the procedure up, however. The entire purification (from making a lysate to collecting the final immunoprecipitate) was accomplished within one day.

To address the second idea, we have just constructed a "kinase dead" version of Cdc28 in which the crucial catalytic residue Lys40 has been replaced by Arg. The mutant, inactive protein kinase is expressed from the GAL promoter. We have just found that when the *GAL-cdc28K40R* mutant is turned on by addtion of galactose, the cells in the culture cease to proliferate. Thus, *cdc28K40R* is a dominant negative allele, and is indeed very probably binding and titrating out important proteins such as substrates. Within the next few weeks we will repeat our affinity purification in the strain carrying *GAL-cdc28-K40R*; the cells will be grown with *GAL-cdc28K40R* off, and synchronized; the cells will be released, and when they pass Start, *GAL-cdc28K40R* will be turned on. We will then purify the *Clb2-cdc28K40R* complexes, with any associated substrates, and submit purified, unidentified proteins for analysis by mass spec. We will repeat this procedure with other affinity-tagged cyclins, especially the G1 cyclins Cln1, Cln2, and Cln3.

B. Directed, Genome-Wide Two-Hybrid Screens.

The two-hybrid screen is a method for finding proteins interacting with a protein of interest. It is thought that cyclins confer substrate specificity to Cdc28 in part because cyclins bind to substrates, and so direct the Cdc28 to the substrate. Thus, there may be cyclin-specific two-hybrid interactions with Cdc28 substrates. Alternatively, it may be that cyclins bind to various proteins to bring Cdc28 to a specific location (e.g., the spindle pole body) where substrates may be found. An important issue is that if indeed cyclins bind directly to substrates, it is not clear that this binding would be tight enough to allow detection in a two-hybrid assay.

There are two unusual features to our two-hybrid screen. First, we are doing it in collaboration with Stan Field's lab in Seattle. They have constructed 6,000 activation-domain fusions that includes every *S. cerevisiae* ORF. Thus, we can screen every yeast protein for interaction with our bait; we call this a "directed, genome-wide" screen. Second, we have two special bait constructs. One is Cdc28 fused to the DNA binding domain; the other is mutant Cdc28K40R. We do the whole screen with both baits, at different concentrations of 3-aminotriazole (3-AT), and look for activation-domain fusions that are either specific for the Cdc28K40R mutant, or which bind it with a higher affinity (as judged by 3-AT resistance), as a sign that the activation domain fusion might include a substrate. So far, this strategy seems to have been very successful, as we have a number of new Cdc28 interactors, including four that are specific for Cdc28K40R.

Results of directed two-hybrid screen:

Genes interacting with both wild-type Cdc28 and with Cdc28K40R:

Known Cyclins:

CLN1 G1 cyclin.
CLN2 G1 cyclin.
CLN3 G1 cyclin.
CLB1 Mitotic cyclin.

Other known interactors:

CAK1 Cdc28 activating kinase, a known interactor.

New Interactors:

PCL7. Pcl7 is a cyclin normally associated with Pho85p. All cyclins have a common structure called the cyclin fold that consists of 5 alpha helices, and a cross complex formation of a Pho85 cyclin with Cdc28 is not unreasonable. Indeed in a two hybrid screen using Pho85, in which *PCL7* was originally isolated, Cln1, Ume3 and Ccl1 were all isolated as interactors, all of which are considered partners for other Cdks (Cdc28, Ume5 and Kin28 respectively) (Measday et al. 1997). Whether this interaction is functionally relevant is however unclear. If in the absence of Pho85 (which is not essential), a *PCL7* deletion results in additional phenotypes, then Pcl7 may have some Pho85 independent functions.

SAP155. Sap155 associates with Sit4p, a phosphatase involved in cell cycle regulation (Luke et al. 1996). It is also a phosphoprotein *in vivo*, and the complex it forms with Sit4 persists from late G1 to late M-phase. Despite much work on Sit4, its role in the cell cycle, and its connection to Cdc28, have not been elucidated. The interaction of Sap155 with Cdc28 may be the crux of this connection.

STB1. A protein that binds Sin3p, a transcriptional regulator. Stb1 contains 9 potential Cdc28 consensus phosphorylation sites, which out of over 6000 yeast proteins places it 19th on the list of most sites. In fact it has the 5th highest density of Cdc28 sites of all yeast proteins.

SPC42. Spindle pole body component. Spc42 is at the core of the spindle pole body; it is the component around which other components are organized.

ARP8. Actin related protein. Nothing much is really known about ARP8. It has 3 potential Cdc28 phosphorylation sites, and of all the actin-related proteins in yeast, it has the largest number of insertions between the actin conserved blocks of homology. It is known that Cdc28 activity can lead to a re-organization of the actin cytoskeleton.

YKR077W. This protein is a good candidate for a Cdc28 substrate because it contains 7 potential Cdc28 phosphorylation sites. We also have evidence from part (C) below that shows that the expression of YKR077W peaks at the G1/S phase boundary, suggesting a cell cycle role. We have found that YKR077W is non-essential, and the deletion has little phenotype. However, there is homologue, YOR066W. YOR066w also has a large number of potential Cdc28 phosphorylation sites, and it is cell cycle regulated. In the near future, we will examine the double knockout ykr077w yor066w.

YPL014W. Novel.
YPL070W. Novel.
YKL014C. Novel.

Proteins interacting specifically with the Cdc28K40R mutant:

Zds1. 2 potential Cdc28 phosphorylation sites in a 100 kDa protein. *ZDS1* was isolated as a high copy suppressor of the plasmid loss defect of a *cdc28*-1N allele, a defect that couldn't be suppressed by a close homologue of *ZDS1*, *ZDS2*. More recently it has been suggested (Mizunuma et al. 1998) that Zds1 acts to transcriptionally repress *SWE1*, which encodes a tyrosine kinase that inhibits Cdc28 activity. A physical interaction between Zds1 and Cdc28 may therefore set up a negative feedback loop to regulate Cdc28 activity.

YDR130C. Novel. This protein contains 5 potential phosphorylation sites, and is predicted to form a coiled-coil structure, which is often indicative of structural proteins. We have found the transcript is regulated, and that it peaks somewhere in S/G2, again suggestive of a role in the cell cycle.

YIL122W Novel. 3 Cdc28 phosphorylation sites in a 39 kDa protein.

YJL193W Novel. 1 potential Cdc28 phosphorylation site in a 42 kDa protein.

So far, we have pursued two of the two-hybrid positives. First, we have worked with Stb1, because the lab of Dr. Brenda Andrews found Stb1 as a protein physically interacting with the transcription factor Swi6, and Swi6 is known to be activated in a Cdc28-dependent manner. It now appears that (1) Stb1 is important for the transcriptional function of Swi6; (2) that Stb1 is a phosphoprotein *in vivo*; (3) Stb1 is an excellent substrate for Cdc28 phosphorylation in vitro; and (4) Stb1 phosphorylation *in vivo* is dependent on Cdc28 activity. We now believe it is very likely that Stb1 is a new substrate of Cdc28, and that it is invovled in transcription.

Second, we have worked on the spindle pole component Spc42. Not only did we get this as a two-hybrid positive with Cdc28, but we also got it in a two-hybrid screen with the S-phase cyclin Clb5. We have never gotten it in any other two-hybrid screen, so it is probably not just a sticky protein. We tested the Spc42-activation domain hybrid against Cln2, Cln3, Clb2, Clb3, and Clb5 fusions with the DNA binding domain; it gave a positive interaction with all three Clbs and a negative result with the two Clns. Finally, we have shown using GFP fusions that Clb2, Clb3, and Clb5 are all localized (in part) at the spindle pole body. Thus, we are fairly certain that Cdc28-Clb complexes are in fact at the spindle poles, and it seems likely that they arrive there because of an interaction with Spc42. Spindle pole body duplication is known to be Start dependent, and spindle pole body separation and spindle formation is known to be Clb-Cdc28 dependent (Fitch et al. 1992) so there is good reason to think that Cdc28 has important substrates at the spindle poles. These substrates, however, are not necessarily Spc42 or any other spindle pole structural protein.

C. Characterization of cell cycle regulated transcription.

As a collaboration with the laboratories of Dr. David Bostein and Dr. Pat Brown, we have characterized the pattern of transcription of all S. cerevisiae open reading frames (ORFs). The Botstein and Brown labs have developed arrays of PCR-amplified ORFs covering essentially the whole genome. These can then be hybridized with fluorescently-labelled cDNAs representing mRNA populations from specific experiments. My lab has done five relevant experiments to obtain synchronized cells: an alpha factor block-and-release, an elutriation, and a cdc15 block and release. The synchronized cells gave us cell cycle specific populations of mRNAs, and we used these to probe the ORF arrays. Full results are presently being written up for publication, but suffice it to say here that we have identified about 700 cell cycle regulated genes, and these include some that are very likely substrates for Cdc28 on the basis that (1) they peak in expression at critical times; (2) they contain clusters of potential Cdc28 phosphorylation sites; and (3) they have been identified as possible candidates in other screens, such as the two-hybrid screen.

D. Future plans.

We are working hard to confirm that candidate substrates found by two-hybrid are in fact in vivo substrates, and part of the evidence for this will come from 2D gel and antibody studies. At present, our three most interesting candidates are Stb1, Spc42, and a putative transcription factor we believe may encode "SFF", a key, Cdc28-activated regulator of mitotic gene expression. A short term step will be to mutate potential sites of phosphorylation in some of these mutants to see if there is a phenotype. A longer term problem is that even if such mutants do have phenotypes, it does not prove that the phenotype is due to the lack of phosphorylation as such—it could be due to the change in primary amino acid sequence. We are working on ways to re-introduce phosphates at mutant sites without exactly restoring the primary sequence in the region of the mutation, and this should allow true confirmation that phosphorylation is important for function.

7. Conclusions.

Without some enrichment step for substrates of the desired type, the 2D gel approach is probably not practical for non-abundant substrates. Nevertheless, it has helped us find one relevant substrate (Sic1) and probably two others described last year (Mcm3 and Cdc54). This year, we have used additional methods to enrich for substrates, and these seem to be working very well. These additional methods will also be applicable to mammalian cells.

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