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A pathway in the yeast cell division cycle linking protein kinase C (Pkc1) to activation of Cdc28 at START

Nicholas J.Marini[1,2], Eric Meldrum[1,3], Ben Buehrer[4], Andrew V.Hubberstey[2], David E.Stone[5], Alexis Traynor-Kaplan[6] and Steven I.Reed[1,7]

[1]Department of Molecular Biology, MB7, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037, [4]Department of Biochemistry/Biophysics, University of North Carolina, Chapel Hill, NC 27599, [5]Department of Biology, University of Illinois-Chicago, Chicago, IL 60607, [6]Department of Medicine, Division of Gastroenterology, University of California, San Diego, CA 92103, USA and [2]Department of Medical Biochemistry, University of Calgary HSC, Calgary, Alberta, Canada T2N 4N1

[3]Present address: Glaxo Institute for Molecular Biology, 1228 Plan-les-Ouates, Geneva, Switzerland

[7]Corresponding author

In an effort to study further the mechanism of Cdc28 function and cell cycle commitment, we describe here a genetic approach to identify components of pathways downstream of the Cdc28 kinase at START by screening for mutations that decrease the effectiveness of signaling by Cdc28. The first locus to be characterized in detail using this approach was *PKC1* which encodes a homolog of the Ca^{2+}-dependent isozymes of the mammalian protein kinase C (PKC) superfamily (Levin *et al.*, 1990). By several genetic criteria, we show a functional interaction between *CDC28* and *PKC1* with *PKC1* apparently functioning with respect to bud emergence downstream of START. Consistent with this, activity of the MAP kinase homolog Mpk1 (a putative Pkc1 effector) is stimulated by activation of Cdc28. Furthermore, we demonstrate a cell cycle-dependent hydrolysis of phosphatidylcholine to diacylglycerol (a PKC activator) and choline phosphate at START. Diacylglycerol production is stimulated by Cdc28 in cycling cells and is closely associated with Cdc28 activation at START. These results imply that the activation of Pkc1, which is known to be necessary during bud morphogenesis, is mediated via the *CDC28*-dependent stimulation of PC-PLC activity in a novel cell cycle-regulated signaling pathway.

Keywords: Cdc28/cell cycle/phosphatidylcholine/protein kinase C/*Saccharomyces cerevisiae*

Introduction

In the budding yeast *Saccharomyces cerevisiae*, entry into the mitotic cell cycle is regulated at a point late in the G_1 interval operationally defined as START (reviewed in Pringle and Hartwell, 1981). Cells in early G_1, prior to START, can exit the mitotic cell cycle in order to conjugate (if exposed to mating pheromone) or to enter a metabolically quiescent 'stationary phase' (if there are insufficient nutrients to support cell division). However, once cells pass the START control point they are committed to enter S phase and complete the next cell cycle. Passage through START requires the activation of a 34 kDa protein kinase (a member of the cyclin-dependent kinase superfamily) encoded by the *CDC28* gene (Reed *et al.*, 1985; Wittenberg and Reed, 1988). Activation of Cdc28 for the START transition requires association with positive regulatory subunits known as G_1 cyclins or Clns (reviewed in Reed, 1992). In *S.cerevisiae*, there are three known G_1 cyclins encoded by the *CLN1*, *CLN2* and *CLN3* genes (Cross, 1988; Nash *et al.*, 1988; Hadwiger *et al.*, 1989; Richardson *et al.*, 1989). All of the possible single and double *cln* deletions are viable but the *cln1 cln2 cln3* triple deletion is inviable and arrests in G_1 with a START defect (Richardson *et al.*, 1989; Cross, 1990). Cdc28 is also required for progression through S phase and the G_2/M transition but these involve association with and activation by different classes of cyclins (B-type cyclins or Clbs; Ghiara *et al.*, 1991; Surana *et al.*, 1991; Epstein and Cross, 1992; Richardson *et al.*, 1992; Schwob and Nasmyth, 1993).

The concept of a cell cycle commitment event occurring at START has been useful in describing yeast cell physiology, but the underlying molecular basis for 'cell cycle commitment' is still unclear. Passage through START triggers several changes in the cell including DNA replication, duplication of the spindle pole bodies (the major microtubule organizing centers in yeast) and bud emergence (Pringle and Hartwell, 1981). Presumably, these changes arise as a result of phosphorylations catalyzed by Cln/Cdc28 complexes. However, the substrates that mediate these changes are unknown. In order to understand further cell cycle commitment in *S.cerevisiae* (and ultimately all eukaryotes), these substrates must be identified and the pathways linking Cdc28 activation at START to its ultimate cell biological consequences elucidated.

While definitive identification of *in vivo* G_1 substrates of the Cdc28 kinase is lacking, two proteins (Far1, $p40^{sic1}$) have been shown to associate with Cdc28 complexes and can be phosphorylated *in vitro* (Mendenhall *et al.*, 1987; Peter *et al.*, 1993; Tyers and Futcher, 1993). The *FAR1* gene product is specifically required for pheromone-induced cell cycle arrest and seems to mediate this effect through inhibition of G_1 cyclin function (Chang and Herskowitz, 1990; Valdivieso *et al.*, 1993). Although the Far1 protein can be phosphorylated in a *CDC28*-dependent fashion, phosphorylation is stimulated by the presence of mating pheromone and is therefore associated with exit from the cell division cycle rather than cell cycle progression (Chang and Herskowitz, 1990; Peter *et al.*, 1993; Tyers and Futcher, 1993). Thus, Far1 does not appear to be a good candidate for a substrate that mediates the

proliferative signal generated by Cdc28 at START. $p40^{sic1}$ is thought to interact with Clb/Cdc28 kinase complexes rather than Cln/Cdc28 complexes and is likely to play an inhibitory role in down-regulating Cdc28 activity and entry into S phase (Mendenhall, 1993; Nugroho and Mendenhall, 1994; Schwob *et al.*, 1994). While recent evidence suggests Cln/Cdc28-dependent proteolysis of $p40^{sic1}$ may be an important event for S phase entry (Dirick *et al.*, 1995), the role of Cdc28 in this process and whether $p40^{sic1}$ is phosphorylated *in vivo* are still unknown.

Although no direct evidence exists, it is likely that one role of the Cdc28 kinase in G_1 is to activate directly transcription factors involved in regulating genes periodically transcribed in the cell cycle. Several classes of genes in *S.cerevisiae* (including the G_1-specific cyclins *CLN1* and *CLN2*, the *HO* endonuclease and most genes encoding proteins involved in DNA synthesis) exhibit a START-dependent G_1 periodicity of expression. Although *CLN1*, *CLN2* and *HO* are cell cycle-regulated via a different promoter element (the Swi4/6 Cell cycle Box or SCB) from the DNA synthesis genes (the MluI Cell cycle Box or MCB; reviewed in Andrews and Herskowitz, 1990; Merrill *et al.*, 1992), both MCB- and SCB-specific transcription can be induced simply by activating the Cdc28 kinase in the absence of protein synthesis (Marini and Reed, 1992). Furthermore, the amount of Cln protein present, which presumably governs the level of Cdc28 kinase activity achieved, is rate limiting for this induction.

In an effort to study further the mechanism of Cdc28 function and cell cycle commitment, we describe here a genetic approach to identify components of pathways downstream of Cdc28 by screening for mutations that decrease the effectiveness of signaling by the Cdc28 kinase. This screen has thus far allowed us to identify several loci that, when mutated, can make the phenotype of a temperature-sensitive *cdc28* allele more severe. The first locus to be characterized at the molecular level using this approach was *PKC1*. The *PKC1* gene, essential for yeast cell viability, encodes a homolog of the Ca^{2+}-dependent isozymes of the mammalian protein kinase C (PKC) family of enzymes (Levin *et al.*, 1990). Although there appear to be several independent requirements for *PKC1* gene product function, at least one of these is during the budded phase of the cell cycle for maintaining cell wall integrity and preventing cell lysis. (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992; Errede *et al.*, 1995; Kamada *et al.*, 1995). At least part of Pkc1 function is mediated through a protein kinase cascade involved in the activation of a mitogen-activated protein (MAP) kinase homolog, Mpk1 (reviewed in Errede and Levin, 1993). By several genetic criteria, we show a functional interaction between *CDC28* and *PKC1* with *PKC1* apparently functioning downstream of START. Consistent with this, Mpk1 activity is stimulated following activation of the Cdc28 kinase. Furthermore, we demonstrate a cell cycle-dependent hydrolysis of phosphatidylcholine (PC) to diacylglycerol (a PKC activator) and choline phosphate, suggesting the activation of a PC-specific phospholipase C (PC-PLC). Diacylglycerol induction is dependent on *CDC28* in cycling cells and is closely associated with Cdc28 activation at START. These results imply that the activation of Pkc1 required for bud morphogenesis in the yeast cell cycle is mediated in a novel fashion via the *CDC28*-dependent stimulation of PC-PLC activity.

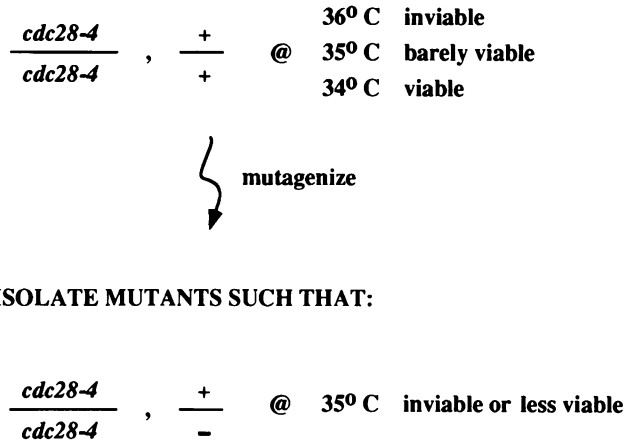

Fig. 1. Scheme for isolating *cdc28* enhancer mutations. The growth characteristics of diploid yeast cells homozygous for the temperature-sensitive *cdc28-4* allele. Cells were mutagenized with UV irradiation and those with increased temperature sensitivity were genetically analyzed (see Materials and methods) to identify loci that enhance the $cdc28^{ts}$ phenotype when heterozygous in a diploid cell. + indicates a wild-type copy of an allelic locus, – indicates a mutationally inactivated copy of the same locus.

Results

Genetic screening for Cdc28 targets

It is likely that the proliferative signal generated by the Cdc28 kinase at START is transmitted through a number of different pathways that collectively ensure the proper timing and coordination of the cell division cycle. For example, there are at least three genetically distinguishable pathways, based on morphological and gross biochemical criteria, which diverge from the Cdc28 execution point in late G_1. These general pathways are defined solely by functionally mapping cell division cycle (*cdc*) mutations and lead respectively to bud emergence, initiation of DNA synthesis and spindle pole body duplication (Pringle and Hartwell, 1981). It is almost certain these pathways are further subdivided and the Cdc28 kinase must phosphorylate a number of substrates to orchestrate these events. Furthermore, other pathways may also exist that cannot be defined by such criteria. The goal of this genetic screen is to identify Cdc28 targets by simply identifying genes that display a dosage-dependent interaction with the Cdc28 kinase. The strategy assumes only that when a *cdc28* temperature-sensitive mutant is forced to grow at the maximal semi-permissive temperature possible (the transitional temperature), the cell has a severely compromised kinase and the limiting factor for growth is, therefore, phosphorylation of G_1 substrates. This screen takes advantage of the fact that loss of Cdc28 function with most temperature-sensitive alleles confers primarily a defect at G_1 rather than at the G_2/M transition.

Diploid cells homozygous for a $cdc28^{ts}$ mutation (*cdc28-4*) were mutagenized and, by replica plating, clones were identified that could no longer grow at the original transitional temperature (Figure 1). One class of these new mutants is likely to harbor heterozygous null (or temperature sensitive) mutations in genes corresponding

to substrates or downstream signaling elements. The rationale is that signaling by the Cdc28 kinase at the transitional temperature is barely adequate so that even 2-fold reductions in the abundance of gene products acting downstream might disrupt an essential signaling event and have a severe impact on growth. Thus, a recessive loss-of-function mutation in such a gene might yield a dominant growth defect phenotype even though it only inactivates one copy of the gene. A conceptually similar approach has proved to be successful in identifying downstream elements in the Sevenless receptor tyrosine kinase signal transduction pathway in *Drosophila* (Rubin, 1991; Simon *et al.*, 1991).

Diploid cells were chosen for mutagenesis because they allow for a more sensitive and specific genetic screen in which mutations are identified based on reduction of dosage rather than complete loss of function. Therefore, even mutational inactivation of essential genes does not affect viability since there is always a second copy of the wild-type gene. Thus, this strategy should eliminate mutations that affect the overall health of a *cdc28*^{ts} cell without affecting Cdc28 function directly.

From ~3×10^4 mutagenized diploid cells, seven mutants were originally isolated that displayed an enhanced temperature sensitive phenotype in a homozygous *cdc28-4* background and, upon subsequent genetic analysis (sporulation, tetrad dissection, back-crossing), all were found to contain a recessive lesion in a single allelic locus. They are referred to as TME-1 through 7 (for Cdc Twenty-eight Mutational Enhancer). So far, only the defect associated with the *tme3* mutation has been molecularly characterized and is discussed below.

tme3 is a temperature-sensitive allele of PKC1

The enhancement of the temperature-sensitive *cdc28* phenotype by *tme3* is shown in Figure 2. The top panel shows patches of diploid yeast cells grown at the indicated temperatures. Note that cells heterozygous for *TME3* function grow significantly less well at 35°C, the original transitional temperature of the parent strain, but are apparently unaffected at lower temperatures. Thus, by reducing the dosage of wild-type Tme3 by roughly one-half, Cdc28-mediated signaling (already weak) is further compromised, indicating a functional interaction between the two gene products. It should be noted that *cdc28*^{ts} diploids heterozygous for *tme1-7* show varying degrees of enhanced temperature-sensitivity from slight (as for *tme3*) to some that lower the restrictive temperature by almost 2°C.

The lower panel of Figure 2 shows haploid cells grown at various temperatures and demonstrates that *tme3* is itself a temperature-sensitive allele of an essential gene. The restrictive temperature of the *cdc28-4 tme3* double mutant is significantly lower (31°C) than either *cdc28-4* (37°C) or *tme3* (35°C) singly mutated cells, further suggesting a cooperative interaction between the two proteins.

Microscopic examination of log phase *tme3* cells incubated at a restrictive temperature (36°C) for 3 h revealed that nearly 80% of the cells had undergone cell cycle arrest, appearing as cells with very small (barely visible) buds. The cells were not unusually large nor did they continue to grow during prolonged incubations at restrictive temperatures. Thus, the temperature-sensitive defect

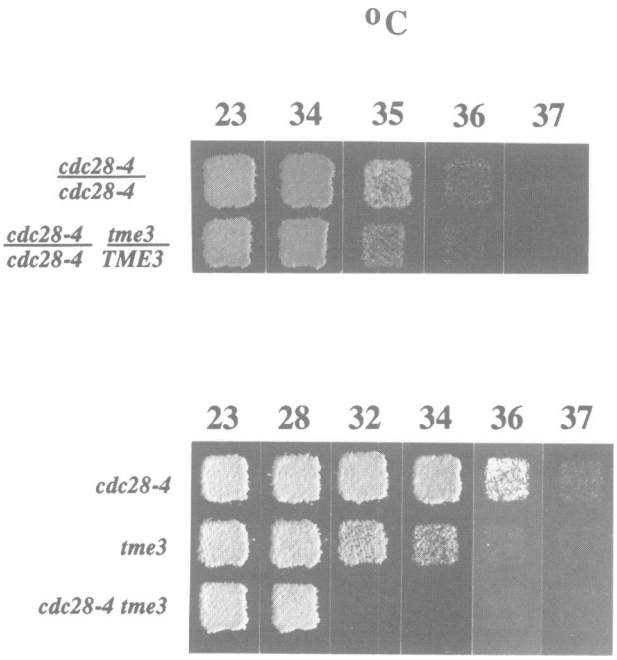

Fig. 2. The temperature-sensitive phenotypes of the *cdc28* enhancer mutation *tme3*. *Saccharomyces cerevisiae* cells of the indicated genotypes were patched on YEPD plates and replica-plated to the various temperatures. The top panel shows diploid cells where the upper row is the parent strain used for mutagenesis (D4u-d) and the second row is the same strain background but heterozygous for *tme3*. The bottom panel shows haploid cells with either mutation alone or in combination. All plates were incubated 20 h at the indicated temperatures.

associated with *tme3* displays both a cell division cycle (*cdc*) and a growth phenotype. Furthermore, many of the cells appeared as non-refractile 'ghosts' suggesting that cell lysis was occurring at the restrictive temperature. A similar phenotype has been reported for yeast cells that lack a functional *PKC1* gene, which encodes a homolog of the Ca^{2+}-dependent isozymes of the mammalian PKC superfamily (Levin *et al.*, 1990; Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992).

In fact, *PKC1* on a centromere plasmid rescued the temperature-sensitive *tme3* defect as did growth on media osmotically stabilized with sorbitol (which also suppresses the cell lysis defect of *pkc1* mutations; data not shown). In addition, we created an integrating plasmid to tag the *PKC1* locus with a selectable *LEU2* marker [YIplac128-(*PKC1*)] and transformed a wild-type strain (15Dau) to leucine prototrophy. This strain was mated to *tme3* cells and two independently derived diploids were induced to sporulate. In 36 tetrads dissected, both the *leu2* and temperature-sensitive phenotypes segregated 2:2 with *leu2* and temperature sensitivity co-segregating in every case indicating genetic linkage of *PKC1* and *tme3*. Based on these data we conclude that *tme3* is a temperature-sensitive allele of *PKC1* and will be referred to henceforth as *pkc1*^{ts}.

Pkc1 functions downstream of Cdc28 in the cell cycle

The *PKC1* gene product is essential for osmotic stability during the budded phase of the cell cycle and, therefore, probably affects the structural integrity of the cell wall and/or cytoskeleton (Levin and Bartlett-Heubusch, 1992;

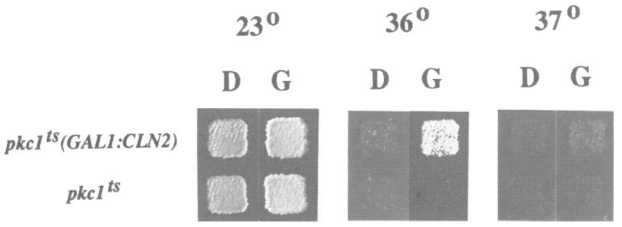

Fig. 3. Overexpression of *CLN2* partially rescues the *pkc1*^{ts} phenotype. *pkc1*^{ts} cells [strain 3(15)] containing an integrated copy of *CLN2* under control of the galactose-inducible *GAL1* promoter or the same cells prior to transformation were patched on YEPD plates and grown overnight. Patches were then replica-plated onto either YEPD or YEPGalactose and incubated for 20 h at the indicated temperatures. D, dextrose; G, galactose.

Paravicini *et al.*, 1992). The signal generated by Pkc1 is mediated in part through a protein kinase cascade resulting in the activation of a mitogen-activated protein (MAP) kinase homolog, Mpk1 (Lee and Levin, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993). In fact, the *MPK1* gene itself was also isolated as a mutational enhancer of the G_1 defect of *cdc28*^{ts} mutants in a similar screen (Mazzoni *et al.*, 1993), further emphasizing the relationship between Cdc28 and the Pkc1/Mpk1 pathway. Defects at any level in this cascade result in cell lysis phenotypes to varying degrees. The roles these kinases play in maintaining cellular integrity during bud formation are unknown, as is the signal that triggers pathway activation.

Given the fact that Pkc1 function is essential during the budded phase of the cell cycle (post-START) and that the gene was isolated in a screen designed to identify Cdc28 targets, it seems plausible to suggest that Pkc1 is activated in a Cdc28-dependent fashion. To test this, we asked whether elevation of Cdc28 activity could overcome the *pkc1*^{ts} defect to any measurable extent. It had been previously determined (Levin and Bartlett-Heubusch, 1992) that several *pkc1*^{ts} alleles could be rescued by exogenously added Ca^{2+} (necessary for PKC activation), which presumably augments the activity of mutant proteins. Thus, if Cdc28 provides an activating signal for Pkc1, it may be possible to rescue the *pkc1*^{ts} defect by increasing this signal.

In Figure 3, *pkc1*^{ts} or *pkc1*^{ts} cells bearing the galactose-inducible *CLN2* allele (*GAL1::CLN2*) were grown at the indicated temperatures on either dextrose (promoter-off) or galactose (promoter-on) media. Since Cln2 is a positive regulator of Cdc28, over-expression from the *GAL* promoter leads to higher than normal levels of kinase activity (Wittenberg *et al.*, 1990). Note that only cells over-expressing *CLN2* are capable of growth under normally restrictive conditions. Thus Cdc28 hyperactivation can partially overcome the temperature-sensitive *pkc1* defect, providing further evidence of genetic interaction. Rescue is not due to functional overlap or bypass since *CLN2* overexpression cannot fully complement the *pkc1*^{ts} defect (Figure 3) nor can it rescue cells deleted for *PKC1* (data not shown). Cdc28-dependent rescue must be dependent, therefore, on some residual activity of the *pkc1*^{ts} allele at higher temperatures.

Based purely on genetic data, it is impossible to order definitively Pkc1 upstream or downstream of Cdc28 in a common pathway or at the same level as Cdc28 with an independent function (i.e. parallel pathways that exert unique activities on common targets). We prefer to order Pkc1 downstream rather than upstream of Cdc28 for several reasons. First, *pkc1*-defective cells arrest growth with a cell cycle phenotype at a point following bud emergence with completely replicated chromosomal DNA (Levin *et al.*, 1990). This G_2 arrest suggests that Pkc1 function is downstream of START and Cdc28 activation. Second, while Cdc28 hyperactivation can partially rescue a *pkc1*^{ts} allele, *PKC1* overexpression has no effect on *cdc28*^{ts} alleles. This is presumably because Cdc28 exerts multiple functions at START with Pkc1 activation being only one of these. Third, in *cdc28-4 pkc1*^{ts} double mutant cells (both haploid and the original *pkc1*^{ts} heterozygous diploid) the enhancement of the *cdc28* phenotype by the *pkc1*^{ts} allele is abrogated by growth in osmotically-stabilized medium. This effect is due to suppression of cell lysis and not to direct rescue of the *pkc1*^{ts} allele, since *pkc1*^{ts} cells grown at a restrictive temperature in media containing 1 M sorbitol lyse rapidly upon shift to hypo-osmotic media. Thus, it is the cell lysis aspect of *pkc1* deficiency that accounts for enhancement of the *cdc28* phenotype. Cellular lysis occurs following the polarization of cell growth necessary for bud formation (Levin and Bartlett-Heubusch, 1992), which is strictly a Cdc28-dependent process. It should be noted that *cdc28*^{ts} *pkc1*^{ts} double mutant cells arrest under restrictive conditions as primarily unbudded cells that have lysed. The likely reason for this is that during the unusually long G_1 intervals in Cdc28-compromised cells, polarized cell growth occurs in the absence of START and bud initiation (Lew and Reed, 1993). This abnormal polarized growth, like normal polarized growth, apparently makes the cell susceptible to lysis under conditions of limiting Pkc1 activity.

Furthermore, whereas Pkc1 displays a dosage-dependent interaction with Cdc28 in diploids still containing one copy of the wild-type *PKC1* gene (Figure 2), *CDC28* dosage has no observable effect on *pkc1*^{ts} phenotypes. It is perhaps noteworthy that *cdc28*^{ts} diploids heterozygous for a *pkc1* deletion (*PKC1/pkc1*Δ) show essentially identical phenotypes to the *pkc1*^{ts} heterozygotes, supporting the idea that the *ts* allele isolated represents a loss of function at the restrictive temperature. Although we cannot definitively rule out independent function at the same level, we believe the above findings, taken together, indicate that Pkc1 functions downstream of Cdc28 rather than in a parallel pathway. Additional support for this comes from experiments described below measuring second messenger activators of PKC, as well as Mpk1 kinase activity (a Pkc1 effector), as a function of Cdc28 activation.

Cdc28-dependent stimulation of diacylglycerol production in the cell cycle

If Pkc1 activation is *CDC28*-dependent, the role of Cdc28 may either be direct (presumably by phosphorylation of Pkc1) or indirect by regulating other activators of Pkc1 activity. For example, members of the PKC family are activated by the co-factor diacylglycerol (DAG) which is released by enzyme-mediated hydrolysis of phospholipids (Nishizuka, 1988, 1992). As in metazoans, *S.cerevisiae* Pkc1 contains consensus DAG binding sites (Levin *et al.*, 1990; see Discussion regarding the significance of DAG in Pkc1 activation). We have, therefore, assayed DAG

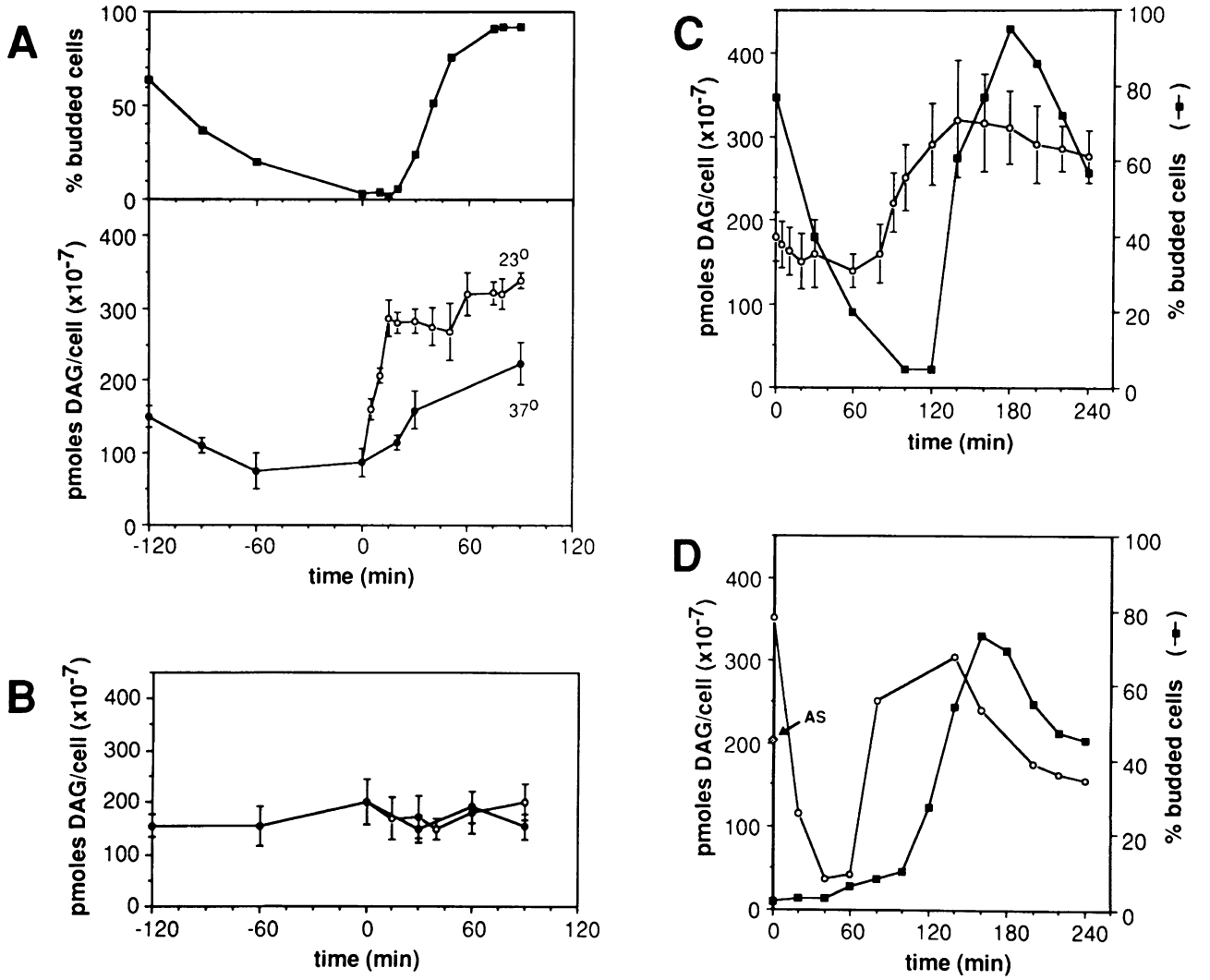

Fig. 4. *CDC28*-dependent accumulation of diacylglycerol. (**A**) D13au (*cdc28-13*) cells (10^7/ml) were arrested at START by incubation at 37°C for 2 h and then shifted to 23°C or maintained at 37° C for an additional 2 h. The cells were analyzed for budding index (top panel) and diacylglycerol (DAG) content (lower panel) at the indicated times. DAG levels 23°C (○), DAG levels 37°C (●). Each point is the average of triplicate samples with standard deviations shown by error bars. (**B**) 15Dau (wild-type) cells were grown and treated exactly as in panel A. DAG levels 23°C (○), DAG levels 37°C (●). (**C**) GCY11 (*bar1*Δ) cells were treated with α-factor (40 ng/ml) at t = 0. DAG levels were measured at the indicated times during the subsequent arrest and synchronous recovery from pheromone treatment. (**D**) 15Dau (wild-type) small daughter cells were isolated by centrifugal elutriation and inoculated into fresh media at t = 0. Total cellular DAG content was measured at the indicated times. AS indicates the asynchronous DAG level prior to elutriation (stippled diamond on the y-axis).

levels across the yeast cell cycle and determined the role of Cdc28 in DAG production. The results from these experiments demonstrate a *CDC28*-dependent burst of DAG accumulation late in G_1 prior to budding, the presumptive period of Pkc1 activation (Figure 4).

In Figure 4A, cells bearing a *cdc28-13* allele (D13au) were arrested at START by inactivation of Cdc28 at the restrictive temperature followed by shift to the permissive temperature (and Cdc28 re-activation). At the indicated times, samples were analyzed for DAG levels as well as for cell cycle synchrony by determining budding index. The results showed that following an initial decrease in DAG levels concomitant with Cdc28 inactivation, there was a 3-fold increase in DAG levels prior to budding that was Cdc28-dependent and occurred rapidly following Cdc28 re-activation. As shown in Figure 4B, the observed fluctuation of DAG levels is not simply an artifact of temperature shock since wild-type cells similarly treated showed no significant changes in DAG levels with temperature shift. We have also observed a comparable burst of DAG accumulation in cells re-entering the cell cycle following recovery from G_1 arrest induced by mating pheromone (Figure 4C). As with the *cdc28*^{ts}-block, DAG levels drop during G_1 arrest and rise prior to the subsequent round of budding. Furthermore, as shown in Figure 4D, early G_1 cells obtained by centrifugal elutriation also display a burst of DAG production in late G_1 just prior to budding. These data, therefore, strongly suggest a post-START Cdc28-dependent stimulation of DAG production in the yeast cell division cycle. It should be noted that the elevation of DAG levels to similar extents in other systems is capable of activating PKC through signal transduction pathways (see Discussion).

It is interesting that very early G_1 cells (t = 0, Figure 4D) contain relatively high levels of DAG which rapidly fall as the cells progress through the G_1 interval. While

this may be an effect of elutriation, it may also reflect the timing of DAG turnover in the cell cycle. Since the osmoremedial function of Pkc1 is apparently necessary through the entire budded phase of the cell cycle (S, G_2 and M phases; Levin and Bartlett-Heubusch, 1992), one would predict activators of Pkc1 to be maintained at elevated levels through the same period and subsequently lost in the next G_1 interval as the data in Figure 4D suggest. Consistent with this, cellular levels of DAG remain high after the initial increase triggered by Cdc28 activation (Figure 4A).

In cells synchronized by either mating pheromone or *cdc28* block, the most striking effect of Cdc28 activation on DAG levels (~2.5- to 3-fold) is seen only in the first round of budding when cells are highly synchronous. Since DAG levels remain high throughout the entire budded phase of the cell cycle and mother and daughter cells rapidly lose budding synchrony in subsequent cell division cycles, DAG oscillations become difficult to detect. In elutriated cells, however, DAG production in late G_1 appears more striking (~8-fold) and may more accurately reflect true changes in the cell cycle. In addition, recovery from elevated DAG levels can be seen as the cells re-enter G_1 at later time points.

It should also be noted that, in Figure 4A, cells maintained at 37°C displayed a gradual increase in DAG levels with time, apparently independent of *CDC28*. This increase is probably a function of cell growth unrelated to cell cycle progression since *cdc28*^{ts}-arrested cells continue to enlarge though blocked in G_1. Thus each successive time point contains a greater mass of membrane fraction per cell and, consequently, a greater source of DAG. Consistent with this, this gradual increase is not seen in the absence of cell growth by protein synthesis inhibitors (see below). For this reason, the drop in DAG levels seen with Cdc28 inactivation is likely to be an underestimate and may also explain the greater-fold increase seen in elutriated cells. The rapid rise in DAG levels following shift to 23°C cannot be attributable to cell growth since there is little appreciable growth during this short period of time.

Modulation of Mpk1 activity by Cdc28

Both the genetic interaction between *PKC1* and *CDC28* as well as the Cdc28-dependent production of DAG suggest that Pkc1 activation lies downstream of Cdc28 at START. An obvious prediction, therefore, is that effectors of Pkc1 function would also be sensitive to changes in Cdc28 activity. Although the precise roles of Pkc1 in maintaining cellular integrity are unknown, at least some of Pkc1 function is mediated via a protein kinase signaling cascade (reviewed in Errede and Levin, 1993). In this pathway, Pkc1 is thought to activate the MEK kinase homolog, Bck1 which in turn activates a pair of MEK homologs (Mkk1, Mkk2) ultimately resulting in the activation of the MAP kinase homolog, Mpk1 (Lee and Levin, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993).

To test whether this pathway is affected by Cdc28 activation, a multi-copy vector containing Mpk1 tagged with the influenza hemagglutinin epitope [YEp352(MPK1-HA); Kamada *et al.*, 1995] was used to transform a *cdc28-13* strain. Mpk1 kinase activity in immunoprecipitates was then assayed against myelin basic protein as a function of

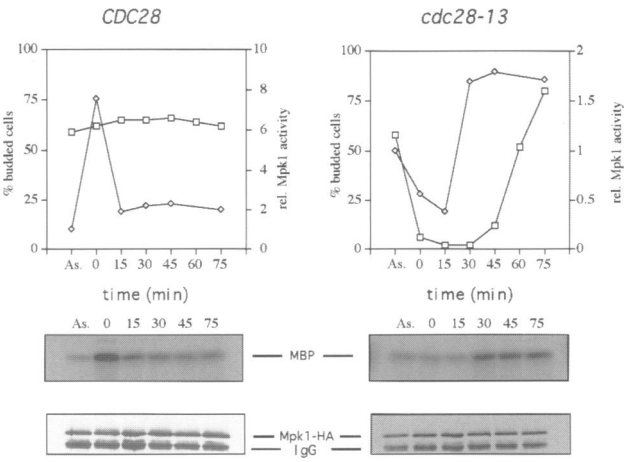

Fig. 5. Stimulation of Mpk1 activity following Cdc28 activation. D13au (*cdc28-13*) and isogenic wild-type (*CDC28*) cells were transformed with YEp352[*MPK1*-HA]. *cdc28-13* cells were arrested at START by incubation at 37°C for 2 h (t = –120 to t = 0) and then shifted to 25°C for 90 min. The cells were analyzed for budding index and Mpk1 activity (against myelin basic protein; MBP) at the indicated times. Wild-type cells were treated identically. MBP kinase assays and Western blot analysis of Mpk protein in immune complexes each represent half of the material recovered from protein A beads after the kinase assay was performed. Mpk1 activity is expressed relative to the asynchronous time point and normalized to the amount of Mpk1-HA recovered in each sample based on laser scanning of both the autoradiograph and Western blot. In control experiments performed on lysates from cells not containing the *MPK1*-HA plasmid, neither MBP kinase activity nor Mpk1 protein was detected using anti-HA antibody (data not shown). % budded cells (□); Mpk1 kinase activity (◇).

Cdc28 activation (Figure 5). The results showed that, as with DAG (cf. Figure 4), there was a 2- to 3-fold decrease in Mpk1 activity during incubation at the restrictive temperature (Cdc28 inactivation) and a subsequent increase (4-fold) following shift to the permissive temperature and Cdc28 re-activation. The Cdc28-dependent stimulation of Mpk1 activity occurred prior to the onset of budding, the presumptive period of Pkc1 pathway activation. These fluctuations were not seen in wild-type cells subjected to the same temperature shifts. In contrast, wild-type cells showed an increase (7.5-fold) in Mpk1 activity following shift to 37°C and a subsequent decrease during incubation at 23°C.

These results are in good agreement with those of Zarzov *et al.* (1995) who have recently demonstrated cell cycle regulation of Mpk1 kinase activity. By measuring tyrosine phosphorylation of Mpk1, the kinase was activated during bud formation (post-START) following recovery from pheromone-induced cell cycle arrest. Furthermore, Mpk1 activity was partially dependent on Cdc28 since tyrosine phosphorylation was significantly reduced following a temperature shift in *cdc28*^{ts} mutants, similar to the results shown here.

It is worth noting that heat-dependent activation of Mpk1 in wild-type cells has been previously reported (Kamada *et al.*, 1995) and this activation occurs via Pkc1. However, no such activation is seen in cells where Cdc28 is inactive (compare asynchronous and 0 time lanes in Figure 5). This further underscores the requirement for Cdc28 signaling in Pkc1/Mpk1 activation, especially in light of the fact that heat shock does not cause significant changes in the cell cycle distribution of wild-type cultures.

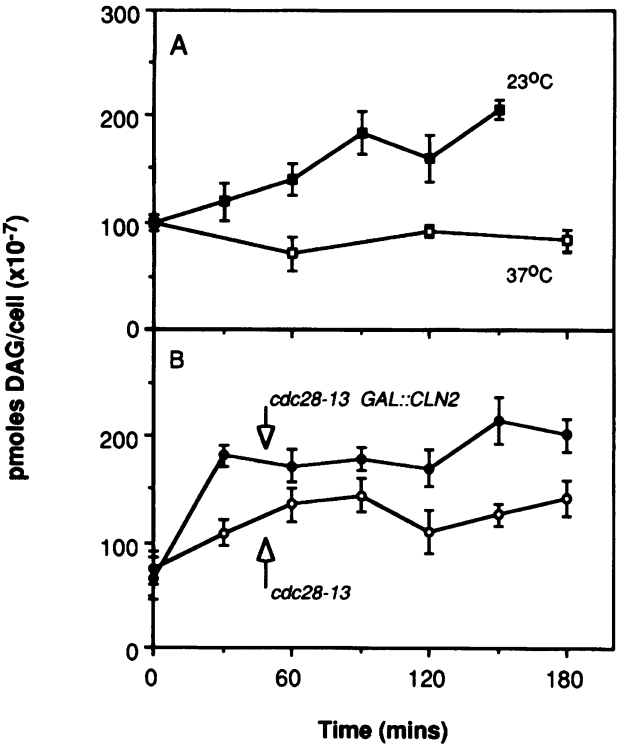

Fig. 6. Direct induction of DAG by the Cdc28 kinase. (**A**) D13au (*cdc28-13*) were grown and arrested as in Figure 5. Cycloheximide was then added to 10 μg/ml, and the cells were incubated further for 10 min at 37°C. The culture was then split and half was shifted to 23°C while the other half was maintained at 37°C. DAG levels were assayed at the indicated times following temperature shift at 23°C (■) or 37°C (□). Each point is the average of triplicate samples with standard error shown. (**B**) D13au (○) or D13au *GAL1::CLN2* (●) were grown to 10^7/ml in YEPRaffinose and shifted to 37°C for 3 h. Galactose (final conc. 2%) was then added to both cultures and incubation at 37°C was continued for 1 h. Cycloheximide was then added (10 μg/ml) to each culture, and after 10 min, both cultures were shifted to 23°C. DAG levels were assayed at the indicated times following temperature shift. Each point is the average of three samples with standard deviations shown. Galactose induction of *CLN2* was assayed functionally in control cultures not treated with cycloheximide for the earlier onset of budding.

Thus it appears, based on genetic interactions, kinetics of DAG production and Mpk1 kinase activation, that Pkc1 is downstream and dependent on the activation of Cdc28 at START. Cdc28-dependent activation of Pkc1 subsequently activates the MAP kinase cascade which plays an important role in maintaining the osmotic integrity of the cell during budding.

Direct induction of DAG production by Cdc28

Since START-dependent production of DAG in the yeast cell cycle may provide the biochemical link between Cdc28 and Pkc1, it is of interest to characterize this phenomenon further. To address whether DAG production is a direct consequence of Cdc28 activation or occurs via more indirect mechanisms, we asked whether protein synthesis was required for DAG accumulation following release from *cdc28^{ts}*-arrest. An experiment similar to that shown in Figure 4A was performed, except that cycloheximide was added to the culture 10 min prior to shift to the permissive temperature (see Materials and methods). As shown in Figure 6A, DAG levels were still induced in a Cdc28-dependent fashion under these conditions, albeit to a lesser extent and at a slower rate (cf. Figure 4A). As alluded to above, there was no detectable increase in DAG levels in the absence of protein synthesis in cells held at 37°C where Cdc28 remains inactive.

The delayed and gradual accumulation of DAG in this experiment is to be expected, in fact, if DAG accumulation is directly dependent on Cdc28 activity. The addition of cycloheximide at the *cdc28* arrest point prevents the maximal production of Cln protein, which occurs via a Cdc28-dependent positive transcriptional feedback loop (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991) but also stabilizes the relatively low level of Cln protein already present (Marini and Reed, 1992). Since Clns bind to and activate Cdc28, the result is that Cdc28 activity is low but chronic throughout the duration of the experiment following shift to the permissive temperature. Cdc28-dependent events thus display slower, more gradual kinetics than those in a normal cell cycle. Indeed, in the presence of cycloheximide, both Cdc28-dependent transcriptional induction (Marini and Reed, 1992) and actin polarization (Lew and Reed, 1993) occur with similarly altered kinetics as DAG accumulation.

Thus it appears that all the factors necessary for Cdc28-dependent stimulation of DAG production are present at the *CDC28* execution point. Since no intervening protein synthesis is necessary, we suggest that activation of the phospholipases involved must be closely linked to Cdc28 kinase activation and may be a direct result of phosphorylation by Cdc28.

Consistent with this suggestion, the extent of DAG production is directly correlated with the level of Cdc28 activity achieved. Overexpression of Cln2, which hyperactivates Cdc28 and partially rescues the *pkc1^{ts}* allele, elevates the levels of Cdc28-dependent DAG accumulation (Figure 6B). Cells overexpressing Cln2 (*cdc28-13 GAL1::CLN2*) or not (*cdc28-13*) were released from a *cdc28^{ts}*-block in the presence of cycloheximide as in Figure 6A. As shown in Figure 6B, *CLN2* overexpression markedly increased DAG levels (1.5–2.0-fold) triggered by Cdc28 throughout the duration of the experiment. The effect of Cln2 overexpression on DAG production further argues the rate-limiting role of Cdc28 kinase activity in this process. We have also observed a similar effect in cells released from a *cdc28*-block in the absence of cycloheximide. Cells overexpressing *CLN2* showed up to 3-fold higher levels of DAG than cells not overproducing *CLN2* (data not shown). Taken together, these data provide strong evidence for a direct relationship between the Cdc28 kinase and DAG production at START and suggest phospholipase activation may be a direct consequence of phosphorylation by Cdc28, although a more indirect mechanism of activation is also possible.

Cdc28-dependent hydrolysis of phosphatidylcholine

DAG as a second messenger for PKC activation can be produced by several mechanisms. The most common pathways are either: (i) PLC activity on phosphatidylinositol-4,5 bisphosphate (which also yields inositol 1,4,5 trisphosphate; Berridge and Irvine, 1984) or PC which also yields choline phosphate (McKenzie *et al.*, 1992), or (ii) phospholipase D (PLD) activity on PC (Billah and

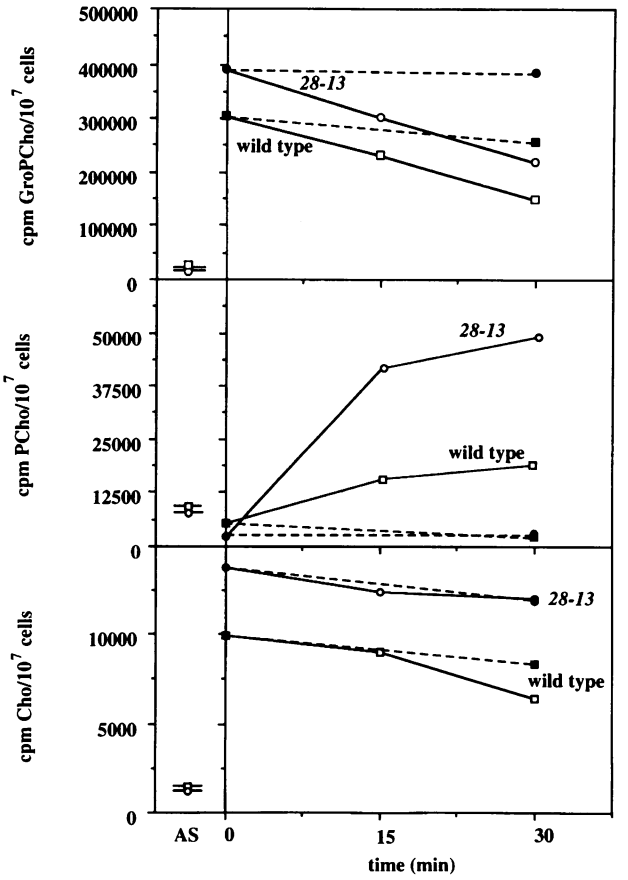

Fig. 7. *CDC28*-dependent hydrolysis of phosphatidylcholine. *cdc28-13* (D13au) and an isogenic wild-type strain (15Dau) were labeled with [^{3}H]choline as described in Materials and methods. Mid-log phase cells were incubated at 37°C for 2h and then shifted to 23°C (t = 0) or maintained at 37°C for an additional 30 min. At the indicated times cells were analyzed for glycerophosphocholine (GroPCho, top panel), choline phosphate (PCho, middle panel) and choline (Cho, bottom panel). AS indicates the asynchronous levels of choline metabolites in the cultures prior to shift to 37°C. *cdc28-13* 23°C (○), 37°C (●); wild-type 23°C (□), 37°C (■).

Anthes, 1990). The products of the PLD reaction are choline and phosphatidic acid, which can then be hydrolyzed to DAG through the action of phosphatidic acid phosphohydrolase. While measurement of total DAG levels demonstrate a Cdc28-dependent increase, this analysis is unable to indicate the probable phospholipid source and the type of phospholipase being activated. We have therefore assayed the abundance of specific phospholipid metabolites as yeast cells traverse START.

Yeast cellular phospholipids were labeled *in vivo* with either [^{3}H]choline or [^{3}H]inositol and phospholipid head group release was measured as a function of Cdc28 activity. Consistent with previous results (Hawkins *et al.*, 1993), we were unable to detect any significant Cdc28-dependent generation of inositol polyphosphate in inositol-labeled cells traversing START (even at very short times following Cdc28 activation, data not shown). There was, however, a marked increase in choline phosphate levels in similar experiments with choline-labeled cells (Figure 7). Choline phosphate levels increased ~20- to 25-fold following recovery from *cdc28^{ts}*-arrest. Furthermore, the kinetics were consistent with that of the DAG increase (cf. Figure 4A).

It is noteworthy that similarly treated wild-type cells showed only a modest increase (~4-fold) in choline phosphate levels seemingly due to temperature shift. Indeed, it appears PC metabolism is sensitive to changes in temperature based on the dramatic increases of both glycerophosphocholine (phospholipase A product) and choline (phospholipase D product) following culture shift to 37°C. It is unlikely, however, that the non-Cdc28-dependent increase in choline phosphate is a direct result of PC hydrolysis but is rather the result of some other step in choline metabolism, since we have never observed any temperature-dependent effect on total DAG levels (Figure 4B).

The data in Figure 7 are from a single experiment that has been repeated six times. In every case synchronized cells traversing START show significantly greater levels of choline phosphate (unlike other choline metabolites) than wild-type cells treated similarly. Furthermore, overproduction of Cln2 led to an enhancement of choline phosphate production, supporting the Cdc28-specificity of this process. Finally, we have also observed a stimulation of choline phosphate in cells traversing START following recovery from mating pheromone-arrest (data not shown). Thus, as with DAG production, these data indicate a cell cycle-related phenomenon.

Since there was no detectable increase in free choline levels, PC hydrolysis is likely to result solely from PLC activity. These data, along with the absence of detectable Cdc28-dependent inositol phosphate generation, suggest that the Cdc28-dependent accumulation of DAG in the yeast cell cycle occurs via stimulation of PC-PLC activity.

Discussion

In this study, we have demonstrated a functional interaction between the Cdc28 kinase and Pkc1 by several genetic and biochemical criteria. The most plausible linkage of Pkc1 function to Cdc28 activation at START is via a cell cycle-regulated, Cdc28-dependent increase in DAG levels (Figure 4), an activator of mammalian PKC (Nishizuka, 1988, 1992). The fold stimulation seen in these experiments is of the same magnitude seen in other systems where receptor-stimulated phospholipid hydrolysis has been studied. For example, the addition of bombesin to Swiss 3T3 cells (Cook *et al.*, 1990), vasopressin to hepatocytes (Bocckino *et al.*, 1985) and thrombin to fibroblasts (Wright *et al.*, 1988) all give rise to ~2- to 3-fold stimulation of DAG levels and subsequent PKC activation. We therefore believe, that the Cdc28-dependent stimulation of DAG production described in this manuscript is of a magnitude capable of activating Pkc1 signaling pathways.

Although a direct relationship between DAG and Pkc1 activity in *S.cerevisiae* has not been documented, all PKC isozymes structurally related to Pkc1 are DAG-stimulable and Pkc1 contains consensus DAG-binding sites (Levin *et al.*, 1990). Furthermore, at least one other PKC activity in yeast cell lysates is stimulated by DAG (Ogita *et al.*, 1990). Two recent studies, however, have demonstrated that affinity purified Pkc1 was not stimulated *in vitro* by the DAG species sn-1,2-dioleoylglycerol, unlike its mammalian counterparts (Antonsson *et al.*, 1994; Watanabe *et al.*, 1994). It is a concern, however, that

a small, atypical fraction of the Pkc1 population was biochemically analyzed (5% of total cellular Pkc1; Antonsson *et al.*, 1994). For example, the Pkc1 isolated has a far greater specific activity than its mammalian counterparts in the absence of allosteric activators while the majority that remains in the insoluble multi-protein complex is inactive (M.Payton, personal communication).

It is also possible that the Pkc1 activity measured in these reports represents a basal, unactivated level since Pkc1 was constitutively activated by mutational incapacitation of an autoregulatory domain which results in the relief of co-factor dependence (Pears *et al.*, 1990; Watanabe *et al.*, 1994). Thus, Watanabe *et al.* (1994) concluded that the appropriate conditions for Pkc1 activation have not yet been determined and that Pkc1 activity is enhanced by co-factors yet to be identified.

We also suggest that the lack of activation under these conditions does not indicate that Pkc1 is DAG-unresponsive, but rather illustrates the need for a different class of DAG co-factor. In this regard, a different subset of DAG species is produced in response to Cdc28 activation than in stimulated mammalian cells (T.Pettitt and M.J.O.Wakelam, unpublished observations). In fact, sn-1,2-dioleoylglycerol level is not elevated as a function of Cdc28 in these kinds of cell cycle paradigms. While the regulatory region of Pkc1 clearly contains the hallmark sequences shown to be required for DAG and phorbol ester binding (Levin *et al.*, 1990), small differences in this region between Pkc1 and its mammalian counterparts perhaps predict a different DAG species as co-factor (Antonsson *et al.*, 1994). Based on the similarities in structure between Pkc1 and the Ca^{2+}-dependent PKC isozymes, it still seems likely that Pkc1 is stimulated by DAG. Further experimentation is required, however, to fully address this issue.

We have also demonstrated that the Cdc28-dependent burst of DAG production correlates with choline phosphate production implicating the activation of a PC-PLC (Figure 7). PC hydrolysis is intimately related to Cdc28 activation at START since: (i) DAG production occurs in the absence of protein synthesis following re-activation of the Cdc28 kinase (Figure 6A), and (ii) the extent of PC hydrolysis is directly correlated with the level of activity of Cdc28 (Figure 6B).

We think it likely that PC-PLC activity is involved for several reasons. First, *in vivo* PC labeling experiments show a Cdc28-dependent stimulation of choline phosphate production, the product of PLC activity (Figure 7). The absence of choline production (and, therefore, PLD activity) in these experiments suggests PLC as the sole contributor to PC hydrolysis. Similarly, we think it unlikely that PI-PLC is involved based on the absence of inositol phosphate generation in response to Cdc28. In accord with this latter point, Hawkins *et al.* (1993) also noticed no cell cycle effects on inositol phosphate levels.

Further evidence for discounting PI turnover comes from studies with *PLC1*, which encodes the only PI-PLC isolated from yeast (Flick and Thorner, 1993; Payne and Fitzgerald-Hayes, 1993; Yoko-o *et al.*, 1993). First, deletion of *PLC1* does not affect total cellular DAG levels nor does it affect the Cdc28-dependent oscillation of DAG (N.J.M., E.M. and S.I.R., unpublished observations). Second, *PLC1* does not interact genetically with *PKC1*, which also suggests that PI turnover (at least *PLC1*-driven PI turnover) is not involved in this pathway (Flick and Thorner, 1993; N.J.Marini, E.Meldrum and S.I.Reed, unpublished observations).

Thus, based on the genetic interactions between *CDC28* and *PKC1*, the Cdc28-dependent stimulation of Mpk1 activity and the Cdc28-dependent hydrolysis of PC in the cell cycle, we propose that one role of the Cdc28 kinase at START is to activate a PC-PLC which then leads to the production of DAG via PC hydrolysis and the subsequent activation of Pkc1. The observation that *CLN2* overexpression increases DAG to higher than normal levels provides a biochemical explanation for the partial rescue of the $pkc1^{ts}$ phenotype by overexpression of *CLN2* and provides further support for this pathway. A similar effect has been observed for other $pkc1^{ts}$ alleles (Levin and Bartlett-Heubusch, 1992), where exogenously added Ca^{2+} seems to rescue the defect by hyperactivation of the mutant proteins. It is unclear, though, if this effect is direct, by binding to Pkc1, or indirect by stimulation of Ca^{2+}-activated PLC to generate abnormally high concentrations of DAG. The finding does, however, confirm temperature-sensitive rescue by upstream activating elements.

Pkc1 mediates at least some of its function in maintaining cellular integrity via a protein kinase signaling cascade (reviewed in Errede and Levin, 1993). In this pathway, a series of kinases are sequentially activated resulting in the activation of a MAP kinase homolog, Mpk1. In this report we have shown that Mpk1 kinase activity is sensitive to modulation by Cdc28 (Figure 5). In addition, Zarzov *et al.* (1995) have also demonstrated Mpk1 activation during bud formation in the cell cycle (post-START) which is at least partially dependent on Cdc28 activation. Thus activation of this signaling pathway is apparently one manifestation of passage through START. How this pathway precisely functions to promote osmotic stability is unknown. It is likely, though, that at least part of the function of Pkc1 is in regulating cell wall composition and/or modification since *pkc1*-deficient cells display severe structural abnormalities (Paravicini *et al.*, 1992). Whereas deletion of *PKC1* displays a cell lysis defect at all temperatures, deletion of any of the remaining downstream members of the MAP kinase cascade results only in a temperature-sensitive cell lysis phenotype (Lee and Levin, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993). This indicates that Pkc1 may have other osmo-stabilizing roles outside of the MAP kinase cascade. We suggest that all pathways regulated by Pkc1 involved in osmotic stabilization during bud emergence and growth are ultimately triggered by Cdc28 activation at START via PLC activation.

All the cell lysis phenotypes associated with *PKC1* or other members of the MAP kinase cascade only manifest themselves at a particular point in the cell cycle when cells begin to bud. Thus, there must be some aspect of the transition from the uniform growth across the entire cell surface that occurs in unbudded, pre-START cells to the localized and directional growth that gives rise to the bud in post-START cells, that makes such cells particularly sensitive to lysis. The Pkc1 pathway(s) apparently marshalls this event and is responsible for making at least some of the changes necessary to maintain cellular integrity. This would also explain the need for pathway

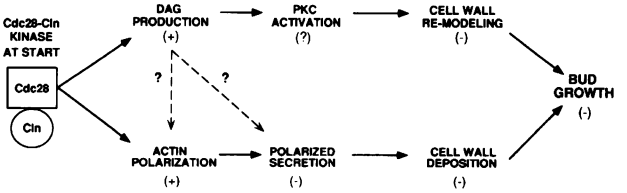

Fig. 8. Cdc28-dependent pathways leading to bud growth. There appear to be at least two separate pathways triggered by the Cdc28 kinase at START that are necessary for productive bud growth. See text for explanation. Cdc28-dependent events that occur in the absence of protein synthesis following Cdc28 re-activation (primary events) are designated (+); those that require protein synthesis (secondary events) are designated (–).

activation during pheromone-induced projection formation which is also a switch to polarized cell growth (Errede *et al.*, 1995; Zarzov *et al.*, 1995). The role of Pkc1 during budding may be direct, by affecting the proper function of cell wall components or modifying enzymes, or indirect, by altering their expression or transport to the bud site. In any event, it seems likely that such regulatory pathways would be a target for the START machinery in cycling cells. This is underscored by the genetic interaction displayed between *CDC28* and both *PKC1* (this report) and *MPK1* (Mazzoni *et al.*, 1993) as well as the START-dependent stimulation of Mpk1 activity (Zarzov *et al.*, 1995; this report).

It should be noted that stimulation of the Pkc1 pathway can also occur outside of the normal cell cycle by mechanisms that are independent of Cdc28 activity. For example, in Cdc28-inactivated cells Mpk1 activity is stimulated 8-fold during projection formation induced by mating pheromone (Errede *et al.*, 1995; Zarzov *et al.*, 1995). Furthermore, Mpk1 activity is increased in response to heat shock (10- to 150-fold; Kamada *et al.*, 1995, Figure 5). This Pkc1-dependent heat induction can occur in alpha-factor arrested cells in the absence of Cdc28 activity, although the data in Figure 5 suggest that heat induction in cycling cells is dependent on Cdc28 activity. Nevertheless, these results do indicate that Cdc28-independent modes of Pkc1 pathway activation also exist within the cell. In fact, the START-dependent stimulation of Mpk1 seems small relative to the potential for pathway activation seen in these Cdc28-independent contexts. The role of Cdc28 may simply be to reset pathway signaling at an elevated level to generate the increased output necessary during the budded phase of the cell cycle.

Thus it appears we can now invoke two pathways that emanate from Cdc28 at START that lead, in combination, to successful bud formation and growth (Figure 8). The biochemical and genetic evidence presented here suggest that one branch leads to the activation of Pkc1 via a PLC activity. Pkc1 then triggers one or more downstream pathways responsible for cell wall re-modeling or maintenance as cells enter the budded phase of the cell cycle. The second Cdc28-dependent pathway branch initially involves polarization of the cortical actin cytoskeleton to the bud site (Lew and Reed, 1993). Actin polarization then leads to polarization of the secretory apparatus which results in localized cell membrane and wall deposition at the bud site and, consequently, bud formation.

Although the Pkc1 pathway branch contains classical signaling molecules that are traditionally pleiotropic, we have positioned it as a distinct, parallel pathway since Pkc1 is not likely to be involved in bud formation: *pkc1Δ* cells are capable of forming buds (Levin *et al.*, 1990). It is quite possible, however, that PLC activity may have more widespread functions and may influence factors affecting polarized cell growth. For example, DAG may also activate other members of the PKC family (Ogita *et al.*, 1990). However, a thorough cell cycle analysis of the role of PC hydrolysis can only be achieved by isolation and identification of the gene(s) for the PLC(s) involved.

Cdc28-dependent polarization of the actin cytoskeleton can occur in the absence of protein synthesis following release from *cdc28^{ts}*-arrest (Lew and Reed, 1993). Like DAG production (this report) and G_1-specific transcriptional induction (Marini and Reed, 1992), these events must all be intimately related to Cdc28 activation and may be the result of direct phosphorylation events. It is becoming clear that Cdc28-dependent events can be classified into two categories: primary [those that can occur in the absence of protein synthesis following Cdc28 re-activation; designated (+) in Figure 8] and secondary [those that require protein synthesis; (–) in Figure 8]. Thus, where bud formation would be a secondary Cdc28-dependent event, DAG production and actin polarization would be primary events; where DNA replication would be secondary, transcriptional induction of DNA replication genes would be primary. The ultimate goal then becomes to molecularly link primary events in these pathways to the Cdc28 kinase as well as to downstream secondary events.

The existence of a putative pathway involving Cdc28, PLC and Pkc1 is significant since it provides the first evidence that classical receptor-stimulated signaling elements can operate within a pathway governed by the major cell cycle regulatory kinase in the absence of extracellular stimuli. In light of the high degree of conservation in both structure and function of the cell cycle regulatory machinery, it will be interesting to see if similar pathways exist in higher eukaryotes. The identification of the PLC gene(s) involved will help to shed light on this question and help us better understand the roles of phospholipid hydrolysis during the cell cycle.

Materials and methods

Yeast strains and plasmids

All yeast strains used in this study were derivatives of BF264-15D, *MATα ade1 his2 leu2-3,112 trp1-1a* (Reed *et al.*, 1985). The genotypes of the strains used were: 15 Dau, *MATa ura3ΔNS* (Cole *et al.*, 1990); GCY11, 15Dau *bar1::LEU2*; D13au, 15 Dau *cdc28-13*; D4au, 15 Dau *cdc28-4*; D4u-d, isogenic MATa/α diploid of D4au homozygous for all loci; TME-3, D4u-d *pkc1^{ts}/PKC1*; 3/28, 15Dau *cdc28-4 pkc1^{ts}*; 3(15), 15 Dau *pkc1^{ts}*. To create strains that conditionally overexpress *CLN2*, *GAL1^{P}::CLN2* was integrated at the *leu2* locus by transformation with YIpG2[*CLN2*] (Marini and Reed, 1992) linearized at the *Bst*EII site. All yeast transformations were carried out by the alkali cation method described by Ito *et al.* (1983).

The plasmid YCplac33(*PKC1*) was created by inserting the 10 kb *Pst*I fragment from YEp352(*PKC1*) (Levin *et al.*, 1990; a generous gift of K.Matsumoto) into the *Pst*I site of YCplac33 (Gietz and Sugino, 1988). An integrating version of *PKC1* was constructed by cloning the 4.2 kb *Sph*I fragment of YCplac33(*PKC1*) into the *Sph*I site of YIplac128 (Gietz and Sugino, 1988). The resulting plasmid, YIplac128(*PKC1*), could be integrated at the *leu2* locus by cleavage with *Bst*EII or at the *PKC1* locus by cleavage with *Spe*I. A *PKC1* deletion construct was created by replacing the 1.3 kb *Stu*I fragment within the *PKC1* coding region (Levin *et al.*, 1990) in YIplac128(*PKC1*) with the 1.4 kb *Hin*dIII fragment of *URA3* (filled-in) from YIpEG28 (Giniger and Ptashne,

1988). Digestion of the resulting plasmid, YIplac128(*pkc1*Δ), with *Eco*R1 yielded a 2.4 kb fragment containing the *pkc1*Δ::*URA3* construct used to transform yeast.

Growth conditions and cell cycle synchrony

Yeast cultures were grown in YEPD (1% yeast extract, 2% bacto-peptone, 0.005% adenine, 0.005% uracil, 2% glucose) unless otherwise noted. When plasmid selection was required, cells were grown in minimal medium supplemented with appropriate amino acids and/or bases (Sherman *et al.*, 1982). Where indicated, sorbitol was added to the media (either plates or liquid) to a final concentration of 1 M. Galactose induction experiments in liquid were performed by growing cells in YEP supplemented with raffinose (2%) and adding galactose to 2% at the indicated times.

To obtain synchronously growing cultures following release from *cdc28*^{ts} -arrest, cells bearing a *cdc28-13* allele were grown to mid-log phase, incubated at 37°C for 2 h then shifted to 23°C. Where indicated, *de novo* protein synthesis was inhibited prior to Cdc28 re-activation by adding cycloheximide (final concentration 10 μg/ml) 10 min prior to shift to the permissive temperature. This concentration of cycloheximide was determined to inhibit 96–97% of total cellular protein synthesis in both asynchronous and *cdc28*-arrested cultures as measured by TCA-precipitable incorporation in cells labeled with [^{35}S]methionine (Marini and Reed, 1992). To obtain cell cultures synchronized by mating pheromone, GCY11 cells (0.5–1.0×10^7 cells/ml) were treated with α-factor at 40 ng/ml. Following uniform arrest, cultures synchronously recovered and progressed through subsequent cell cycles. Small daughter cells (15Dau) were collected by centrifugal elutriation essentially as described (Lew *et al.*, 1992). Collected cells were re-inoculated in fresh YEPD.

Genetic screening for cdc28^{ts} enhancer mutations

Strain D4u-d (MATa/α, cdc28-4/cdc28-4) was plated on YEPD plates at 250 cells/plate and mutagenized with UV irradiation to ~50% survival of an isogenic haploid strain (D4au). The plates were incubated in the dark at room temperature for 3 days and then replica-plated to YEPD plates at 34°C, 35°C and 36°C. Colonies that grew more slowly at 34–36°C (not 23°C) were isolated and re-tested several times for increased temperature sensitivity over the parent strain. Mutant diploids were sporulated, asci were dissected and the temperature sensitivity of spores was determined relative to a *cdc28-4* haploid strain. In some cases (see Results), the mutational enhancer seemed to segregate (2:2) with lethality. In most cases, tetrad dissection gave rise to three or four viable spores with some exhibiting an enhanced *ts* phenotype over a *cdc28-4* haploid. These were back-crossed to *cdc28-4* cells and the resulting diploids were re-tested for enhancement of the *cdc28*^{ts} phenotype. If positive, these were repeatedly sporulated and analyzed as above until the phenotype either was lost or segregated cleanly as a single allele in a 2:2 fashion.

The dominance or recessiveness of mutational enhancers was tested by crossing mutant segregants with an isogenic wild-type strain (15Du) yielding diploids heterozygous for the mutation as well as for *CDC28*, or simply by transforming cells with a single copy of the wild-type *CDC28* gene. This was necessary since the mutations display a phenotype (enhanced temperature sensitivity) when heterozygous in a *cdc28*^{ts} background which is the basis of the screen. All mutations to date are recessive with respect to Cdc28 and have no observable phenotype when heterozygous in a *CDC28* background. Complementation tests between mutants were performed by mating mutant segregants and assaying temperature sensitivity of the resulting diploids. Although these cells are more temperature sensitive than the parent strain (D4u-d), the phenotype is significantly more severe when the mutant allele is homozygous and thus easy to distinguish.

Preparation of yeast protein extracts

Cultures of strain D13au (*cdc28-13*) expressing HA-tagged *MPK1* from the plasmid YEp352-*MPK1*-HA (Kamada *et al.*, 1995) were grown at 25°C in minimal selective media to ~3×10^6/ml. Cells were then re-suspended in YEPD and grown for one generation prior to shifting to 37°C by the addition of an equal volume of YEPD equilibrated to 50°C. Cultures were maintained at 37°C until cells were arrested as monitored by the number of unbudded cells. The cultures were then quickly shifted to the permissive temperature by the addition of an equal volume of chilled YEPD and maintained at 25°C for the duration of the experiment. Protein extracts were prepared by the method of Kamada *et al.* (1995). Protein concentrations were determined with the Bio-Rad protein assay reagent kit.

Immunoprecipitations and Mpk1 kinase assays

Immunoprecipitations and kinase assays were performed essentially by the method of Kamada *et al.* (1995). Briefly, 100 μg of protein extract were incubated with 2 μl of Anti-HA raw Ascites fluid for 60 min at 4°C. Fifteen microliters of packed and washed protein A–Sepharose beads (Pharmacia-LKB) were incubated with this antibody/protein mixture for 60 min at 4°C. Following extensive washing of the beads, kinase buffer and 5 μg of myelin basic protein were added to each assay mixture. The reactions were started by adding 2.2 μl of ATP (2 μl of 1 mM ATP and 0.2 μl of [γ-^{32}P]ATP) and incubated at 30°C for 30 min. The kinase reactions were stopped by the addition of SDS–PAGE sample buffer. The proteins were released from the adsorbed complex by boiling, the supernatant divided into two equivalent samples and each fractionated by SDS–PAGE. One gel was dried and subjected to autoradiography and the other subjected to Western analysis using anti-HA antibody. Mpk1 kinase activity was quantitated by laser densitometry and normalized to the amount of Mpk1-HA recovered by Western analysis. In control experiments using extracts from cells not containing the *MPK1*- HA plasmid, no detectable myelin basic protein kinase activity was recovered with anti-HA antibody (data not shown).

Quantitative assay for total cellular diacylglycerol (DAG)

Briefly, ~3×10^7 cells were harvested for each time point, washed and cell pellets stored at –70°C until processing. Typically, triplicate samples were taken for each time point. Cell extracts were prepared on ice by resuspending cells in 1 ml methanol and adding 0.5 ml chloroform and 0.9 ml water. This resuspension was sonicated for 10 s and an additional 0.5 ml chloroform added. After vortexing and centrifugation (1500 r.p.m. clinical centrifuge for 15 min), the interphase and lower phase together were washed twice with 1 ml water:methanol:chloroform (16:16:1). After the final wash, 0.5 ml lower phase was dried under vacuum and used for DAG determination using the DAG kinase linked assay of Preiss *et al.* (1986). The amount of DAG in each sample was determined using a calibration curve generated with pure sn-1,2-diacylglycerol (Sigma) over a range of 0.2–3.0 μmoles each time the assay was performed.

Assay for the generation of [^{3}H]choline metabolites

Yeast cells were grown overnight to logarithmic phase in 'semi-minimal' media (synthetic complete media supplemented with 0.2% yeast extract) containing 2 μCi/ml [methyl-^{3}H]choline chloride (80 Ci/mmol, New England Nuclear). Cells were synchronized at START with the *cdc28-13* allele as described above. Approximately 5×10^7 cells were taken per sample. Washed cell pellets were extracted with 0.5 ml methanol for 30 min on ice prior to addition of 0.5 ml chlorofom and 0.45 ml H_2O. Glycerophosphocholine, choline phosphate and choline in the aqueous methanolic layer were separated on Dowex 50X8 H^+ (200–400 mesh, Bio-Rad) essentially as described (Cook and Wakelam, 1989; McKenzie *et al.*, 1992) and quantitated by scintillation counting.

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