Inhibitor-1 (I-1) is a specific inhibitor of protein phosphatase-1 (PP1). We assayed the ability of I-1 to inhibit *Saccharomyces cerevisiae* PP1, Glc7p, in vivo. Glc7p like other PP1 catalytic subunits associates with a variety of noncatalytic subunits, and Glc7p holoenzymes perform distinct physiological roles. Our results show that I-1 inhibits Glc7p holoenzymes that regulate transcription and mitosis, but holoenzymes responsible for meiosis and glycogen metabolism were unaffected. Additionally, we exploited a genetic screen for mutants that were dependent on I-1 to grow. This scheme can identify processes that are negatively regulated by Glc7p-catalyzed dephosphorylation. In this paper I-1-dependent *gfa1* mutations were analyzed in detail. *GFA1* encodes glutamine-fructose-6-phosphate transaminase. One or more phosphorylated proteins activate *GFA1* transcription because the pheromone response and Pkc1p/mitogen-activated protein kinase pathways positively regulate *GFA1* transcription. Our findings show that an I-1-sensitive Glc7p holoenzyme reduces *GFA1* transcription. Therefore, *GFA1* is a member of a growing list of genes that are negatively regulated by Glc7p dephosphorylation.

Protein phosphatase-1 (PP1), a major eukaryotic protein serine/threonine phosphatase, regulates a wide variety of physiological processes, including protein synthesis, cell cycle progression, carbohydrate metabolism, ion channel regulation, and gene transcription (1–3). In the budding yeast *Saccharomyces cerevisiae*, PP1 is encoded by a single gene *GLC7*, and it has >80% identity at amino acid level with its mammalian counterparts (4, 5). *GLC7* is essential for growth and is involved in multiple physiological processes including glycolysis, metabolism, sporulation, cell cycle progression, chromosome segregation, protein synthesis, and glucose repression (4–9).

Most if not all intracellular PP1 exists in association with noncatalytic regulatory subunits (1–3). At least nine noncatalytic subunits have been identified in *S. cerevisiae* by molecular genetics (10, 11). The holoenzymes pair the single PP1 catalytic subunit, Glc7p, with alternative noncatalytic subunits. Each Glc7p holoenzyme is likely to have distinctive functions in vivo. PP1 noncatalytic subunits dictate substrate dephosphorylation in part by controlling the subcellular localization of the holoenzyme. The traits of noncatalytic subunit mutations in *S. cerevisiae* have provided preliminary evidence about the physiological processes regulated by each Glc7p holoenzyme. For example, the Glc7p/Gac1p holoenzyme regulates glycerol metabolism (12), Glc7p/Sds22p governs mitosis (13, 14), Glc7p/Gip1p is involved in sporulation (11), and Glc7p/Reg1p modulates glucose repression (8). All Glc7p noncatalytic subunits studied to date act positively because mutations in noncatalytic subunit genes mimic the traits of *glc7* loss of function alleles.

In mammalian cells, endogenous inhibitors, inhibitor-1 (I-1), DARPP-32, inhibitor-2, ribosomal protein, RIPP-1, and nuclear protein, NIPP-1 also regulate PP1 activity (15–19). The homologous proteins I-1 and DARPP-32 inhibit PP1 activity via multiple interactions with PP1. Phosphorylation of threonine 35 in these inhibitors is critical since they are only functional inhibitors after phosphorylation by cAMP-dependent protein kinase (16, 20). Additionally, the N-terminal tetrapeptide sequence KIQF of these inhibitors is also required for PP1 inhibition (21, 22). X-ray crystallography data of the PP1 catalytic subunit bound to a regulatory subunit identified this tetrapeptide sequence as a recognition motif that is shared by many PP1-binding proteins (23).

To date, no negative regulators for Glc7p have been identified in *S. cerevisiae*. The yeast genome does not encode recognizable homologs to I-1, DARPP-32, or NIPP-1. Moreover, Glc8p, which has sequence similarity to inhibitor-2, acts more like a positive regulator of Glc7p in *S. cerevisiae* (24, 25). However, Glc7p activity must be tightly regulated in yeast because great alterations are detrimental to cell viability. Depletion of Glc7p leads to arrest in the G1 or M-phase of the cell cycle (7, 25, 26). In contrast, overexpression of *GLC7* causes hyperpolarized growth and cell death (25–27).

Phosphorylated I-1 is a potent Glc7p inhibitor in vitro (28). Indeed I-1 inhibition is a defining criteria used to classify protein phosphatases as type 1 protein phosphatases (1–3). Furthermore, I-1 interacts with Glc7p in vivo (29). In this study, we tested the ability of human I-1 to inhibit yeast Glc7p holoenzymes in vivo. Since there are a number of *glc7* traits, some of which can be attributed to specific holoenzymes, these analyses surveyed the ability of I-1 to inhibit a spectrum of Glc7p holoenzymes. Our data indicate that I-1 can inhibit some but not all Glc7p holoenzymes. Finally, we isolated mutants that required I-1 expression to grow. We found that some *gfa1* mutants required I-1 or some other means of Glc7p inhibition to grow. A thorough analysis revealed that Glc7p activity inhibits transcription of *GFA1*. By inhibiting Glc7p activity, *GFA1* transcription increased sufficiently in the isolated *gfa1* mutants to overcome their glucosamine auxotrophy trait.

*This paper is available on line at http://www.jbc.org.*
Glc7p Regulation of GFA1

**TABLE I**

| Plasmid | Relevant markers | Source or Ref. |
|---------|------------------|----------------|
| p2341   | hck2::URA3       | This work      |
| p2168   | 2-μm URA3 GLC7(1-207) | This work    |
| p2368   | URA3 GFA1(1-73)-lacZ | This work    |
| p2397   | LEU2 GFA1(1-73)-lacZ | This work    |
| p1791   | 2-μm URA3 SNI1   | 5             |
| pA26    | 2-μm LEU2 GFA1(2-207) | 9             |
| pBM1966 | reg1::LEU2       | 37            |
| pBM2800 | 2-μm URA3 HXT4-lacZ | 37            |
| pBW112  | 2-μm LEU2 HKX2   | 36            |
| pg-3    | 2-μm TRF1 GPD1p1 | 32            |
| pGEMZIK-1-I | reg1::I-1     | 22            |
| pGEMZIK-1-I-T35A | I-1-T35A (22)   | 22            |
| pN41    | mig1::URA3      | 39            |
| pJZ203  | 2-μm ADE3 LEU3 GPD1p1-I-1 | This work    |
| pJZ204  | 2-μm TRF1 GPD1p1-I-1 | This work    |
| pJZ205  | CEN URA3 GPD1p1-I-1 | This work    |
| pJZ206  | 2-μm GPD1p1-I-1  | This work      |
| pJZ207  | 2-μm TRF1 GPD1p1-I-1-T35A | This work    |
| pJZ208  | CEN URA3 GPD1p1-I-1-T35A | This work    |
| pJZ209  | 2-μm GPD1p1-I-1-T35A | This work    |
| pJZ501  | CEN URA3 GFA1    | This work      |
| pJZ504  | CEN URA3 GFA1    | This work      |
| pJZ507  | 2-μm LEU2 GFA1   | This work      |
| pKC886  | CEN TRP1 GLC7    | 5             |
| pRSH    | 2-μm URA3 PBS2  | George Boguslawski |
| pRS316  | CEN URA3        | 82            |
| pRS426  | 2-μm URA3       | 83            |
| pTSV30A | 2-μm ADE3 LEU2  | John Pringle   |
| pYact1  | ACT1            | 43            |
| YEp50-HA-GLC7 | CEN URA3 HA-GGLC7 | 84            |
| YEp52-RHO1 | 2-μm URA3 RHO1 | David Levin    |
| YEp352-PKC1 | 2-μm URA3 PKC1 | David Levin    |
| YEp352-BCK1 | 2-μm URA3 BCK1 | David Levin    |
| YEp352-MKK1 | 2-μm URA3 M KK1 | David Levin    |
| YEp352-MPK1 | 2-μm URA3 MPK1 | David Levin    |
| YIp566  | LEU2 lacZ       | 85            |

**EXPERIMENTAL PROCEDURES**

*Strains, Media, and Growth Conditions*—Yeast strains CH1305 (MATa ade2 ade3 leu2 ura3 lys2 can1), JC746-9D (MATa ade2 leu2 his3 trp1 can1), and JC746 (MATa ade2 leu2 his3 trp1 can1) have been described previously (5, 30). JC1053-3D is an isogenic MATa derivative of CH1305 obtained by HO-induced mating type conversion. Strains JC1053-18B (MATa ade2 ade3 leu2 ura3 lys2::HIS3 ipl1-1), JC746 (MATa ade2 ura3 leu2::HIS3 ipl1-1 can1), and JC746-9D (MATa ade2 ura3 leu2::HIS3 ipl1-1 can1) have been described previously (5, 30). JC1053-3D is an isogenic MATa derivative of CH1305 obtained by HO-induced mating type conversion.

Yeast were grown in rich YEP-glucose medium or synthetic medium supplemented with amino acids (31). The alternative carbon sources, galactose, raffinose, or fructose, were used in some media at 2% (w/v). For scoring the colony-sectoring trait, low adenine medium was used that contained 0.5 μg of adenine per ml. For growing gfa1-97 and gfa1::URA3 strains, filter-sterilized D-glucosamine was added to rich or synthetic media at a final concentration of 1–2.5 mg/ml. D-glucosamine/ml. Desired transformants were recognized by their colony-sectoring phenotype and were confirmed by Southern analysis. The hck2::URA3 mutation in p2341 was constructed by insertion of a 1-kilobase pair into a HindIII fragment from JC1053-18B into the HindIII site within HXX2 in a subclone of pBW112 (36). Correct integration of hck2::URA3 in yeast transformants was confirmed by polygram chain reaction analysis of yeast genomic DNA. Disruptions reg1::LEU2, reg2::URA3, and mig1::URA3 were introduced into yeast by transformation with fragments from pBM1966, BS-reg2::URA3, or pN41, respectively, as described (37–39).

**Plasmid Constructions**—Plasmids used in this work are summarized in Table I. Plasmid p2JZ204 was constructed by transferring a NcoI-1 fragment from plasmid pGEMZIK-1-I (22) into the pKan and Sall sites of pG-3 (32), placing I-1 under control of the GPD1, glyceraldehyde-3-phosphate dehydrogenase promoter. The Sall-HindIII fragment from p2JZ204 carrying a GPD1 promoter-driven I-1 gene was further subcloned into pTSV30A, pRS316, and pRS426 to generate p2JZ203, p2JZ205, and p2JZ206, respectively. Cloning the mutant I-1 (T35A) KpnI-SalI fragment from pBE3M5ZIK-I-T35A into the p6-3 fragment generated plasmid p2JZ207. The Sall-HindIII fragment carrying GPD1-driven I-1-T35A was further subcloned into pRS316 and pRS426 to make p2JZ208 and p2JZ209. Plasmid p2JZ208 was constructed in several steps. It contains the truncated GLC7 gene from pA26 (9) encoding residues 1–207 of Glc7p in the pRS426 vector.

Plasmid p2JZ501 was isolated by complementation of the gfa1-97 mutation in JC1007-97. Derivatives of p2JZ501 were made to localize the complementing region of DNA. GFA1 is the only known open reading frame on the gfa1-97 complementing plasmid, pJZ504, which has a 3.7-kilobase pair EcoRI fragment in pRS316. Integrative plasmids, p2368 and p2397, contain the YSC1-GFA1 intergenic region (SalI-EcoRI or SalI-BamHI fragments) cloned into YIp56 and YIp366, respectively (85), to make GFA1-lacZ fusions. Derivatives of pJZ504 were isolated by complementation of the gfa1-97 mutation in JC1007. Derivatives of pJZ504 were made to localize the
Western Immunoblotting—Cells were grown in synthetic media with or without glucosamine. To determine the effect of growth phase on the level of Glc7p, samples from middle logarithmic to stationary phase were harvested, and crude protein extracts were prepared by vortexing with glass beads. Typically, 50 μg protein per lane was loaded and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% w/v acrylamide). The resolved proteins were transferred to nitrocellulose membranes and blocked with 5% dehydrated skim milk in phosphate-buffered saline (8 mg of NaCl, 0.2 mg of KCl, 1.44 mg of Na2HPO4, and 0.24 mg of KH2PO4 per ml, pH 7.4). Overnight incubation at 4 °C with anti-HA (1 μg/ml; 12CA5 Roche Molecular Biochemicals), anti-yeast phosphoglycerate kinase (0.5 μg/ml, PKG, Molecular Probes), or anti-I-1 (22) primary antibodies was followed by anti-mouse IgG-conjugated peroxidase (Cappel) secondary antibodies. Bands were finally visualized using luminol chemiluminescence reagents (Pierce) and several exposures to XAR5 (Eastman Kodak Co.) film.

Northern Analysis—Total yeast RNA was isolated, fractionated on formaldehyde-agarose gels, and hybridized with 32P-labeled probes as described (31). The GLC7 probe was the 486-base pair EcoRI-SalI fragment from pKC856. The ACT1 probe was from the plasmid pYact1 (43).

RESULTS

I-1 Inhibits a Subset of Glc7p Holoenzymes in Vivo—Human I-1 was expressed in yeast using the strong, constitutive GPD1 promoter in various low and high copy vectors. I-1 inhibits yeast PPI1 in vivo (28). Surprisingly, we failed to find any growth impairments, which would be expected if I-1 expression severely inhibited Glc7p activity. Anti-I-1 immunoblot analysis of I-1 yeast transformant proteins confirmed expression of the human I-1 protein (data not shown). Therefore, we compared the traits of strains transformed with I-1 expressing and control plasmids to learn whether any Glc7p holoenzymes were modulated by I-1 in vivo.

The Gac1p/Glc7p holoenzyme promotes glycogen synthesis in yeast (12). Null mutations in GAC1 or missense mutations in GLC7 greatly reduce glycogen accumulation (5, 44–46). Therefore, glycogen accumulation provides an assay of the Gac1p/GLC7 holoenzyme activity. First, Glc7p promotes transcription of IME1, encoding the main transcriptional inducer of early meiotic genes. Next, an undefined step after IME1 expression, but before premeiotic S-phase, requires Glc7p activity (46). Finally, packaging of meiotic spores appears to require the Gip1p/Glc7p holoenzyme (11, 46). Diploid JC746 transformed with I-1-expressing or control plasmids was transferred to solid sporulation medium, and the frequency of sporulated cells was counted after 3 days. Remarkably, I-1 expression failed to alter significantly the sporulation frequency (Fig. 1B). High copy plasmids that express truncated GLC7 genes behave like dominant-negatives because they can suppress ipil1 and gen2 protein kinase mutations (6, 9). However, as far as the sporulation functions of Glc7p are concerned, high copy truncated GLC7 had no detectable effect (Fig. 1B). Additionally, sporulation frequency was unaffected by high copy GLC8. Sporulation of yeast diploids normally yields four haploid spores within a tetrad. We previously found that some glc7 alleles yielded a high frequency of dyads (46). Dyads are produced instead of tetrads when one meiotic division fails to occur or spore packaging is incomplete. None of the transformants in Fig. 1B produced more than 2% dyads. Together these data show that neither truncated GLC7, I-1, nor high copy GLC8 inhibit Glc7p sporulation functions.

Some GFA1 Mutants Require I-1 Expression to Grow—Glc7p activity must be tightly controlled because it is essential for growth yet can inhibit growth if overexpressed (6, 24, 26, 49). Since the results of the previous section showed that I-1 inhibits a subset of Glc7p holoenzymes, we took advantage of ectopic I-1 expression to isolate mutants that require I-1 expression for viability. Such mutants would illustrate novel aspects of Glc7p

![Fig. 1. I-1 expression inhibits some Glc7p holoenzymes in vivo.](http://www.jbc.org/content/18072/552/18072/F1.large.jpg)

Supplemental Methods: Two-tailed t test showed that pJZ205 and pJZ206 transformant glycogen levels differed insignificantly from pRS426 control transformants (p = 0.3 and 0.18, respectively). B, three independent JC746 transformants were sporulated for 3 days at 30 °C on SPOR plates. For each of the three sporulated patches, at least 200 cells were counted, and sporulation frequency was calculated from the numbers of ascis. C, the ipil1-1 strains JC1085-18B (top and left) and JC1117-7D (bottom and right) transformed with pJZ204 (2-μm TRP1 I-1) or its parent vector, pG-3 (2-μm TRP1), were streaked on tryptophan-deficient minimal medium and incubated at the indicated temperatures for 3 days.

2. L. Li and J. F. Cannon, unpublished observations.
The Gfa1-97 Mutation Reduces but Does Not Eliminate Glutamine-Fructose-6-phosphate Transaminase Activity—Glutamine-fructose-6-phosphate transaminase catalyzes the formation of glucosamine 6-phosphate, which is the first and rate-limiting step for protein glycosylation and chitin synthesis (51). We confirmed that disruption of the GFA1 gene generates a strain that is auxotrophic for glucosamine (50) by construction of a gfa1::URA3 mutant. We assayed the glutamine-fructose-6-phosphate transaminase enzyme activity of crude extracts from wild-type, gfa1-97, and gfa1::URA3 mutant strains. As shown in Table II, the gfa1-97 mutant had approximately 35% of the wild-type specific activity, whereas activity in a gfa1::URA3 null mutant was undetectable. Despite the reduction in enzyme activity in gfa1-97 (JC1007-97) cells, when transformed with an I-1-expressing plasmid (JC1007-97/pJZ203), they grew at the same rate as wild-type on glucosamine-free minimal medium. This paradox was resolved by experimental results described later. Additionally, the GFA1 genotype significantly influenced the intracellular glucosamine (Table II); however, the mechanism is unknown.

Since glucosamine synthesis in the gfa1-97 mutant was reduced, we tested if extracellular glucosamine could suppress the I-1-dependent trait of gfa1-97. To test this possibility, JC1007-97/pJZ203 cells were streaked on plates that contained 5 mg of glucosamine/ml. On this medium, JC1007-97/pJZ203 mutant cells readily lost the plasmid pJZ203 and became white or sector ed (Fig. 2A). Such gfa1-97 cells without a source of I-1 were dependent on glucosamine to grow. This result indicates that the reduced glucosamine synthesis in JC1007-97 is responsible for its I-1 dependence, and extracellular glucosamine could suppress the I-1 dependence of the gfa1-97 mutation.

Because gfa1::URA3 null mutants had an undetectable glutamine-fructose-6-phosphate transaminase activity, we reasoned that they might also be I-1-dependent. However, since gfa1::URA3 strain can only grow in the presence of glucosamine, the I-1 dependence could not be tested. Expressing I-1 in gfa1::URA3 mutant cells did not relieve the glucosamine requirement for growth.

**Phenotypic Characterization of Gfa1–97—Suppression of the I-1 dependence by growth in medium containing 5 mg of glucosamine/ml provided an easy way to obtain JC1007-97 plasmid-free cells to study traits of gfa1-97 without I-1 present. These plasmid-free JC1007-97 cells produced completely white colonies of Leu+ cells. To distinguish the plasmid-bearing and plasmid-free cells, they will be labeled explicitly as "JC1007-97/pJZ203" and "JC1007-97," respectively.

The gfa1-97 mutation caused temperature-sensitive growth on media without glucosamine. JC1007-97 cells grew as well as wild-type CH1305 cells at temperatures up to 25 °C. However, at 30 °C and higher temperatures, only 0.1% of the cells formed visible colonies after 2 days, the majority of cells either died at the single-cell stage or divided several times to form microcolonies (Fig. 3A). No specific cell cycle arrest was observed for these cells. Cells stopped growing and produced buds of differing sizes. At 37 °C, the growth defect trait was exacerbated; no colonies appeared after 5 days of incubation. The majority of cells did not divide and a large population lysed or had long, slender buds when examined microscopically (Fig. 3B).

Growth of JC1007-97 in liquid cultures showed three impairments compared with wild-type cells (Fig. 3C). First, the JC1007-97 mutant cells were significantly delayed at entering the exponential growth phase. Second, JC1007-97 cells divided at 80% the growth rate of wild-type cells during exponential phase. Third, the maximum cell density of the JC1007-97 mutant cells was 70% of the wild-type. Note that the rich YEP-glucose medium used for liquid culture growth initially contains some glucosamine from the yeast extract ingredient. Growth on fructose was similar to that observed on glucose. However, growth on galactose was unimpaired. In fact, galactose suppressed the I-1 dependence trait of gfa1-97 and partially suppressed the temperature-sensitive trait (Fig. 2).

Gluconamine is important for chitin synthesis and protein glycosylation, which are required for cell wall integrity (51). Mutants with defects in cell wall integrity lyse at restrictive temperatures but can be rescued in media with greater osmolality. Therefore, we tested if the addition of sorbitol or NaCl to the medium could rescue the growth defect of the JC1007-97 strain. JC1007-97 grew well at 30 °C in glucosamine-free media supplemented with 0.8 m sorbitol or 0.25 m NaCl (data not shown). Sorbitol suppressed the I-1 dependence and temperature-sensitivity traits of gfa1-97 (Fig. 2). NaCl suppressed

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**Table II**

| Strain genotype | Glutamine-fructose-6-phosphate transaminase-specific activity | Intracellular glucosamine |
|-----------------|-------------------------------------------------------------|---------------------------|
| GFA1            | 46 ± 4                                                      | 22 ± 3                    |
| gfa1–97         | 16 ± 6                                                      | 140 ± 25                  |
| gfa1::URA3      | <1.0                                                        | 51 ± 12                   |

* Cells with the indicated genotypes (congenic with CH1305) were grown in YEP-glucose media containing 2 mg of glucosamine/ml. Glutamine-fructose-6-phosphate transaminase assays were done as described under “Experimental Procedures.” The glutamine-fructose-6-phosphate transaminase-specific activity was determined as nanomoles of glucosamine per mg of crude extract protein. The data shown are the average of three independent cultures.

† Intracellular glucosamine concentration was expressed as nanomoles of glucosamine per mg of crude protein in the extract.
these traits identically to sorbitol (data not shown). This suppression was consistent with \textit{gfa1-97} causing a defect in cell wall integrity.

\textbf{Inhibition of Glc7p Activity Suppresses Traits of \textit{gfa1-97}}—In addition to the known function of I-1 to inhibit PP1 activity, it is possible that \textit{gfa1-97} results in a reliance on a different I-1 function. Therefore, we tested whether inhibition of Glc7p by some other means could suppress \textit{gfa1-97}. High copy, truncated \textit{GLC7} genes (\textit{GLC7} \textit{D}) behave as dominant-negatives (6, 9). Mutations in \textit{gcn2} and \textit{ipl1} protein kinase genes are suppressed by \textit{GLC7} \textit{D} because these protein kinases phosphorylate known or suspected Glc7p substrates. Therefore, we tested whether the inhibition of Glc7p activity via \textit{GLC7} \textit{D} could substitute for I-1. When \textit{JC1007-97} was transformed with \textit{p2168}, which carries \textit{GLC7} \textit{D} at high copy, the I-1 dependence was relieved (Fig. 4). Additionally, the slow growth of \textit{JC1007-97} at 30 °C was suppressed by \textit{GLC7} \textit{D} (Fig. 3A).

I-1 inhibits PP1 activity only when threonine 35 of I-1 is phosphorylated (20, 22). A mutant form of I-1 in which alanine is substituted for threonine 35 cannot inhibit PP1 activity \textit{in vitro} because it cannot be phosphorylated (20, 22). Wild-type I-1 interacts with Glc7p in the two-hybrid assay, whereas the I-1-T35A mutant does not (29). In addition, I-1 suppresses the \textit{ipl1} temperature-sensitive trait, whereas I-1-T35A does not (Fig. 1 and data not shown). We tested whether I-1-T35A could suppress traits of \textit{gfa1-97}. We found that expression of I-1-T35A could neither suppress the I-1 dependence of \textit{gfa1-97} as scored by the colony sectoring trait (Fig. 4) nor the temperature-sensitive growth at 30 and 37 °C on glucosamine-free media (Fig. 3A). Taken together, these genetic data show that \textit{gfa1-97} was isolated as a I-1-dependent mutation because Glc7p inhibition was required for glucosamine-free growth.

\textbf{Glucosamine Does Not Alter Glc7p Activity or Protein Levels}—Why do \textit{gfa1-97} cells require Glc7p inhibition for growth? The first possibility we considered was that reduced glucosamine biosynthesis in some way increased Glc7p activity, which is toxic (24, 26, 27). This explanation would predict that extracellular glucosamine reduces Glc7p activity because glucosa-

\textbf{Fig. 2. Suppression of \textit{gfa1-97} traits by growth media.} A, \textit{JC1007-97} was streaked on low adenine synthetic complete medium with the indicated carbon sources, glucosamine (5 mg/ml), or sorbitol (0.8 M). Plates were incubated at 30 °C 4 days before photography. Great retention of \textit{pJZ203} produces solid red colonies, which are dark in this figure. In contrast, white colonies or sectors derive from cells that lost \textit{pJZ203}. B, glucosamine or sorbitol suppressed the \textit{gfa1-97} temperature sensitivity. Ten-fold serial dilutions of \textit{CH1305} (\textit{GFA1}) and \textit{JC1007-97} (\textit{gfa1-97}) were spotted on YEP-glucose, YEP-glucose with 5 mg glucosamine/ml, YEP-glucose with 0.8 M sorbitol, YEP-galactose, or YEP-fructose. Plates were incubated at 37 °C for 4 days before photography.

\textbf{Fig. 3. Demonstration of \textit{gfa1-97} traits without I-1.} A, temperature sensitivity caused by \textit{gfa1-97} was apparent from growth of \textit{CH1305} (\textit{GFA1}) and \textit{JC1007-97} (\textit{gfa1-97}) transformants at different temperatures. The genotypes of strains and plasmids are shown in the legend. Plasmids in \textit{JC1007-97} transformants are (starting at top, going clockwise) as follows: \textit{pJZ504}, \textit{pJZ208}, \textit{p2168}, \textit{pJZ206}, \textit{pJZ205}, and \textit{pRS426}, respectively. Transformants were streaked on YEP-glucose plates and incubated for three days at the indicated temperatures. B, the \textit{gfa1-97} mutation caused arrest at all points in the cell cycle on glucosamine-free medium. \textit{CH1305} or \textit{JC1007-97} cells grown at 37 °C for 24 h in YEP-glucose were fixed and photomicrographed. C, the \textit{gfa1-97} cells exhibited a pre-exponential phase growth lag, retarded exponential growth rate, and reduced growth yield compared with wild-type cells in liquid cultures. Growth of liquid 30 °C YEP-glucose (without added glucosamine) cultures of \textit{CH1305} (triangles, dashed lines) and \textit{JC1007-97} (circles, solid lines) were monitored by 600 nm light scattering. Cell counts by hemocytometer showed similar results.
Glc7p Regulation of GFA1

were screened for plasmids that suppressed the I-1 dependence of JC1007-97/pJZ203 by the colony-sectoring assay. Because Glc7p inhibition would increase the phosphorylation of Glc7p substrates, potential high copy suppressors were expected to be protein kinase genes. Therefore, 24 protein kinase genes in 2-μm vectors were also specifically tested. By these routes, we found that RH01, PKC1, BCK1, MKK1, MKK2, MPK1, SNF1, and PBS2 could suppress gfa1-97. Except for PBS2 and SNF1, these genes encode components of the Rhodel/Pkc1 MAP kinase pathway (52–55). This pathway maintains cell wall integrity (56). Except for RH01, all these suppressing genes encode protein kinases. Glucosamine is used in the synthesis of glycoproteins and chitin, which are both components of the cell wall. The isolation of these high copy suppressors suggests that the stress in cell wall biosynthesis caused by gfa1-97 can be relieved by hyperactivity of the Rhodel/Pkc1 pathway. Moreover, these suppression results implicate Glc7p in antagonizing the activity of this pathway.

The PPZ protein phosphatases encoded by PPZ1 and PPZ2 antagonize some aspects of the Rhodel/Pkc1 pathway. Because the catalytic domains of PPZ proteins are very homologous to PP1 enzymes, we considered I-1 might inhibit activity of these PP1 homologues. Therefore, we tested whether I-1 expression recapitulated the increased salt or caffeine resistance traits of ppz1 (57). We found that wild-type yeast transformed with plasmids that express I-1 or I-1-T35A were equally sensitive to caffeine and lithium (data not shown). Therefore, we have no evidence that I-1 inhibits Ppz1p or Ppz2p in vivo.

Glc7p and the Pkc1p Pathway Regulate GFA1 Transcription—The pheromone-responsive transcription factor Ste12p and its coactivator Mcm1p have binding sites in the GFA1 promoter. Both Ste12p and Mcm1p are multiply phosphorylated proteins (58, 59). Because the transcriptional activity of Ste12p and Mcm1p is regulated by their phosphorylation, we explored whether GFA1 transcription was altered in strains with inhibited Glc7p activity. The E. coli lacZ gene was fused to GFA1 at codon 73 and integrated at the GFA1 locus so that GFA1 transcription could be quantitated by β-galactosidase assays (see "Experimental Procedures"). Consistent with pheromone induction of GFA1 mRNA (50), β-galactosidase activity from GFA1-lacZ was induced ~3-fold by pheromone (Fig. 5A). High copy I-1 and Glc7Δ increased GFA1-lacZ β-galactosidase activity 29 and 67%, respectively (Fig. 5B). Although these increases of GFA1 transcription were small, both were significant (p < 0.005 and p < 0.0005, respectively). Enzyme assays showed that I-1 increased glutamine-fructose-6-phosphate transaminase activity from gfa1-97 but not wild-type cells (data not shown). Moreover, elimination of the Glc7p/Reg1p holoenzyme by reg1::URA3, and wild-type cells (data not shown). Therefore, despite increases of glucosamine that might be caused by increases of Glc7p activity, we were unable to document a significant change in Glc7p-specific activity by in vitro assays.

We also examined Glc7p protein levels using immunoblot analysis. Glc7p protein levels were monitored using an HA-GLC7 fusion gene on a centromeric plasmid. This gene complements all GLC7 traits and allowed the relative Glc7p protein levels to be assayed using anti-HA antibody. No consistent differences of Glc7p levels were found between wild-type and gfa1-97 cells. Furthermore, extracellular glucosamine had no effect on the levels of Glc7p in wild-type or gfa1-97 cells (data not shown). Moreover, we found only minor differences of GLC7 mRNA levels in wild-type and gfa1-97 cells harvested at equivalent time points (data not shown). The GLC7 mRNA abundance increased in later growth phase samples for both cultures. This induction in late exponential phase has been noted before (4). These experimental results demonstrate that gfa1-97 cells do not have increased Glc7p activity or protein levels.

High Copy Suppressors of Gfa1-97—To help elucidate the reason gfa1-97 cells require Glc7p inhibition for growth, we sought high copy suppressors of gfa1-97. High copy libraries

Fig. 4. Suppression of JC1007-97 I-1 dependence trait. JC1007-97 transformed with various URA3 plasmids was grown for 6 days on low adenine uracil-deficient medium before photography. White or sectored colonies indicate loss of pJZ203 and suppression of the I-1 dependence trait. The smaller, white colonies visible in the panels are respiratory-deficient clones, which do not turn red, despite the retention of pJZ203. The panels are labeled with the plasmid genotypes. The plasmids used were pRS316, p2168, pJZ205, pJZ208, pJZ206, and pJZ209.

mine relieves the I-1-dependent trait of gfa1-97. Since glycogen synthase is a Glc7p substrate and its activity is dependent on phosphorylation (5), we analyzed glycogen accumulation in wild-type and gfa1-97 mutant cells. A slightly darker iodine staining was observed with gfa1-97 cells, indicating an increase in glycogen accumulation and thus potentially elevated Glc7p activity. Quantitative glycogen assays confirmed that gfa1-97 cells accumulated 20% more glycogen than wild type (data not shown). Although increased glycogen accumulation can be caused by increases in Glc7p activity, many other factors can influence glycogen levels (5). Glc7p activity was specifically assayed in yeast crude extracts by [32P]phosphorylase dephosphorylation in the presence of okadaic acid (11, 27, 28). By this assay we did not find significant differences of Glc7p-specific activity between isogenic gfa1-97, gfa1::URA3, and wild-type cells (data not shown). Therefore, despite increases of glucosamine that might be caused by increases of Glc7p activity, we were unable to document a significant change in Glc7p-specific activity by in vitro assays.

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were screened for plasmids that suppressed the I-1 dependence of JC1007-97/pJZ203 by the colony-sectoring assay. Because Glc7p inhibition would increase the phosphorylation of Glc7p substrates, potential high copy suppressors were expected to be protein kinase genes. Therefore, 24 protein kinase genes in 2-μm vectors were also specifically tested. By these routes, we found that RH01, PKC1, BCK1, MKK1, MKK2, MPK1, SNF1, and PBS2 could suppress gfa1-97. Except for PBS2 and SNF1, these genes encode components of the Rhodel/Pkc1 MAP kinase pathway (52–55). This pathway maintains cell wall integrity (56). Except for RH01, all these suppressing genes encode protein kinases. Glucosamine is used in the synthesis of glycoproteins and chitin, which are both components of the cell wall. The isolation of these high copy suppressors suggests that the stress in cell wall biosynthesis caused by gfa1-97 can be relieved by hyperactivity of the Rhodel/Pkc1 pathway. Moreover, these suppression results implicate Glc7p in antagonizing the activity of this pathway.

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To learn if the high copy suppressors we isolated also suppress gfa1-97 by means of increasing GFA1 transcription, GFA1-lacZ β-galactosidase activity was assayed for cells transformed with suppressor gene plasmids. These assays revealed that all high copy suppressor genes increased GFA1 transcription to various degrees (Fig. 5B and data not shown). Induction of GFA1-lacZ by MPK1/SLT2, the MAP kinase of the cell wall integrity pathway, reveals that suppression by RH01, PKC1, BCK1, MKK1, and MKK2 can be explained by their stimulation of Mpk1p.

Snf1p and Reg1p regulate the transcription of glucose-repressed genes (60). Our results showed that overexpression of SNF1 suppressed gfa1-97 by increasing GFA1 transcription. Inactivation of REG1 also increased GFA1-lacZ expression.
with pTSV30, pJZ203, or p2155 were grown in leucine-, uracil-deficient C, cells transformed with YEp352, YEp352-PKC1 MPK1, or YEp352-A600. The relevant genotype of the plasmids is shown within the bar.

Transformants were performed on three or more cultures derived from independent segregants. The bars show data from CH1305/p2368 or CH1305-A600 grown in YEP-glucose. The data are from CH1305/p2368 cells transformed with pTVS30, three pJZ203, or p2155 grown in leucine-deficient medium. The bars show data from CH1305/p2368 or CH1305-A600 grown in YEP-glucose. The culture was split, and 10 μg of β-factor per ml was added to one culture (triangles) at time 0, and cell samples were assayed for β-galactosidase activity.

Control culture (circles) had no β-factor added. B, the GFA1-lacZ β-galactosidase activity was assayed for exponential phase cultures. The left three data are from CH1305/p2368 cells transformed with pTVS30, pJZ203, or p2155 grown in leucine-deficient medium. The center two bars show data from CH1305/p2368 or CH1305 reg1::LEU2/p2368 cells grown in YEP-glucose. The right three data are from CH1305/p2368 cells transformed with YEp352, YEp352-PKC1, or YEp352-MPK1 grown in uracil-deficient medium. C, CH1305/pBM2800 transformed with pTSV30, pJZ203, or p2155 were grown in leucine-, uracil-deficient medium and assayed for HXT4-lacZ β-galactosidase activity. B and C, the relevant genotype of the plasmids is shown within the bar. Assays were performed on three or more cultures derived from independent transformants.

Fig. 5. Attenuation of Glc7p activity increases GFA1 and HXT4 transcription. A, CH1305/p2368 (GFA1-lacZ) cells were grown to mid-exponential phase (A600 = 0.7–1.0) in YEP-glucose. The culture was split, and 10 μg of β-factor per ml was added to one culture (triangles) at time 0, and cell samples were assayed for β-galactosidase activity. The control culture (circles) had no β-factor added. B, the GFA1-lacZ β-galactosidase activity was assayed for exponential phase cultures. The left three data are from CH1305/p2368 cells transformed with pTVS30, pJZ203, or p2155 grown in leucine-deficient medium. The center two bars show data from CH1305/p2368 or CH1305 reg1::LEU2/p2368 cells grown in YEP-glucose. The right three data are from CH1305/p2368 cells transformed with YEp352, YEp352-PKC1, or YEp352-MPK1 grown in uracil-deficient medium. C, CH1305/pBM2800 transformed with pTSV30, pJZ203, or p2155 were grown in leucine-, uracil-deficient medium and assayed for HXT4-lacZ β-galactosidase activity. B and C, the relevant genotype of the plasmids is shown within the bar. Assays were performed on three or more cultures derived from independent transformants.

DISCUSSION

PP1 holoenzymes achieve diversity by the variety of noncatalytic subunits that can associate with catalytic subunits. The single PP1 catalytic subunit, encoded by GLC7 in S. cerevisiae, is known to regulate a variety of physiological processes. Glc7p binds to at least nine different noncatalytic subunits (10). Mammalian PP1 enzymes are also regulated by inhibitor proteins. Inhibitor I-1 was known to inhibit Glc7p activity in vitro (28). In this work we show I-1 also inhibits Glc7p in vivo, although not all holoenzymes appear sensitive to I-1.

Separate domains of I-1 participate in PP1 binding and inhibition (61, 62). The amino acid sequence KIQF in I-1 is similar to a sequence found in many PP1 noncatalytic subunits, and it is required for I-1 inhibition function (23, 61, 63). This sequence is thought to be an interface for PP1 binding. Because Reg1p and Gac1p noncatalytic subunits contain a sequence similar to KIQF (63), I-1 is unlikely to bind and inhibit Glc7p while Glc7p is bound to either Reg1p or Gac1p. However, depending on relative affinities and protein levels, I-1 could potentially displace Reg1p or Gac1p from Glc7p, thereby reducing the catalytic activity of those holoenzymes. Our data are consistent with in vivo inhibition of the Reg1p/Glc7p holoenzyme by I-1 because expression of I-1 in yeast mimics the reg1 induction of GFA1 and HXT4 transcription (Fig. 5, and see Ref. 37). In contrast, Gac1p/Glc7p holoenzyme inhibition by I-1 is, at best, minor (Fig. 1). Because Sds22p lacks a KIQF-homologous domain (63), the Sds22p/Glc7p holoenzyme could potentially bind I-1 without dissociation. Regardless of the mechanism, I-1 suppression of ipf1 temperature sensitivity argues that I-1 inhibits the Sds22p/Glc7p holoenzyme in vivo (Fig. 1).

I-1 residues around Thr-35 are thought to occupy the PP1-inhibitor binding site and competitively inhibit PP1 substrate access (64). PP1 inhibition by I-1 requires I-1 Thr-35 phosphorylation (20). In mammalian cells cAMP-dependent protein kinase phospho-
We hypothesize that Glc7p is naturally subject to inhibition. Three observations prompt this hypothesis. First, intermediate Glc7p activity is required for normal cellular growth since substantial increases or decreases of the wild-type activity kills yeast cells. Second, several PP1 inhibitors exist in mammalian cells, and if they were evolutionarily ancient would also be found in the unicellular eukaryote, S. cerevisiae. However, only the I-2 homologue Glc8p has been found in yeast, and it appears to predominantly activate Glc7p activity (24, 25). Our data demonstrate no Glc8p influence on Glc7p holoenzymes that regulate sporulation or GFA1 transcription, respectively. Finally, assays of yeast PP1 activity are exquisitely sensitive to crude extract dilution.³ This behavior is similar to mammalian PP1 assays (1–3) and is evidence of PP1 inhibitors present in the crude extract. The prospect of natural Glc7p inhibitors spurred our hunt for mutants that depend on I-1 expression for viability.

Two independent mutants, which required I-1 expression for viability, were isolated and discovered to contain mutations in GFA1, which encodes glutamine-fructose-6-phosphate transaminase, the glucosamine biosynthetic enzyme. These mutants could not grow without glucosamine if I-1-T35A, which cannot inhibit PP1, was expressed. Moreover, inhibition of in vivo Glc7p activity by GLC7Δ revealed that the gfa1 mutants need Glc7p inhibition to grow without glucosamine. Neither gfa1-17 nor extracellular glucosamine influenced Glc7p activity, Glc7p protein, or GLC7 RNA levels (data not shown). Therefore, gfa1-17 mutant cells have a condition where normal Glc7p activity forbids glucosamine-free growth. We considered that Glc7p regulated glutamine-fructose-6-phosphate transaminase, by dephosphorylation because this enzyme from other fungal species is regulated by phosphorylation (51, 67). However, changes in Glc7p activity did not significantly affect enzyme activity in wild-type cells (data not shown). We demonstrated that Glc7p inhibition induces GFA1 transcription (Fig. 5) and that increased GFA1 transcription restores gfa1-17 glutamine-fructose-6-phosphate transaminase to wild-type levels (data not shown). Therefore, gfa1 mutants were isolated as I-1-dependent mutants because they required GFA1 transcription induction that results from Glc7p inhibition.

The mating pheromone response pathway induces GFA1 transcription (50). Pheromone exposure induces haploid cells to pendent mutants because they required induction that results from Glc7p inhibition. Therefore, gfa1 mutants do not increase nor extracellular glucosamine nor suppress because gfa1 genotype had on these levels (Table II). Since I-1 and Glc7p activity regulate the glucose transporter encoded by HXT4 (Fig. 5C), we hypothesize that they also regulate glucosamine transporters in a manner that senses glucosamine biosynthetic capacity. Growth on galactose allows derepression of glucose-repressed genes and can suppress the I-1-dependent trait of gfa1-17 (Fig. 2). Similarly, reg1 mutations or SNF1 overexpression also suppress gfa1-17 (reg1 work) and derepress glucose-repressible genes (60). Glucose repression of GFA1 could explain these observations; however, this possibility has not been tested. If GFA1 transcription were glucose-repressible, it would be novel because mig1 mutations do not increase GFA1 transcription nor suppress gfa1-17-1 dependence. Mig1p is responsible for glucose repression of other genes (60). Furthermore, gfa1-17 and GFA1+ cells grown on rich medium showed a greater difference in glutamine-fructose-6-phosphate transaminase activity than cells grown in minimal medium (data not shown). This finding vividly demonstrates the influence that extracellular nutrient levels and growth rate have on GFA1 regulation.

GFA1 regulates transcription of several genes in S. cerevisiae. Evidence has implicated the Glc7p/Reg1p holoenzyme in regulating transcription of SUC2, INO1, ADH2, HXT2, HXT4, and IME1 (8, 37, 46, 76–78) and Glc7p/Gac1p in regulating CUP1 (79). In some cases a candidate Glc7p phosphoprotein substrate is genetically implicated, but none have been proven by rigorous biochemical analyses. The results in this paper add GFA1 to the list of genes regulated by Glc7p/Reg1p. Similar to INO1 regulation, GFA1 transcription was increased by elevated Snf1p activity but not by mig1 or hsk2 mutations. Curiously, only Mcm1p and Ste12p are known to regulate GFA1 transcription, and these regulators are distinct from the proteins known to regulate INO1 (76, 77). Therefore, to explain the role of Glc7p/Reg1p holoenzyme in transcriptional regulation requires postulating that it dephosphorylates a number of different proteins or that it dephosphorylates proteins that function at many promoters such as TATA-binding proteins or components of RNA polymerase II holoenzyme. One of the other I-1-dependent mutations we isolated was in SP76, which encodes a protein that influences chromatin structure and RNA polymerase elongation (80, 81). Further work will be required to define the Glc7p substrates responsible for these transcriptional effects.

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