The Synthetic Genetic Network around PKC1 Identifies Novel Modulators and Components of Protein Kinase C Signaling in Saccharomyces cerevisiae

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Budding yeast Saccharomyces cerevisiae contains one protein kinase C (PKC) isozyme encoded by the essential gene PKC1. Pkc1 is activated by the small GTPase Rho1 and plays a central role in the cell wall integrity (CWI) signaling pathway. This pathway acts primarily to remodel the cell surface throughout the normal life cycle and upon various environmental stresses. The pathway is heavily branched, with multiple nonessential branches feeding into and out of the central essential Rho1-Pkc1 module. In an attempt to identify novel components and modifiers of CWI signaling, we determined the synthetic lethal genetic network around PKC1 by using dominant-negative synthetic genetic array analysis. The resulting mutants are hypersensitive to lowered Pkc1 activity. The corresponding 21 nonessential genes are closely related to CWI function: 14 behave in a chemical-genetic epistasis test as acting in the pathway, and 6 of these genes encode known components. Twelve of the 21 null mutants display elevated CWI reporter activity, consistent with the idea that the pathway is activated by and compensates for loss of the gene products. Four of the 21 mutants display low CWI reporter activity, consistent with the idea that the pathway is compromised in these mutants. One of the latter group of mutants lacks Ack1(Ydl203c), an uncharacterized SEL-1 domain-containing protein that we find modulates pathway activity. Epistasis analysis places Ack1 upstream of Pkc1 in the CWI pathway and dependent on the upstream Rho1 GTP exchange factors Rom2 and Tus1. Overall, the synthetic genetic network around PKC1 directly and efficiently identifies known and novel components of PKC signaling in yeast.

The cell wall integrity (CWI) pathway acts primarily to maintain cell integrity throughout the life cycle (reviewed in reference 22). The pathway also responds to a variety of external and internal stresses, such as heat shock, hypo-osmotic shock, cytoskeletal defects, cell wall defects, and secretion blocks. The pathway acts to regulate activity and expression of key cell wall synthesis enzymes and to control processes such as actin polarization and polarized secretion to ensure appropriate remodeling of the cell surface.

The small GTPase Rho1 is central to the CWI pathway. Rho1-GTP directly binds to and activates multiple effectors, including the Fks1/2 glucan synthases, the Bni1/Bnr1 formins, and the exocyst component Sec3 (22). One key and direct target is Pkc1, the only Saccharomyces cerevisiae homolog of mammalian protein kinase C and an essential protein required for the maintenance of cell integrity (21).

The CWI pathway is stimulated by a variety of intrinsic and extrinsic signals transduced via a variety of upstream branches (22). Many of these signals, e.g., heat shock and cell wall perturbations, are detected via their effect on the cell surface as sensed by the multiple members of the WSC family of cell surface sensors (11, 15, 38). These putative mechano-sensors bind to and activate the Rho guanine nucleotide exchange factors (GEFs) Rom2 and Tus1 to activate Rho1 (28, 32). However, upstream signaling is even more complex, with multiple additional branches feeding information into the pathway. Some signals are transduced via Rho1 regulators but independently of the WSC sensors, e.g., TOR signaling modulates the Rho1 GEF Rom2 (33), CDK1/Cdc28 directly phosphorlates the Rho1 GEF Tus1 (19), and the mRNA binding protein Mpt5 inhibits expression of the Rho1 GTPase-activating protein Lrg1 (34). Finally, actin cytoskeletal defects appear to be sensed by an unknown mechanism independent of both the WSC sensors and known Rho1 regulators (12).

Here, we focus on the Pkc1 hub of the CWI pathway. Pkc1 controls multiple downstream branches, the best understood of which is the mitogen-activated protein (MAP) kinase cascade terminating in the Slt2 MAP kinase. This cascade is not essential under normal conditions but is required for cells to survive stresses such as high temperature. Slt2 is known to directly activate, among others, the Rlm1 and Swi4 transcription factors, which are important for expression of genes involved in cell surface construction (1, 41).

We set out to identify new regulators and components of the CWI/Pkc1 pathway. We chose a genetic strategy. Synthetic lethal genetic interactions, a type of phenotype enhancement, occur when two loss-of-function mutations in different genes

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cause lethality but only when present together. Such interactions can now be systematically uncovered in yeast for any given “query” mutation by using synthetic genetic array (SGA) analysis (36) or diploid-based synthetic lethality analysis with microarrays (27). Although synthetic genetic interactions tend to occur between genes in parallel “buffering” pathways or processes, mutations in essential genes are thought to preferentially sensitize an organism to a second mutation that affects the same rather than a parallel process (26).

Here, we determined the set of mutations in nonessential genes (null mutations) that confer hypersensitivity to lowered Pck1 activity via a novel modification of SGA analysis, dominant-negative SGA (DN-SGA) analysis. We find that most of the corresponding 21 genes are closely and functionally linked to the CWI pathway, including the uncharacterized YDL203c/ACK1 gene, which encodes a new upstream modulator of basal CWI activity.

MATERIALS AND METHODS

Media, chemicals, and transformation. The rich yeast medium (YPD) consisted of 1% Bacto yeast extract (Difco), 2% peptone (Difco), and 2% glucose. Rich YPGalactose medium was as described for YPD, but with 2% galactose as the sole carbon source. Synthetic derived medium (SD, Gal, Sac, or SRAffinose) contained 0.67% yeast nitrogen base without amino acids (Difco); 2% glucose, 2% galactose, 0.005% sucrose, or 2% raffinose, respectively; and appropriate nutrient supplements and drug additions where appropriate. Solid media were as described above, with the addition of 2% Bacto agar (Difco). Chemicals were from Sigma-Aldrich unless stated otherwise. Restriction enzymes were from New England Biolabs. All transformations were performed using the conventional lithium acetate method (10).

Oligonucleotides. The following primers were used (Sigma-Aldrich): Ack1F (5′-GATCCGATATCAAGCTTATCGATACCGTCGACATGGTTAATC-3′), Ack1R (5′-GGCTGACTAATAATTTGTCAAACTTCGAAGAGC-3′), Tus1F (5′-GAATTCGATATCAAGCTTATCGATACCGTCGACATGGTTAATC-3′), Tus1R (5′-GGCTGACTAATAATTTGTCAAACTTCGAAGAGC-3′). The rich yeast medium (YPD) contained 2% glucose, 1% Bacto yeast extract (Difco), 2% peptone (Difco), and 2% glucose.

Strains, genetic crosses, and plasmids. Most of the S. cerevisiae strains used are derived from the BY4741 or BY4742 yeast strain of the S288c strain background. For the DN-SGA screen, the query strain was MATaΔ::MAP1pr-HIS3 can1Δ his3Δ leu2Δ ura3Δ MET15::HIS7-17 leu2Δ transformed with pGAL-PCK1 (dead). We obtained the wild-type BY4741 strain, with the genotype MATaα his3Δ leu2Δ met15::ura3Δ (Euroscarf), along with its derivative single null mutations αp2Δ::KanMX4, αr1Δ::KanMX4, bck1Δ::KanMX4, bni1Δ::KanMX4, chl1Δ::KanMX4, cik1Δ::KanMX4, cfs1::KanMX4, fkh1::KanMX4, egd1::KanMX4, emm1::KanMX4, has2Δ::KanMX4, hst1Δ::KanMX4, had1::KanMX4, hpr1Δ::KanMX4, rom2a::KanMX4, sec29Δ::KanMX4, spc2::KanMX4, swiΔ::KanMX4, tus1Δ::KanMX4, ump1::KanMX4, and ydl203c::KanMX4. We also obtained the wild-type strain BY4742, with the genotype MATαα his3Δ leu2Δ met15::ura3Δ and the homoygous αp2Δ::KanMX4, αr1Δ::KanMX4, bck1Δ::KanMX4, bni1Δ::KanMX4, cik1Δ::KanMX4, cfs1::KanMX4, fkh1::KanMX4, egd1::KanMX4, emm1::KanMX4, has2Δ::KanMX4, hst1Δ::KanMX4, had1::KanMX4, hpr1Δ::KanMX4, rom2a::KanMX4, sec29Δ::KanMX4, spc2::KanMX4, swiΔ::KanMX4, tus1Δ::KanMX4, ump1::KanMX4, and ydl203c::KanMX4.

RESULTS

We had previously shown that an allele of PCK1 encoding a catalytically inactive full-length protein behaves as a dominant-negative allele (40), i.e., expression of this allele in trans lowers Pck1 activity even in the presence of a wild-type chromosomal copy of the gene (11). We exploited this allele to identify the synthetic genetic network around PCK1.

DN-SGA analysis. We transformed the wild-type query strain with a marked plasmid expressing the catalytically inactive allele of PCK1 [pck1(dead)] under the control of the galactose-inducible GAL1,10 promoter (40). The transformed query strain was crossed with the arrayed haploid deletion
FIG. 1. Chemical-genetic analysis indicates that two-thirds of the PKC1 genetic interaction network comprises “within-pathway” interactions. (A) Example of sorbitol suppression of the hypersensitivity of a null mutant to staurosporine. Wild-type and api2Δ cells were grown in rich medium and then diluted into wells of a 96-well plate containing fresh medium supplemented with staurosporine (range, 0 μg/ml to 50 μg/ml). The cells were incubated either in the presence (+) or in the absence (−) of sorbitol (1 M). (B) Scheme illustrating the functional classification of the PKC1-interacting genes. Fourteen of the genes behave as encoding proteins that act in the Pkc1 pathway (staurosporine sensitivity is suppressed by osmotic stabilization), six of which encode known CWI pathway components (see text). The remaining seven genes behave as encoding proteins that, at least in part, parallel to Pkc1 (staurosporine sensitivity is not suppressed by osmotic stabilization).

Collection, resulting in arrayed, transformed haploid mutant progeny generated by standard SGA methodology (36). The ~5,000 arrayed transformants were then transferred onto medium containing galactose as the sole carbon source (inducing condition), and the growth of the each spot was noted. The sixty-six transformants that grew poorly or not at all on galactose-containing medium in two or three of three independent runs were taken to constitute the set of primary mutants identified by the analysis.

Mutants were eliminated from further analysis when any of the following applied: (i) the untransformed mutants were unable to proliferate on galactose as the sole carbon source, thereby eliminating gal mutants; (ii) sensitivity to expression of Pkc1(dead) was not reproduced upon direct transformation; or (iii) mutants were sensitive to expression of wild-type Pkc1 driven from an equivalent pGAL-PKC1(WT) construct, thereby eliminating those sensitive to overexpression of the protein rather than to lowered activity. In all, 29 mutants survived the chemical treatment: chlorpromazine deforms the plasma membrane (5), calcifluor white disrupts chitin structure in the cell wall (29), and tunicamycin indirectly causes a secretion block by interfering with protein glycosylation (20). Mutants defective in the Pkc1 pathway or in closely allied processes tend to be hypersensitive to one or more of these compounds. We found that all 21 mutants in the PKC1 network were also sensitive to at least one of the chemicals, 12 (57%) were sensitive to two or more, and 6 (29%) were sensitive to all three (Table 2). We conclude that all the mutants in the PKC1-interacting network display one or more phenotypes associated with CWI dysfunction.

The PKC1 network is rich in known components. We expect many of the PKC1-interacting genes to encode components of the branched CWI pathway (26). Indeed, we find that 6 of the 21 interacting genes (29%) encode known components of the Pkc1 pathway (Table 1) (22): Bck1, a direct target of the kinase; the Swi4 and Mig1 downstream transcription factors; the Bni1 formin and Mid1 calcium channel, which seem to act sensitivity cosegregated with the marked deletion in at least 10 tetrads for 21 of the 28 cases in genetic backcrosses, consistent with the idea that these mutations cause sensitivity to lowered Pkc1 activity. The identity of the knockout in each strain was successfully confirmed by PCR. These 21 mutants constitute the PKC1 interaction network (Table 1).

Chemical sensitivity links the interacting genes to the CWI pathway. Pkc1-Slt2 activity is stimulated by multiple different stresses to the cell. Many of these stresses can be caused by chemical treatment: chlorpromazine deforms the plasma membrane (5), calcifluor white disrupts chitin structure in the cell wall (29), and tunicamycin indirectly causes a secretion block by interfering with protein glycosylation (20). Mutants defective in the Pkc1 pathway or in closely allied processes tend to be hypersensitive to one or more of these compounds. We found that all 21 mutants in the PKC1 network were also sensitive to at least one of the chemicals, 12 (57%) were sensitive to two or more, and 6 (29%) were sensitive to all three (Table 2). We conclude that all the mutants in the PKC1-interacting network display one or more phenotypes associated with CWI dysfunction.

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| Gene  | Function or process |
|-------|---------------------|
| API2  | Protein of unknown function. |
| ATG15 | Lipase required for intravacular lysis of autophagosomes. |
| BCK1  | MAP kinase kinase downstream of Pkc1. |
| BNI1  | Formin that nucleates the formation of linear actin filaments. |
| CHL1  | Protein required for establishing sister chromatid pairing in S phase. |
| CIK1  | Kinesin-associated protein required for karyogamy and mitotic spindle orientation. |
| CSF1  | Protein required for low-temp fermentation. |
| CTF19 | Outer kinetochore protein required for mitotic chromosome segregation. |
| EGD1  | Protein involved in de novo cotranslational protein folding. |
| JNMI  | Part of dynactin complex required for proper nuclear migration and spindle partitioning. |
| LAS21 | Protein involved in the synthesis of GPI anchors. |
| MIDI  | Calcium channel. |
| MIG1  | Transcription factor involved in glucose repression. |
| OP13  | Phospholipid methyltransferase. |
| OST3  | Subunit of oligosaccharyltransferase in the ER lumen. |
| PER1  | Protein required for GPI-phospholipase A2 activity. |
| SEC28 | Protein required for ER-Golgi retrograde transport. |
| SPA4  | Component of polarisome that acts as a scaffold Mkk1 and Slt2. |
| SWI4  | Transcription factor involved in late G1 gene expression. |
| UMP1  | Chaperone required for maturation of 20S proteasome. |

TABLE 1. Confirmed genes that are members of the PKC1 network

a Genes encoding known components of the CWI pathway are in bold.

b GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum.
TABLE 2. Chemical sensitivities of null mutants in the PKC1 interaction network

| Mutant | Tunicamycin | Calcofluor white | Chlorpromazine |
|--------|-------------|-----------------|---------------|
| arg15  | +           | +               | +             |
| chl1   | +           | +               | +             |
| cik1   | +           | +               | +             |
| csf1   | +           | +               | +             |
| ctf19  | +           | +               | +             |
| ged1   | +           | +               | +             |
| bck1   | +           | +               | -             |
| imm1   | +           | +               | -             |
| las21  | +           | +               | -             |
| ost3   | +           | +               | -             |
| sec28  | +           | +               | -             |
| opi3   | -           | -               | -             |
| mid1   | -           | -               | -             |
| per1   | +           | -               | -             |
| spa2   | +           | -               | -             |
| ump1   | +           | -               | -             |
| ydr203c| +           | -               | -             |
| api2   | -           | +               | +             |
| bni1   | -           | +               | +             |
| swi4   | -           | +               | +             |
| mig1   | -           | -               | +             |

* + indicates hypersensitivity to the compound relative to the sensitivity of congenic wild-type control cells, – indicates sensitivity indistinguishable from that of congenic wild-type cells.

The majority of interacting genes encode inhibitors of CWI signaling. What functional relationships may link interacting gene products to Pkc1 function? Some gene products may function as inhibitors of CWI signaling. Mutants lacking such proteins could be defective in processes to which the CWI pathway responds, e.g., cell wall construction, with the resulting elevation in pathway activity acting to ameliorate the consequences of the mutations.

We estimated CWI pathway activity in the 21 null mutants by using an established transcriptional reporter in which expression of the bacterial LacZ gene is dependent on the Rlm1 transcription factor (17). This reporter faithfully reflects the activity state of the Pkc1-MAP kinase pathway (17). We found that the majority of mutants (12/21) display high basal Rlm1-LacZ reporter activity (Fig. 2). The mutants with the highest basal reporter activity (>3 times that of wild-type cells) point to the best-known triggers of and roles for CWI signaling: las21Δ, per1Δ, and ost3Δ are defective in cell wall/surface synthesis (2, 7, 18), and bni1Δ is (and api2Δ is thought to be) defective in actin organization/cell morphogenesis (3, 6). A novel trigger is suggested by the high reporter activity seen for opi3Δ mutants. This mutant lacks a phospholipid methyltransferase required for phosphatidylethanolamine (PC) synthesis (23).

The CWI pathway may thus also respond to changes in PC levels, complementing the established role of Pkc1 in directly phosphorylating enzymes involved in PC synthesis (4, 42).

To date, feedback control of CWI signaling has been established for the MAP kinase downstream branch (22). Importantly, three of the mutants in the PKC1 network displaying elevated Rlm1-LacZ reporter activity lack a known component of the pathway. One of these, Swi4, acts downstream of the MAP kinase branch: the others, Bni1 and Mig1, do not (22). These observations support feedback control of the CWI path-
way and indicate that such feedback may be more extensive than previously recognized.

Some interacting genes encode activators of CWI signaling.

Some of the remaining genes in the PKCI network may encode activators of CWI signaling. Mutants lacking such proteins would have low pathway activity, rendering them hypersensitive to further reduction. In total, four mutants displayed low basal Rlm-LacZ reporter activity, consistent with compromised CWI signaling: bck1Δ, egd1Δ, ctf19Δ, and ydl203cΔ (Fig. 2). BCK1 encodes the known MAP kinase kinase kinase that is absolutely required for activity of Slt2 and Rlm1. Rlm1-LacZ reporter activity is minimal in this mutant (~2% of wild-type levels). EGD1 encodes a subunit of the nascent polypeptide-associated complex (30). CTF19, a gene regulated by the CWI pathway (31), encodes a protein involved in accurate mitotic chromosome segregation (13). Both egd1Δ and ctf19Δ mutants display modest reductions in reporter activity (~81% and ~66% of wild-type levels, respectively). Egd1 and Ctf19 may be novel activators of CWI signaling, but their roles remain to be clarified. Finally, mutants lacking the uncharacterized YDL203c gene display substantial reductions in reporter gene activity (down to ~25% of wild-type levels).

Ydl203c/Ack1 is a novel upstream activator of Pkci. Loss of YDL203c results in low Rlm1-LacZ reporter activity. The staurosporine hypersensitivity of ydl203cΔ is suppressed by sorbitol, supporting a role for Ydl203c in the CWI/Pkc1 pathway (Fig. 1B and data not shown). If Ydl203c is indeed important for CWI signaling, then mutants lacking the protein should display an appropriate phenotype. The mutants are not temperature sensitive for growth but are hypersensitive to tunicamycin treatment (Table 2 and data not shown).

If Ydl203c is a bona fide regulator of CWI signaling, then we expect its overproduction or activation to stimulate reporter activity. Indeed, we find that overexpression of YDL203c in wild-type cells elevates Rlm1-LacZ reporter activity by approximately twofold (Fig. 3A).

Is activation of CWI signaling compromised in a ydl203cΔ mutant? We assayed activation of reporter activity in wild-type and ydl203cΔ mutants upon cell surface stresses (calcofluor white or chlorpromazine treatment) or upon actin depolymerization (latrunculin B treatment). We find that the reporter is efficiently activated in the mutant and upon each of the treatments tested (Fig. 3B). Ydl203c is therefore not required for the transduction of cell surface or actin cytoskeleton defects to the CWI pathway. Activation of the reporter activity upon latrunculin B treatment is, however, less efficient than that upon treatment with the cell surface stressors, indicating that Ydl203c may preferentially enhance this input. Overall, the protein appears to selectively affect the amount of CWI signaling under both basal and induced conditions.

If Ydl203c indeed acts in the Pkci pathway, then epistatic analysis should specify a point of action for the protein. We undertook two complementary analyses. First, we examined if activation or overexpression of known pathway components, ranging from the upstream Rom2 and Tus1 Rhok1 GEFs to the downstream Slt2 MAP kinase, could suppress the tunicamycin sensitivity of ydl203cΔ mutants. We found that activation or overexpression of all the corresponding genes can efficiently suppress the mutant defect, consistent with the idea that Ydl203c acts at or upstream of the Rhok1 GEFs in the pathway (Fig. 4A and B). Second, we examined if overexpression of YDL203c can suppress the defects of mutants defective at various levels of the pathway. We found that overexpression of the gene efficiently suppresses the defects of rom2Δ and tus1Δ mutants and wsc1Δ/wsc1Δ mutants lacking a major upstream sensor but not the defects of pkcf110 or slt2Δ/slt2Δ mutants lacking key downstream components. These data point to the possibility that Ydl203c acts in the Pkci pathway and at or upstream of Pkci (Fig. 4C and D). We rename the gene ACK1 (activator of C kinase I).

No clear molecular mode of action is indicated by the sequence for Ack1 save for the presence of a SEL-1 domain, suggesting the possibility of protein-protein interactions (24). Two recent proteomic studies link Ack1 to the Rhok1 GEFs: Ack1 is found in complexes with Rom2 (8, 9) and Tus1 (8), consistent with a role upstream in the Pkci pathway.

Does Ack1 act via the Rhok1 GEFs? Our genetic analysis indicates that Rom2 activity is not wholly dependent on Ack1 and vice versa: overexpression of one suppresses the defect caused by the absence of the other. The same holds for Tus1 and Ack1. There are two possible explanations. First, Ack1 may act independently of the two main Rhok1 GEFs to stimulate Pkci activity. Second, Ack1 may act via both Rhok1 GEFs, with the activity of neither being wholly dependent on Ack1. If the former model applies, then ACK1 should affect CWI signaling even in the absence of both Rom2 and Tus1; if the latter applies, then ACK1 function would be abolished in the absence of these two Rhok1 GEFs. We constructed rom2Δ tus1Δ double mutant haploids by standard genetic manipulations. We found that the double mutant is viable in our strain background but is temperature sensitive and hypersensitive to benomyl.
found that overexpression of ACK1 is unable to suppress any of the defects tested for rom2Δ tus1Δ double mutants (Fig. 4 and data not shown). Whatever its role, Ack1 is a novel upstream activator of CWI-Pkc1 signaling and functionally dependent on the Rho1 GEFs.

**DISCUSSION**

**DN-SGA analysis.** Here, we report an adaptation of SGA methodology, DN-SGA, where the “query” mutation is dominant negative and is expressed in trans from a plasmid under the control of a regulatable promoter. In the case of the PKC1 query, the adapted method identified true positives with a high degree of specificity: >95% of the primary strains identified as robustly sensitive to expression of the dominant-negative allele were also found to be sensitive to the protein kinase C inhibitor staurosporine.

DN-SGA analysis has two strengths. Because the query allele is plasmid borne and hence extrachromosomal, it is not genetically linked to any chromosomal locus (16). The entire genome is thus surveyed in the approach. Second, because lethality is dependent on the conditional expression of the dominant-negative query allele, the terminal phenotype associated with each synthetic lethal interaction can be easily determined. For example, most of the synthetic lethal interactions with PKC1 reported here are accompanied by increased cell lysis, consistent with the idea that a cell integrity defect underlies the majority of these interactions (S. A. Krause and J. V. Gray, unpublished observations). DN-SGA analysis extends the range of alleles that are amenable to systematic genetic analysis and should be a useful addition to the genetic arsenal.

**The synthetic lethal network around PKC1.** The synthetic lethal network around PKC1 is rich in genes functionally and closely linked to Pkc1. All of the 21 null mutants display the appropriate chemical sensitivities (Table 2). An independent chemical-genetic epistasis test places two-thirds of the genes within the functional envelope of Pkc1 (Fig. 1), of which six encode known components of the CWI pathway. We confirm that another gene, YDL203c/Ack1, encodes a novel modulator of the pathway (see below). Our findings are consistent with the long-held expectation that genetic enhancements of hypomorphic alleles efficiently enrich for mutations affecting the same pathway or biological process (26).

If the synthetic lethal network around PKC1 preferentially identifies components of the CWI pathway, why are all the known components of the pathway not represented in the network? There are multiple possible reasons. First, some components, like Rh1, are essential and not covered by this analysis. Second, some components are redundant, e.g., Mkk1 and Mkk2, where mutation of either gene alone causes little or no phenotypic change. Third, many components may be involved in branches and processes that do not contribute to the essential role(s) of Pkc1. Fourth, many components may contribute preferentially to an activated rather than to the basal state of the pathway. Only mutations affecting basal activity will be identified under the nonstressed conditions examined here. Finally, although SGA analysis and its derivatives are systematic and genome-wide, they suffer a high...
rate of false negatives (36). Such genetic screens are not fully saturating.

Functional relationships underlying the genetic network.

Here, we also probed the likely functional relationships that underlie the genetic interactions with PKC1. We estimated Pkc1 pathway activity in each mutant by using an Rlm1-LacZ reporter, a faithful indicator of Pkc1-Slt2 activity state (17). We thereby identified three distinct subgroups within the PKC1 network. We consider each in turn.

We found that the majority (12/21) of mutants in the PKC1 network display high basal reporter activity, consistent with the idea that such mutants cause defects to which the Pkc1 pathway responds. These mutants would be particularly dependent on Pkc1 activity if this high activity were required to ameliorate the phenotypic consequences of the mutations. Such behavior is expected for signaling pathways that affect cell viability and that are under negative-feedback control. Both conditions apply to the Pkc1/CWI pathway: the overall pathway is essential and is known to be under feedback control by the activity state of the nonessential, downstream MAP kinase module (22). Our data point to the possibility that feedback control is more extensive and also embraces the Bni1 and Mgl1 branches.

Six of the mutants display normal basal reporter activity, suggestive of normal Pkc1 pathway activity, as assessed by the activity of an Slt2-Rlm1-dependent reporter. Such mutants could be deficient in any branch of the pathway other than the MAP kinase branch that controls the Rlm1-LacZ reporter. In addition, the CWI pathway activity (as assessed by reporter gene activity) must not be under feedback control in response to loss of these gene products: activity remains normal. Alternatively, these mutants may be deficient in some process or pathway that acts in parallel to the CWI pathway and whose absence is again not surveyed by the pathway. Examples of both types appear to be represented in this mutant class: the chemical-genetic epistasis test (Fig. 1) places three of the six proteins within the envelope of Pkc1 function. Two of these are known components of the pathway: Spa2 and Mid1. Spa2 is important for proper localization of the Slt2 MAP kinase, but this localization is not important for activation of the Rlm1 transcription factor by Slt2. Mid1 is a putative calcium channel lying on a separate branch relative to the MAP kinase module, leaving Slt2-Rlm1 communication fully intact.

Finally, four null mutants display compromised reporter activity, suggestive of low Pkc1-Slt2-Rlm1 activity. Such mutants could be hypersensitive to reduction in Pkc1 activity because the activity of Pkc1 (as assessed by an Slt2-Rlm1-dependent reporter) in unstressed mutants is closer to the minimal threshold activity of the kinase required to support cell viability. These mutants should lack positively acting components of the pathway. Indeed, all four genes behave in a chemical-genetic epistasis test as part of the Pkc1 pathway (Fig. 1). The mutant with the lowest reporter activity lacks Bck1, a kinase known to be required for communication between Pkc1 and Slt2. In contrast, mutants lacking EGD1 and CTF19 display only a modest reporter defect. The relationships between Edg1 and Ctf19 and CWI signaling are not as yet established, although CTF19 is a transcriptional target of the pathway (31). The fourth mutant, lacking YDL203c/ACK1, displays an intermediate reporter defect. We find that this gene indeed encodes a novel activator of CWI signaling (see below).

Ack1 is a new upstream component of the Pkc1 pathway. Multiple lines of evidence place the uncharacterized protein Ydl203c/Ack1 in the Pkc1 pathway. First, ack1Δ mutants are hypersensitive to lowered Pkc1 activity. Second, this hypersensitivity is efficiently suppressed by osmotic stabilization (Fig. 1), placing Ack1 function within the envelope of Pkc1 in vivo activity. Third, the CWI pathway is compromised in ack1Δ mutants: the mutants display low Rlm1-LacZ reporter activity (Fig. 2) and are hypersensitive to tunicamycin (Table 2), and this hypersensitivity is suppressed by overexpression of many pathway components (Fig. 4A). Fourth, overexpression of ACK1 is sufficient to stimulate CWI pathway activity: overexpression is sufficient to suppress the defects of some mutants defective in upstream signaling (Fig. 4C) and to elevate basal Rlm1-LacZ reporter activity (Fig. 3A). Finally, standard epistasis analysis places Ack1 within the pathway and indicates that it acts upstream of Pkc1 (Fig. 4).

We do not yet fully understand how Ack1 modulates CWI signaling. The protein has been found in complex with the Rho1 GEFs Rom2 (8, 9) and Tus1 (8). Indeed, our genetic analysis indicates that both Rom2 and Tus1 are required for Ack1 to affect CWI signaling: overexpression of ACK1 can suppress the defects of rom2Δ and tus1Δ mutants but not those of the double mutant (Fig. 4C). Ack1 may thus be a modulator of or a scaffold for Rho1 GEF complexes. Whatever its role, it does not appear to grossly alter the stability of the proteins or their subcellular localization (S. A. Krause and J. V. Gray, unpublished observations). Our investigations are ongoing.

Ack1 is clearly important for setting activity level of the pathway but does not appear to be required for gross activation of the pathway in response to cell surface or cytoskeletal defects (Fig. 3B). Whatever its mode of action, Ack1 joins an ever increasing number of components, including the PUF protein Mpt5 (34) and the ubiquitination enzyme Ubp3 (39), that point to unanticipated complexity in setting and modulating pathway activity.

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