Genetic Analysis of the Relationship Between Activation Loop Phosphorylation and Cyclin Binding in the Activation of the *Saccharomyces cerevisiae* Cdc28p Cyclin-Dependent Kinase

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ABSTRACT

We showed recently that a screen for mutant *CDC28* with improved binding to a defective Cln2p G1 cyclin yielded a spectrum of mutations similar to those yielded by a screen for intragenic suppressors of the requirement for activation loop phosphorylation (T169E suppressors). Recombination among these mutations yielded *CDC28* mutants that bypassed the G1 cyclin requirement. Here we analyze further the interrelationship between T169E suppression, interaction with defective cyclin, and G1 cyclin bypass. DNA shuffling of mutations from the various screens and recombination onto a T169E-encoding 3′ end yielded *CDC28* mutants with strong T169E suppression. Some of the strongest T169E suppressors could suppress the defective Cln2p G1 cyclin even while retaining T169E. The strong T169E suppressors did not exhibit bypass of the G1 cyclin requirement but did so when T169E was reverted to T. These results suggested that for these mutants, activation loop phosphorylation and cyclin binding might be alternative means of activation rather than independent requirements for activation (as with wild type). These results suggest mechanistic overlap between the conformational shift induced by cyclin binding and that induced by activation loop phosphorylation. This conclusion was supported by analysis of suppressors of a mutation in the Cdk phosphothreonine-binding pocket created by cyclin binding.

CYCLIN-dependent kinases have two requirements for enzymatic activation: cyclin binding and activation loop phosphorylation. Enzymatic activation involves the refolding of the kinase catalytic subunit from its inactive form as unphosphorylated monomer to its fully active, cyclin-bound form in which it assumes a fold found in other active protein kinases (Jeffrey *et al.* 1995). It is likely that in addition to simple enzymatic activation, cyclin binding also contributes to substrate targeting (Schulman *et al.* 1998; Cross *et al.* 1999; and references therein).

Cyclin binding and activation loop phosphorylation are independently required for full enzymatic activation of most Cdks, with extremely low (but nonzero) activity either for cyclin-bound unphosphorylated kinase or for phosphorylated, cyclin-free monomer (Russo *et al.* 1996; Brown *et al.* 1999). One exception to this pattern is Cdk7, which can be activated by cyclin H in the absence of phosphorylation or by phosphorylation alone (Fisher *et al.* 1995). Cdk2 has been characterized structurally in all four states [with and without cyclin, with and without phosphorylation (Brown *et al.* 1999; Pavletich 1999]. It is clear from these studies that cyclin binding is sufficient to induce most of the conformational changes that are required for conversion of Cdk2 into an active enzyme; nevertheless, the subtle additional changes in structure upon phosphorylation of cyclin-bound Cdk2 result in a very large increase in enzymatic activity. Phosphorylation of the cyclin-free monomer may result in greater flexibility of the activation loop and yields an extremely low level of kinase activity that is nevertheless higher than that of unphosphorylated monomer (Brown *et al.* 1999). Thus, while the requirement for cyclin binding to induce Cdk enzymatic activity is well understood structurally, the role of activation loop phosphorylation is less clear.

Cak1p phosphorylates threonine 169 in the activation loop of the Cdc28p cyclin-dependent kinase (Espinoza *et al.* 1996; Kaldis *et al.* 1996; Thuret *et al.* 1996). Threonine 169 cannot be substituted with glutamic acid to give a functional *CDC28* (Lim *et al.* 1996; Cross and Levine 1998). This is probably due not to a regulatory requirement for activation loop phosphorylation, but rather to a simple requirement for a dianionic residue to coordinate basic residues in a binding pocket (Johnson *et al.* 1996; Russo *et al.* 1996). This is suggested by the finding that directed evolution of Cdc28p mutants containing the T169E substitution resulted in T169E-containing Cdc28p that could carry out all cell cycle functions tested with appropriate regulation (Cross and Levine 1998).

We showed recently that a screen for mutant Cdk with improved binding to a defective Cln2p G1 cyclin yielded a spectrum of mutations overlapping with the T169E

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TABLE 1

Strains used

Name	Genotype	Source	Reference
KL244	*MATα bar1 cln1Δ cln2Δxs cln3Δ leu2::LEU2::GAL1::CLN3 cdc28-csr1 trp1 ura3 ade1 his2 pURA3/cln2-KA,EA*	Lab stock	Levine *et al.* (1999)
SY80	*MATα cak1::HIS3 trp1 leu2 ura3 his3 ade2 can1 pURA3/CAK1*	Ann Sutton	Kaldis *et al.* (1996)
FC23-8	*MATα bar1 leu2::LEU2::GAL1::CLN2 trp1 ura3 ade1 his3 cdc28::HIS3 pURA3-GAL-CDC2-hs*	Lab stock	Cross and Levine (1998)
1817-2	*MATα bar1 trp1 leu2 ura3 ade1 his3 cdc28::HIS3 pURA3/CDC28-csr1*	Lab stock	Levine *et al.* (1999)

suppressors (Levine *et al.* 1999). Recombination among these mutations using a recombinant pool "DNA shuffling" approach yielded *CDC28* mutants that bypassed the G1 cyclin requirement (Levine *et al.* 1999). These results suggested a connection between Cdk activation loop phosphorylation and cyclin binding. Here we pursue this connection by characterizing mutants from the recombinant pool that suppress T169E.

MATERIALS AND METHODS

Yeast strains and genetic methods: Yeast strains were all described previously (Table 1). Screens for *CDC28* function were as previously described (Cross and Levine 1998; Levine *et al.* 1999) and are outlined below.

Function of mutant *CDC28* with or without overexpressed wild-type *CLN2* was assayed in strain FC23-8: *cdc28::HIS3 leu2::LEU2::GAL1::CLN2 pGAL1::CDC2-hs/URA3*. Ability of mutant *CDC28* to complement *cdc28::HIS3* was assayed in transformants of this strain by assaying ability to lose the *CDC2* plasmid on fluoroorotic acid (FOA)-D medium. Ability of mutant *CDC28* to complement *cdc28::HIS3* was also assayed in strain 1817-2 (*cdc28::HIS3 pURA3/CDC28-csr1*) by the same assay. [The *csr1* allele of *CDC28* (Levine *et al.* 1998) was present on the plasmid in this strain; this was not essential for the experiments and reflected only available material for strain construction.] Ability of mutant *CDC28* to function in combination with overexpressed *CLN2* was assayed by examining the ability of such transformants (after selection on FOA-D) to resist mating-factor-induced cell cycle arrest on galactose medium (*GAL1::CLN2* on) but not on glucose medium (*GAL1::CLN2* off). Specificity of this response for Cln2p-Cdc28p interaction has been established previously (Oehlen and Cross 1994; Levine *et al.* 1998).

Function of mutant *CDC28* in combination with the defective *cln2-K129A,E183A* allele (Levine *et al.* 1999) was assayed in strain KL244: *cln1Δ cln2Δ cln3Δ leu2::LEU2::GAL1::CLN3 pcln2-K129A,E183A/URA3*, by the viability of transformants with mutant *CDC28* on YEPD medium (*GAL1::CLN3* off). The ability of such transformants to function in the complete absence of the three *CLN* G1 cyclins was assayed by the viability of transformants on FOA-D medium, selecting against the *pcln2-KA,EA/URA3* plasmid.

Ability of mutant *CDC28* to bypass the *CAK1* requirement was assayed in strain SY80 (*cak1::HIS3 pCAK1/URA3*) from Ann Sutton by assaying the viability of transformants on FOA medium selecting against maintenance of the *CAK1* plasmid.

DNA methods: Construction of point mutants and of recombinant pools by splice-overlap PCR was as described (Cross and Levine 1998; Levine *et al.* 1999). Vent thermostable DNA polymerase (New England Biolabs, Beverly, MA) was used throughout, with the buffer provided by the manufacturer. For the first recombinant pool, plasmids containing the various mutations indicated in Figure 1, as well as wild-type *CDC28*, were pooled for template using oligonucleotides at spaced intervals within the *CDC28* gene. These 20-mer oligonucleotides were in matched complementary pairs, so that adjacent amplification products could then be "stitched together" by splice-overlap PCR following gel purification. Modified PCR conditions (10 min annealing time at 55° instead of 1 min; 15 cycles instead of 25 cycles) were used to minimize amplification of contaminating full-length product independent of inclusion of both fragments in the reaction.

The splice-overlap recombinant PCR experiments described in Figures 3 and 7 were performed similarly except that the *CDC28* coding sequence was amplified complete from the initial mutants. These PCR products were then mixed in various ratios and amplified using the oligos flanking small segments of *CDC28*. This had the effect of allowing a more efficient and predictable yield of recombinants. The mutations indicated in the various segments were made equimolar at the level of input template for the PCR reaction; wild-type DNA was also always included at a proportion of at least one-third of the input.

RESULTS

DNA shuffling to improve bypass of the requirement for activation loop phosphorylation in Cdc28: It has been shown that allowing recombination between different modules of a protein with independent partial solutions to a functional challenge can yield highly effective recombinant molecules (Stemmer 1994). Previously we used this strategy to recombine *CDC28* mutations that suppress a defective *CLN2* G1 cyclin, *CDC28* mutations that suppress a glutamate-for-phosphothreonine substitution (*CDC28-T169E*; Cross and Levine 1998), and *CDC28* mutations suppressing another activation loop mutation (L157A). DNA shuffling yielded mutant *CDC28* genes that could bypass the *CLN* G1 cyclin requirement (Levine *et al.* 1999). Since T169E suppressors (Cross and Levine 1998) were one source of mutations used as input to the pools, we wanted to evaluate the effectiveness of mutants in the recombinant pool at activation loop phosphorylation bypass.

We screened members of the recombinant pool, fused to a T169E-containing 3′ end, for the ability to function in the *CDC28* assays described previously for selection and characterization of T169E suppressors

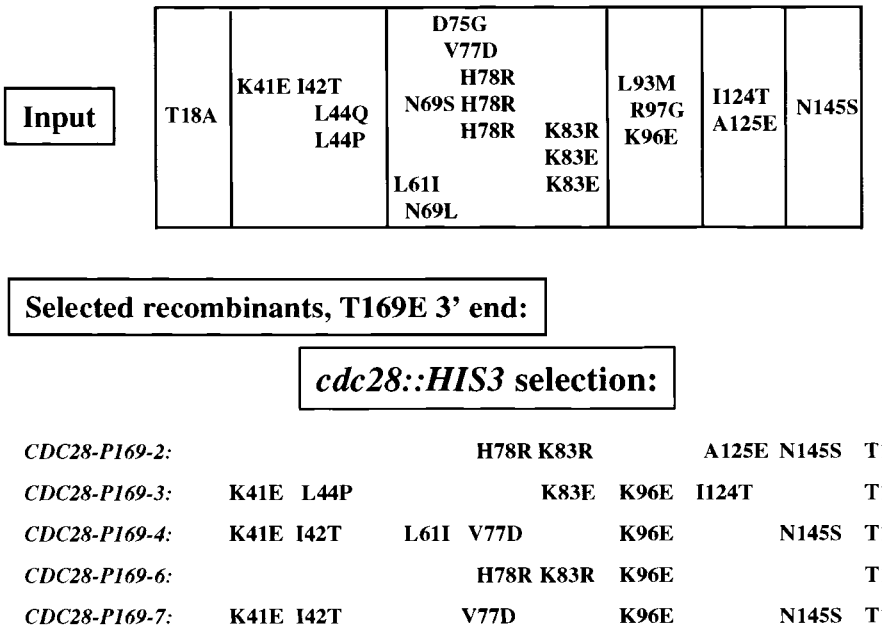
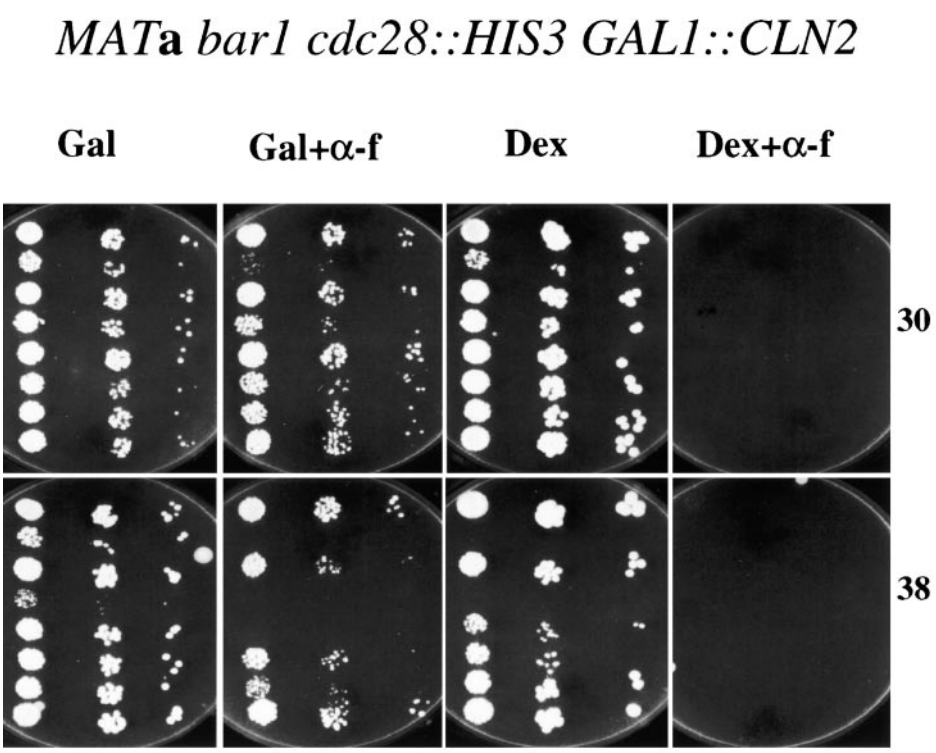


Figure 1.—Sequences of recombinant pool 1 input and selected T169E suppressors. The construction of this recombinant pool was described previously (Levine *et al.* 1999). The indicated segments containing the pooled templates shown (including wild type in all segments) were spliced together by splice-overlap PCR to make a recombinant pool. The pool was recombined with a T169E-encoding 3′ end by splice-overlap PCR and tested for CDC28 function in the assays described (Cross and Levine 1998): complementation of *cdc28::HIS3* and a cyclin-specific function inhibiting mating factor sensitivity in conjunction with overexpressed *CLN2* (Oehlen and Cross 1994). See Figure 2 for examples of the assays. Five strong alleles isolated from the pool were sequenced.

(Cross and Levine 1998). We assayed for the ability to efficiently support viability in the absence of a wild-type *CDC28* gene, and we also assayed a cyclin-specific function: the ability to confer mating-factor resistance when the G1 cyclin *CLN2* was overexpressed (Oehlen and Cross 1994; Levine *et al.* 1998). These assays were conducted at 30° and at 38°, since we found previously that the higher temperature selected effectively for better mutants (Cross and Levine 1998). Some highly active mutants selected from recombinant pool 1 (Figure 1) were sequenced.

We found potent T169E suppressors that consisted of a backbone of previously identified weak T169E suppressors identified in the course of sequential mutagenesis (Cross and Levine 1998) recombined with various mutations derived from the other screens. Figure 2 is a reconstruction of the effects of additional mutations on previously identified weak T169E suppressors: compare 96/169-2 to P169-3 and 169-53 to P169-7, P169-4 (Figure 2). The additional mutations [derived from the defective cyclin suppression scheme or the L157A suppression scheme (Levine *et al.* 1999)] significantly increased biological activity. Thus the recombinant pool experiment, carried out using second-generation T169E suppressors (Cross and Levine 1998), yielded recombinants that were comparably active to the fourth-generation T169E suppressors (Cross and Levine 1998) generated by sequential selection and mutagenesis (Figure 2).

Selection for *cak1* bypass: The selection for T169E suppression used to identify suppressors from recombinant pool 1 (Figures 1 and 2) yielded rather disparate sequences (for example, there is no single point mutation in common among them), suggesting that the assays were not stringent enough to identify the best solutions from the recombinant pool. Therefore, we carried out a second recombinant pool construction. The input to the second recombinant pool is shown in Figure 3. All of the active clones identified in Figure 1 could have been generated also in the second recombinant pool. This pool is predicted to contain 5040 distinct sequences. We recombined the pool with a T169E 3′ end and screened for highly efficient suppressors. To do this in a more selective way than was done in the first experiment (Figures 1 and 2) we made use of the observation that efficient T169E suppressors will bypass the *CAK1* requirement for viability (Cross and Levine 1998). We observed previously that efficient *CAK1* bypass by T169E-containing mutants is strongly enhanced by the deletion of the wild-type *CDC28* gene. This is presumably due to competition between unphosphorylated wild-type Cdc28 (unphosphorylated due to the lack of the Cak1-activating kinase) and the T169E mutants (which cannot be Cak1 phosphorylated) for some essential factors (which could be cyclins, substrates, or other factors). Thus, requiring efficient *CAK1* bypass in the presence of wild-type *CDC28* should require efficient T169E suppression as well as efficient competition with unphosphorylated wild-type Cdc28, and this could tighten the selection to identify optimal T169E suppressors. We therefore selected members of the pool that could efficiently rescue a *cak1::HIS3 pURA3-CAK1* strain to viability on 5-fluoroorotic acid (selecting against maintenance of the *CAK1* plasmid). We readily obtained strong positives from the recombinant pool in this assay (Figures 3 and 4), although the original fourth-generation *CDC28-T169E* mutants were only very weakly positive in this assay (Cross and Levine 1998; Figure 5).



CDC28-wt							T169T
96/169-2:			L44P		K96E		T169E
P169-3:	K41E		L44P	K83E	K96E	I124T	T169E
169-53:				V77D	K96E		T169E
P169-7:	K41E I42T			V77D	K96E	N145S	T169E
P169-4:	K41E I42T		L61I	V77D	K96E	N145S	T169E
169-5331:				V77D	K83E K96E	I124V	T169E
169-53319:	T18S			V77D	K83E K96E	I124V	T169E

Figure 2.—Sequences recovered from the recombinant pool as strong T169E suppressors compared to T169E suppressors recovered by sequential mutagenesis. FC23-8 (Table 1; genotype at top of figure) was transformed with the indicated plasmids (see below for sequences, arranged from top to bottom in the same order as they appear in the photographs). Transformants were plated on FOA-D medium (selecting against the *URA3/GAL-CDC2-hs* plasmid) to select for cells in which *CDC28* function was solely due to the transformed plasmid. [Vector and *CDC28-T169E* were negative in this assay (Cross and Levine 1998)]. Transformants were tested for efficiency of *CDC28* function by plating 10-fold.serial dilutions of equal optical densities of stationary phase culture on YEPD medium and incubating at 30° or 38°. The mutants were also tested for a cyclin-specific function: the ability to confer mating factor resistance when the G1 cyclin *CLN2* was overexpressed from the *GAL1* promoter (Oehlen and Cross 1994; Cross and Levine 1998, Levine *et al.* 1998). Some of these mutants were selected from previous work (Cross and Levine 1998) and previously were found to exhibit varying levels of T169E suppression. The addition of mutations derived from other screens (underlined) by the recombinant PCR process in mutants PI 69-3, -4, and -7 (Figure 1) to these mutants significantly improved the T169E suppression activity of the mutants. Included for comparison are *CDC28-169-5331*, one of the most active fourth-generation mutants recovered by sequential mutagenesis, and a T18S-containing derivative with increased activity (Cross and Levine 1998).

We sequenced four strong alleles (Figure 3). These sequences were quite similar: all had a mutation at T18 (either T18A or T18S) and all contained L61I, V77D, and K96E. S53I, K83E, and I124V were also found in three out of four clones sequenced. Therefore, the more stringent *cak1* bypass selection appears to have identified a small class of solutions to the problem of T169E suppression from the recombinant pool. *cak1* bypass suppression requires T169E; reversion to T169T eliminates suppression (Figure 5), as described previously for other mutants (Cross and Levine 1998), presumably because there is a requirement for at least some negative charge (either phosphothreonine or glutamic acid) at this position for Cdc28 function.

None of the suppressors were as efficient at rescuing this strain as a *TRP1-CAK1* plasmid (data not shown), probably because Cak1p has other important but nonessential functions (Cross and Levine 1998), such as phosphorylating and activating the Kin28p protein kinase (Espinoza *et al.* 1998; Kimmelman *et al.* 1999). None of the T169E-containing suppressors could support viability in the absence of *CAK1* at 37° (data not shown).

Suppression of a binding-defective Cln2p G1 cyclin by T169E-containing *CDC28* mutants: We observed overlap between mutations that allowed suppression of a defective G1 cyclin, *cln2-K129A,E183A*, and mutations that suppressed T169E (Levine *et al.* 1999). Suppression of the defective *cln2*, with mutations in the presumptive Cdk-binding interface that cripple Cdc28p binding (Levine *et al.* 1996, 1999), may reflect an increased pro-

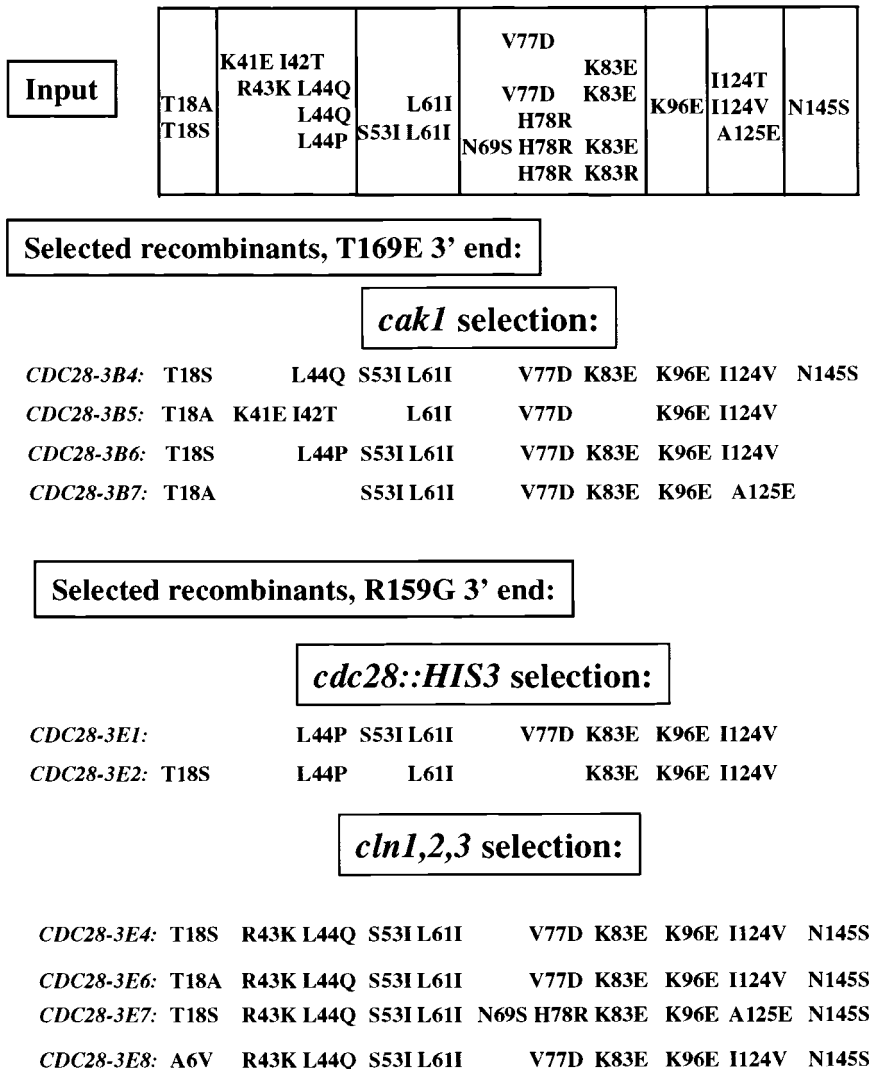


Figure 3.—Sequences of recombinant pool 2 input and selected T169E and R159G suppressors. Recombinant pool 2 (Levine *et al.* 1999) was constructed from the indicated input as in Figure 1. The pool was recombined with a T169E-encoding or R159G-encoding 3′ end. The T169E-containing pool was tested for bypass of the *cak1* requirement (*i.e.*, allowing FOA resistance in transformants of a *cak1::HIS3 pURA3/CAK1* strain; see Figure 4 for assay). The R159G-containing pool was tested for *CDC28* function in FC23-8 (Table 1; see Figure 9) and tested for G1 cyclin bypass by rescue of 1607-2D (*cln1 cln2 cln3 GAL1::CLN3*) on glucose medium, as described (Levine *et al.* 1999; see Figure 10). The most active clones in the various assays were sequenced.

pensity to adopt the enzymatically active Cdk conformation (Levine *et al.* 1999). To pursue this overlap, we tested mutants 43244 and 53319, the strongest of the mutants derived by sequential selection for T169E suppression (Cross and Levine 1998) in the *cln2-KA,EA* suppression (SKE) screen but found no detectable activity (Figure 6). This screen requires function of *cln2-KA,EA* since it is the only potentially functional G1 cyclin present under conditions of assay. In contrast, the strong T169E suppressor mutants derived by the recombinant pool approach (3B4, 3B5, 3B6, 3B7) (Figure 3) were all positive in this assay, indicating that they could function together with the defective G1 cyclin (Figure 6). The defective G1 cyclin was required for viability because these transformants were inviable on FOA medium selecting against the *pcln2-KA,EA/URA3* plasmid (data not shown; see below). We performed a third recombinational experiment (Figure 7) to examine more closely the correlation between T169E suppression in the *cak1* bypass assay and performance in the SKE assay requiring interaction with the defective Cln2p cyclin. The input to this pool was restricted on the basis of the best T169E suppressor clones identified previously (Figure 3). We selected recombinants from this pool on the basis of efficient suppression of *cak1* or on the basis of an efficient SKE phenotype. Upon isolation of recombinants functional in each assay, we sequenced them and cross-tested them in the reciprocal assay. We found a very similar set of sequences selected from the recombinant pool by the two selections (Figure 7), and all of them cross-tested as highly efficient in the reciprocal assay (Figures 4 and 6 and data not shown). Thus at least for the mutations available in this recombinant pool, this approach detects similar structural requirements for efficient *cak1* bypass and for rescue of the *cln2-KA,EA* defect. Due to the preselection of input to the pool, though, these results do not imply the absence of alternative solutions to one challenge that might not solve the other. Indeed, the best T169E suppressors identified in previous work [*CDC28-169-43244* and

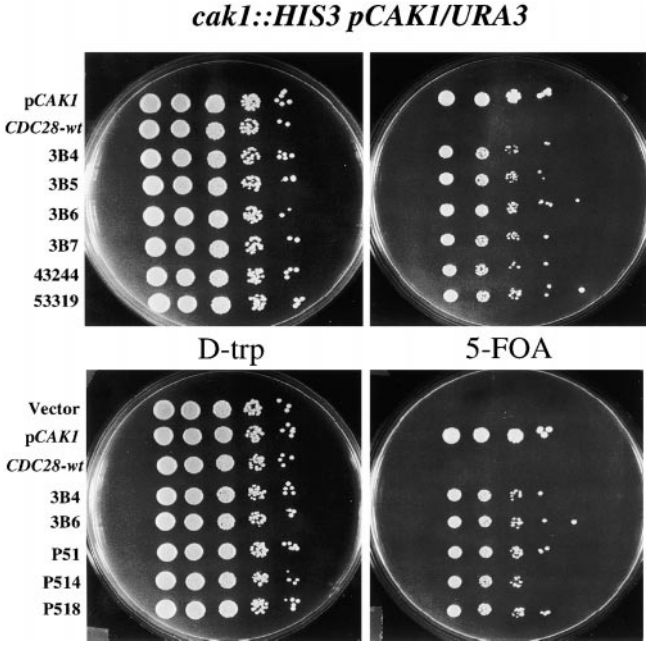


Figure 4.—Efficient *cak1* bypass with *CDC28-T169E* mutants derived from recombinant pool 2. SY80 (*cak1::HIS3 pURA3/CAK1*; Table 1) was transformed with the indicated plasmids (see Figures 1, 3, 7) and 10-fold serial dilutions of transformants spotted on ScD-trp or on FOA-D to select against the *pUR3/CAK1* plasmid.

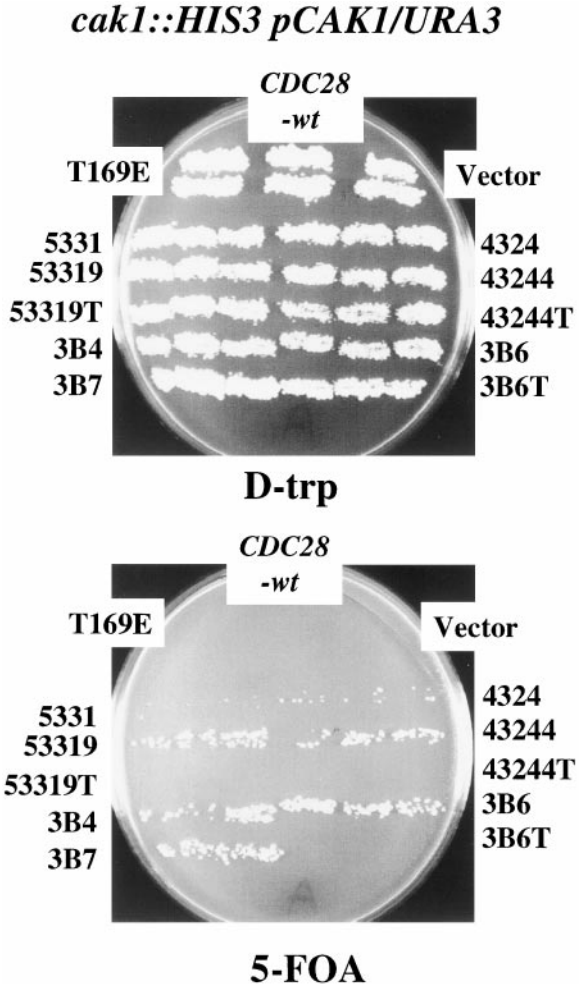


Figure 5.—Negative charge at position 169 is required for Cdc28p function even in T169E-suppressor backgrounds. SY80 (Table 1; *cak1 pURA3/CAK1*) was transformed with the indicated plasmids. *cak1* bypass is indicated by growth on 5-FOA, selecting against the *URA3/CAK1* plasmid. 43244 and 53319 are T18S-containing versions of 4324 and 5331 (Cross and Levine 1998). 3B4, 3B6, and 3B7 sequences are presented in Figure 3. 43244T, 53319T, and 3B6T are T169T, revertants of 43244, 5331, and 3B6, respectively.

-53319, the T18S-containing versions of *CDC28-169-4324* and *-5331* (Cross and Levine 1998)] appeared about as efficient as the mutants in Figure 3 at *cak1* bypass but were negative in the SKE assay (Figures 4 and 6).

Reversion of T169E to T169T in strong T169E suppressors allowed bypass of the G1 cyclin requirement: Although some of the most efficient T169E suppressors could suppress the defective G1 cyclin *cln2-KA,EA* (Figures 6 and 8), none were able to bypass the G1 cyclin requirement, since transformants were inviable on Dex-FOA (selecting against the *cln2-KA,EA* plasmid) (Figure 8). In contrast, reversion of T169E to T169T (clones labeled T) allowed G1 cyclin bypass in strong T169E bypass suppressor mutants (Figure 8).

The fact that mutations selected for T169E suppression yield bypass of the *CLN* G1 cyclins when placed in a T169T context argues that these mutations may promote the active conformation without the aid of cyclin binding. These same mutants are unable to bypass the G1 cyclin requirement with T169E. Thus they have cyclin-dependent activity with T169E (*i.e.*, providing the G1 cyclin requirement when provided with a defective G1 cyclin, *cln2-KA,EA*; also, the T169E versions of these mutants rescue *cdc28::HIS3* in the presence of a normal complement of cyclins) and potentially cyclin-independent activity with T169T (*i.e.*, rescuing a strain lacking all three *CLN* G1 cyclins). It may be useful to consider that these mutants convert the requirement for both cyclins and activation loop phosphorylation to a redundant requirement for either cyclin binding or activation loop phosphorylation for activation. Binding of cyclin H and MAT1 or activation loop phosphorylation are each independently sufficient to activate Cdk7p kinase activity (Fisher *et al.* 1995; Martinez *et al.* 1997, No. 6687), providing a possible natural analogy to these mutants. A difference is that Cdk7p bound to cyclin H/MAT1 is active with unphosphorylated threonine (Fisher *et al.* 1995), while the T169E suppressors probably still require the glutamic acid substitution, since they do not bypass the *CAK1* requirement when T169E is reverted to T169T (see above).

The requirement for the phosphothreonine-binding pocket is reduced by T169E suppressor mutations: The T169E suppressors might work by a global conformational change, or they could work by specifically promot-

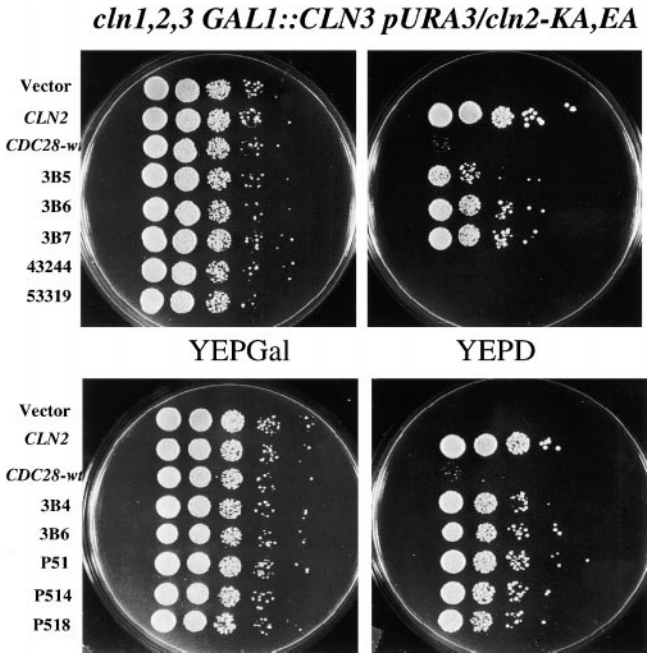


Figure 6.—Suppression of the defective G1 cyclin *cln2-KA,EA* by T169E-containing *CDC28* mutants. A *cln1 cln2 cln3 pcln2-KA,EA/URA3 GAL1::CLN3* strain (KL244; Table 1) was transformed with the indicated plasmids (see Figures 1, 3, 7 for sequences) and tested for viability on galactose (*CLN3* on) *vs.* glucose (*CLN3* off) medium.

ing the acceptance of glutamic acid. This could happen, for example, if the mutations created new contact points specific for this amino acid as distinct from phosphothreonine.

The phosphate on threonine 160 in the Cdk2 activation loop (equivalent to Cdc28p T169) interacts with three arginine residues that form a binding pocket (Russo *et al.* 1996). These arginine residues are in critical structural regions of Cdk2. One is in the PSTAIRE helix, which moves dramatically upon cyclin binding to reposition the ATP binding site. One is in the catalytic loop adjacent to a probable catalytic Asp residue. The third is in the αL12/β9 region of the activation loop, which also shifts position strikingly upon cyclin binding (Jeffrey *et al.* 1995). These residues are all conserved in Cdc28p. We reasoned that if the T169E suppressors were specific for glutamic acid at position 169, then they should fail to suppress phenotypic consequences of the loss of the binding pocket residues in the presence of phosphothreonine. R159 is equivalent to the pocket arginine in the activation loop. Previously we showed that mutation of R159 to glycine inactivated most Cdc28p biological functions and eliminated enzymatic activity (Levine *et al.* 1998). We asked if the T169E suppressors, or combinations of mutations in the recombinant pools, could rescue *cdc28-R159G*.

We recombined R159G with the recombinants in pool 2 and found that many clones from this pool when

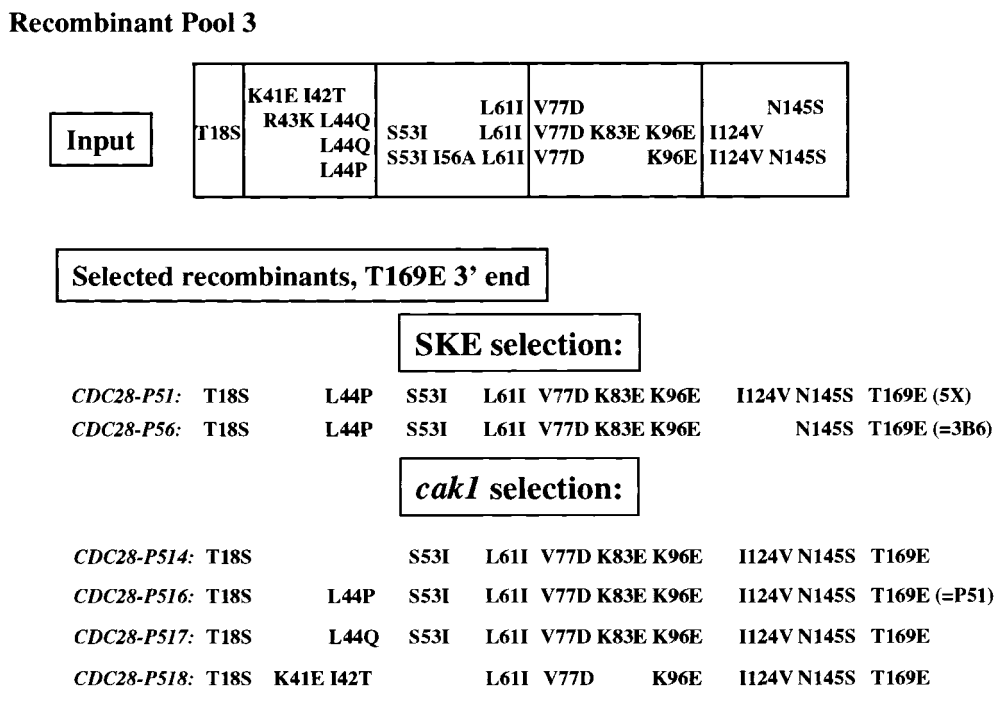


Figure 7.—Selection of similar sequences by selecting *cak1* bypass or by selecting for suppression of the defective G1 cyclin *cln2-KA,EA* by T169E-containing *CDC28* mutants. Recombinant pool 3 was constructed using the input sequences shown. All sequences used contained T169E 3′ ends. The pool was tested for efficient SKE suppression (*i.e.*, viability of a *cln1 cln2 cln3 pcln2-KA,EA GAL1::CLN3* strain on glucose medium; this selection was tightened by requiring efficient suppression at 35° for efficient *cak1* bypass (*i.e.*, allowing FOA resistance in transformants of a *cak1::HIS3 pURA3/CAK1* strain). The most efficient clones identified in each screen were sequenced. Two clones from each selection are not included in the tabulation because they contain additional mutations derived from PCR misincorporation during pool synthesis. 5X, *CDC28-P51* was isolated five times in this experiment.

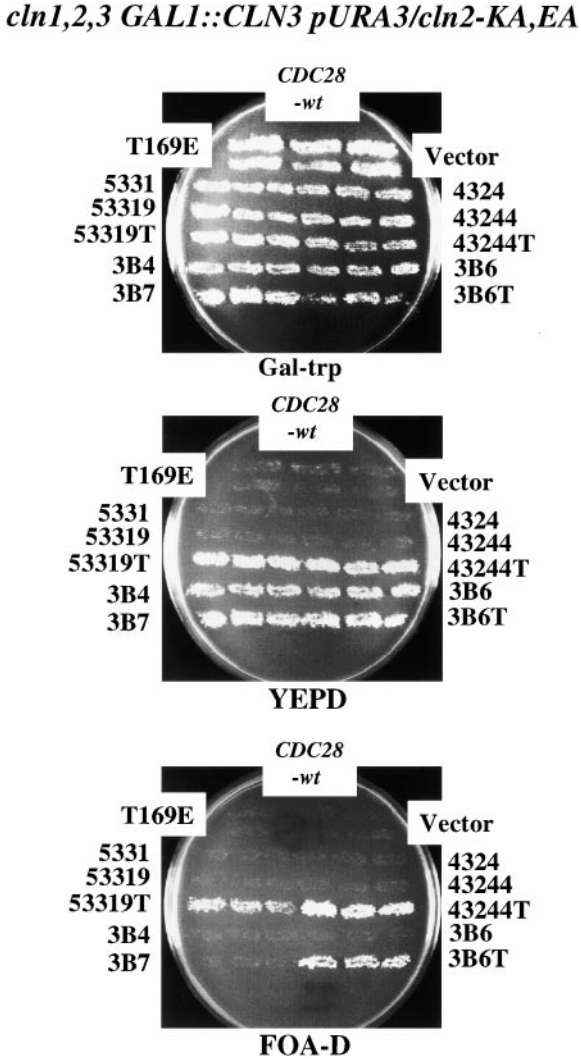


Figure 8.—T169E is incompatible with G1 cyclin bypass. The *CDC28* mutants tested in Figure 5 were transformed into KL244 (Table 1; *cln1 cln2 cln3 LEU2::GAL1::CLN3 cdc28-csr1 pURA3/cln2-KA,EA*). Suppression of the defective Cln2p-KA,EA cyclin was tested on YEPD medium, turning off *GAL1::CLN3*. Bypass of the G1 cyclin requirement was tested on FOA-D medium, turning off *GAL1::CLN3* and also selecting against maintenance of the *pURA3/cln2-KA,EA* plasmid.

combined with R159G could support viability of a *cdc28::HIS3* strain. We isolated two of the most efficient of these clones (3E1 and 3E2; Figure 9 and data not shown) and determined their sequences (Figure 3). The sequences isolated displayed a strong similarity to the most efficient T169E suppressors (Figure 3), indicating that the T169E suppressors do not function by a mechanism specifically requiring glutamic acid substitution. Rather, they may work by reducing the requirements for phosphothreonine interactions with the rest of the protein, including interaction of the phosphate with R159. This is interesting because R159 is equivalent to Cdk2 R150, which is part of the αL12/β9 region; this region carries out a helix-to-sheet transition that is central to the refolding of inactive monomer to active cyclin-bound Cdk2 (Jeffrey *et al.* 1995).

We also tested the recombinant pool constructs with R159G for the ability to rescue *cln1,2,3* inviability and found positive clones for this activity (Figures 3 and 10). These clones also were able to rescue *cdc28::HIS3* (Figure 9). 3E1 and 3E2, the clones identified by solely requiring *CDC28* function, were almost or completely negative in the *cln1,2,3* assay, although they were able to suppress the defective *cln2-KA,EA* cyclin (Figure 10). All clones that we have identified as causing G1 cyclin bypass also cause resistance to mating factor in a *cdc28::HIS3* background (Levine 1999); this may be expected since the G1 cyclins are the most likely targets of the mating-factor-induced cell cycle arrest signal. This correlation was also observed in the R159G-containing mutants (Figures 9 and 10).

We recombined the 3E4 sequence with R159R T169T, with R159R T169E, or with R159G T169T. We found that all of these mutants rescued *cdc28::HIS3* except for the R159G T169E version; only R159G T169T and R159R T169T could rescue the *cln1,2,3* strain (data not shown). We also failed to identify any clones in the recombinant pool that could rescue function of the R159G T169E 3′ end in the *cdc28::HIS3* assay (data not shown). The R159G suppressors were also completely negative in the *cak1* bypass assay (data not shown). All of these results indicate that the R159G suppressors retain an essential requirement for negative charge at position 169 and further that the R159G mutation creates an absolute requirement for phosphothreonine that cannot be substituted with glutamic acid (at least in the context of this set of suppressor mutations).

The R159G mutation almost completely eliminates Cdc28p-associated histone H1 kinase activity (Levine *et al.* 1998). We found that the R159G suppressor mutations restored significant (although generally less than wild type) levels of protein kinase activity (data not shown), confirming biochemically that these mutations restored function to *cdc28-r159G*.

DISCUSSION

Activation loop phosphorylation can be viewed as part of a transition from cyclin-free, unphosphorylated Cdk to cyclin-bound and phosphorylated Cdk (Pavletich 1999). Consistent with this view, a similar spectrum of mutations suppress both the defective activation loop in the T169E mutant and interaction with the defective G1 cyclin *cln2-KA,EA* (Levine *et al.* 1999). The initial mutants were unable to solve both challenges simultaneously. This presumably reflects an increased cyclin requirement for activation due to the T169E mutation, consistent with previous results (Cross and Levine 1998). We show here that further selection for efficient T169E suppression yields combinations of suppressor mutations able to rescue *cln2-KA,EA* even while re-

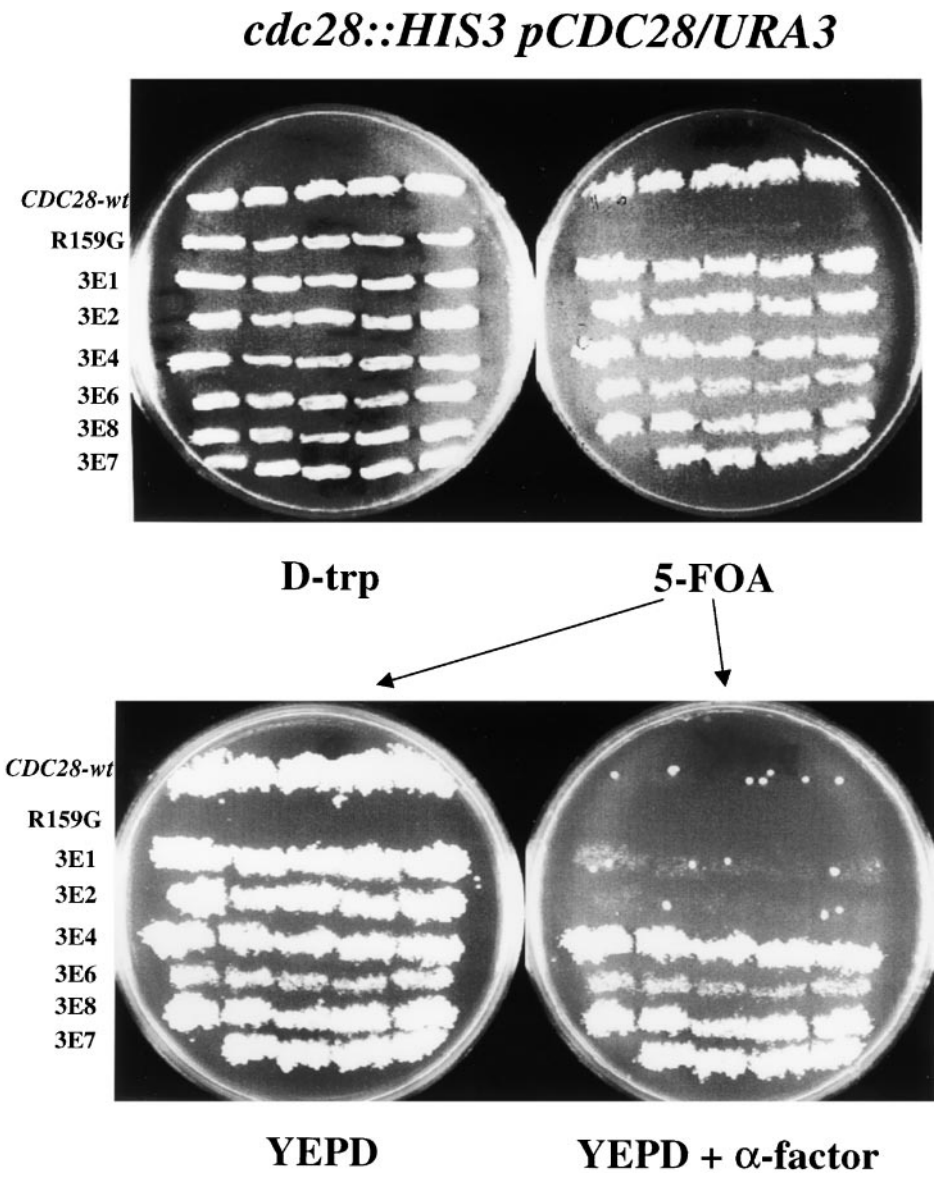


Figure 9.—Sequences from recombinant pool 2 suppress lack of *CDC28* function due to the R159G mutation. 1817-2 (*cdc28::HIS3 pCDC 28/URA3*; Table 1) was transformed with wild-type *CDC28* (SF19), with *cdc28*-R159G (KL059; Levine *et al.* 1998), or with the indicated R159G-containing suppressor mutants derived from recombinant pool 2 (Figure 3). Transformants were replica plated to 5-FOA to test for *CDC28* function. After 5-FOA selection, transformants were tested for mating factor resistance. Only clones able to bypass the G1 cyclin requirement (3E4, 3E6, 3E7, and 3E8; see Figure 10) are able to drive proliferation in the presence of mating factor.

taining the T169E mutation. The T169E mutation remained incompatible with complete bypass of the G1 cyclin requirement, indicating that *cln2-KA,EA* rescue was proceeding by interaction with the defective cyclin. Interestingly, reversion to T169T in these mutants did yield G1 cyclin bypass (Figure 8). This suggests that in these mutants, activation loop phosphorylation and cyclin binding may be alternative routes to activation, rather than nonredundant requirements for activation as in wild type. The incompatibility of T169E with G1 cyclin bypass could reflect a specific functional requirement for phosphothreonine at this position, for example, to allow substrate interaction in the absence of cyclins. A simpler explanation would be that phosphothreonine is more effective than glutamic acid at coordinating the structural changes required for kinase activation (Johnson *et al.* 1996).

Our finding that a common set of mutations is able to reduce or eliminate the requirements for both cyclin binding and activation loop phosphorylation for Cdk activation suggests that these requirements are functionally coupled, both supporting the same structural transition.

We proposed previously that these suppressor mutations might function by reducing a barrier to achieving the active conformation of Cdc28 (Levine *et al.* 1999). This idea readily explains the reduced requirements for cyclin binding. The energy available to the Cdk-cyclin complex upon cyclin binding is probably mainly derived from the burying of a large hydrophobic interface in the complex. This energy is presumably normally used both to drive the conformational shift of the Cdk to an enzymatically active form and to make the complex tight and stable. If previous mutation renders the conformational shift to the active form more readily achieved, then more of the energy is available to support stable

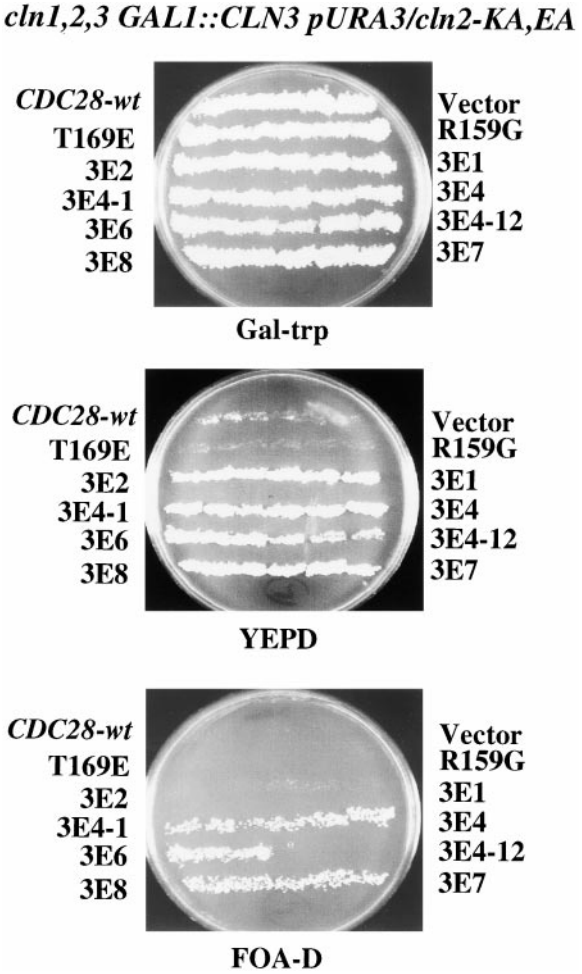


Figure 10.—Suppression of *cln2-KA,EA* and G1 cyclin bypass by R159G mutants. Mutants suppressing R159G (Figures 3 and 9) were transformed into KL244 (Table 1; *cln1 cln2 cln3 leu2::LEU2::GAL1::CLN3 cdc28-csr1 pURA3/cln2-KA,EA*) and tested as in Figure 8. Sequences are presented in Figure 3. 3E4-1 is an R159R version of 3E4; 3E4-12 is an R159R T169E version of 3E4.

binding to the cyclin, which could rescue a partially binding-defective cyclin.

Similarly, reduction of a barrier to the active conformation of Cdc28 also could result in the suppression of T169E. In the wild-type Cdk, the dianionic phosphate group may be required for precise coordination and alignment of the PSTAIRE helix, the catalytic loop and β9, since these are the locations of the arginines in the phosphothreonine-binding pocket (Russo *et al.* 1996). It is notable that the major motions of the Cdk driven by cyclin binding involve precisely the PSTAIRE helix and β9. These motions result in nearly the exact configuration required to generate the phosphothreonine-binding pocket (for images of this pocket in active and inactive Cdk2 conformations, see http://www.rockefeller.edu/labheads/crossf/activation_loop.html). Activation loop phosphorylation may then "cement" the final structure. The monoanionic glutamic acid residue may be unable to perform this coordination, but mutations that lead the Cdk to adopt the correctly aligned position spontaneously may allow acceptance of glutamic acid in place of phosphothreonine.

Very similar reasoning could explain the mutations leading to the loss of the requirement for R159, which is one residue of the phosphothreonine-binding pocket. If the function of the binding pocket-phosphothreonine interactions is the correct alignment of the parts of the protein contributing to the pocket, this requirement could be reduced or eliminated by phosphate-independent alignment of these regions due to mutation.

Unlike T169E, the R159G phosphothreonine-binding pocket mutation is compatible with efficient G1 cyclin bypass when combined with appropriate suppressor mutations. The role of R159 in conjunction with phosphothreonine-169 could be to help fix the β9 strand in its appropriate position in the active configuration. The amino acids making up β9 are largely α-helical in monomeric Cdk2, and cyclin binding induces a shift to β-sheet (Jeffrey *et al.* 1995), positioning R150 (equivalent to R159) correctly for binding to the phosphate (Russo *et al.* 1996). Identification of R159G-containing *CDC28* mutants allowing G1 cyclin bypass could imply correct positioning of β9 in the absence of cyclin and of the R159-phosphate interaction, supporting the idea that the suppressor mutations promote the active conformation, in which position 159 is in β-sheet.

There are evolutionary considerations relevant to our results. Mitogen-activated protein kinases (MAPK), the closest relatives to the cyclin-dependent kinases (Hanks and Hunter 1995), are activated by activation loop phosphorylation (as are the Cdks), but do not require binding of a cyclin-like activator. Activation loop phosphorylation of MAPK results in a motion of a key helix that realigns the catalytic cleft (Canagarajah *et al.* 1997); this motion is quite similar to the motion of the PSTAIRE helix in Cdk2 that occurs upon cyclin binding. It seems likely that the enzyme precursor to Cdks and MAPKFs was a proline-directed kinase (as are both MAPKs and Cdks) requiring activation loop phosphorylation but not requiring cyclin binding. The primordial Cdk enzyme could initially have been activated enzymatically solely by phosphorylation, with cyclins providing targeting functions (Cross *et al.* 1999). Since the cyclin binds specifically to the active conformation of the Cdk enzyme, appearance of cyclin activators could set the stage for addiction of the Cdk to cyclin binding to produce the enzymatically active conformation by mutational degeneration of the intrinsic cyclin-independent folding capacity of the Cdk. Primary regulation of the system by proteolytic control of cyclin abundance could then emerge to generate the modem eukaryotic system.

It is interesting that many of these suppressor mutations are in residues that are quite conserved in the Cdk family but quite divergent in the MAPK family (data not shown). In the absence of direct structural analysis,

though, we cannot interpret the mechanism of action of these suppressor mutations. Modeling based on the Cdk2 structures does not give a clear picture of how the mutations work, and they are not predicted to cluster in specific regions of the protein (data not shown).

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