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A protein complex of translational regulators of *GCN4* mRNA is the guanine nucleotide-exchange factor for translation initiation factor 2 in yeast

(eIF-2/eIF-2B/GCN3/GCD protein/general amino acid control)

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ABSTRACT **In *Saccharomyces cerevisiae*, phosphorylation of the α subunit of translation initiation factor 2 (eIF-2) by protein kinase GCN2 stimulates translation of *GCN4* mRNA. In mammalian cells, phosphorylation of eIF-2α inhibits the activity of eIF-2B, the GDP–GTP exchange factor for eIF-2. We present biochemical evidence that five translational regulators of *GCN4* encoded by *GCD1*, *GCD2*, *GCD6*, *GCD7*, and *GCN3* are components of a protein complex that stably interacts with eIF-2 and represents the yeast equivalent of eIF-2B. *In vitro*, this complex catalyzes guanine nucleotide exchange on eIF-2 and overcomes the inhibitory effect of GDP on formation of eIF-2·GTP·Met-initiator $tRNA^{Met}$ ternary complexes. This finding suggests that mutations in *GCD*-encoded subunits of the complex derepress *GCN4* translation because they mimic eIF-2α phosphorylation in decreasing eIF-2B activity. Our results indicate that translational control of *GCN4* involves a reduction in eIF-2B function, a mechanism used in mammalian cells to regulate total protein synthesis in response to stress.**

Phosphorylation of the α subunit of translation initiation factor 2 (eIF-2α) on Ser-51 is a prominent mechanism for down-regulating protein synthesis in mammalian cells. The phosphorylated form of eIF-2 inhibits the conversion of eIF-2·GDP to eIF-2·GTP at the completion of each cycle of translation initiation, a reaction catalyzed by the guanine nucleotide-exchange factor eIF-2B. Only the GTP-bound form of eIF-2 can form a stable ternary complex with the initiator $tRNA^{Met}$ ($tRNA_i^{Met}$) and catalyze new rounds of translation initiation (for review, see ref. 1).

GCN2 is an eIF-2α kinase in *Saccharomyces cerevisiae* that stimulates expression of the transcriptional activator GCN4 in response to amino acid starvation (2, 3). Translation of GCN4 under nonstarvation conditions is inhibited by four short upstream open reading frames (uORFs) in the *GCN4* mRNA leader. Genetic results indicate that ribosomes scanning from the 5′ end of *GCN4* mRNA translate the first uORF encountered (uORF1), resume scanning, and under nonstarvation conditions, reinitiate translation at the second, third, or fourth uORFs instead of at the GCN4 ORF (2, 4). Dever *et al.* (3) showed that phosphorylation of eIF-2α by GCN2 increases under conditions of amino acid starvation and that this phosphorylation is required for increased translation of GCN4 under these conditions. By analogy with mammalian systems, it was proposed that phosphorylation of eIF-2α reduces the concentration of eIF-2·GTP·Met-$tRNA_i^{Met}$ ternary complexes by inhibiting the recycling of eIF-2·GDP to eIF-2·GTP by the yeast equivalent of eIF-2B. Consequently, many ribosomes that have translated uORF1 and resumed scanning would not reacquire the ternary complex until after scanning past uORFs 2–4, allowing reinitiation to occur at the GCN4 start codon instead (3, 4).

Mutations have been identified in the α and β subunits of eIF-2 that lead to constitutive derepression of *GCN4* independently of GCN2 kinase function (5). The same phenotype has been observed for mutations in multiple *GCD* genes that reduce the efficiency of general translation initiation (6–8). Based on the finding that GCD1 and GCD2 are components of a high-molecular-weight complex that binds a fraction of the eIF-2 in the cell (8), we suggested that GCD1 and GCD2 are subunits of the yeast equivalent of eIF-2B. According to this model, mutations in *GCD1* and *GCD2* stimulate GCN4 translation by reducing guanine nucleotide exchange on eIF-2, thereby decreasing the concentration of eIF-2·GTP·Met-$tRNA_i^{Met}$ ternary complexes. At odds with this hypothesis, a previous study suggested that conversion of eIF-2·GDP to eIF-2·GTP in yeast does not require an exchange factor (9). We present strong biochemical evidence that yeast eIF-2 forms a stable complex with GDP *in vitro* and that the high-molecular-weight complex containing GCD1 and GCD2 is the yeast equivalent of mammalian eIF-2B.

MATERIALS AND METHODS

Plasmid and Strain Constructions. Site-directed mutagenesis (10) was used to insert 9 codons encoding the hemagglutinin (HA) epitope N-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-C at the C terminus of the *GCD1* coding sequence. The 2.3-kb *Bam*HI DNA fragment containing the HA-tagged *GCD1* allele was inserted into low-copy-number *TRP1* plasmid pUN10 (11), producing plasmid pBM31; pBM10 contains the corresponding fragment with wild-type *GCD1*. *GCD1* disruption plasmid pBM26 was constructed by inserting the 2.3-kb *Bgl* II fragment containing *LEU2* at the *Bgl* II site in the *GCD1* gene on YIp5-Sc4040 (12). Diploid strain MC1046 containing the *gcd1::LEU2* disruption was generated by transforming (13) MC1038 (*MATa/MATα ura3-52/ura3-52 leu2-3,-112/leu2-3,-112, GAL2/GAL2, trp1Δ63/trp1Δ63,* ⟨*HIS4–lacZ*⟩/⟨*HIS4–lacZ*⟩) to Leu^+ with the 6.1-kb *Bam*HI fragment from plasmid pBM26. Sporulation and tetrad analysis of the transformant revealed 2+:2-segregation for viability, and all viable spores were Leu^-. MC1046 was transformed to Trp^+ with pBM31 or pBM10. Transformants were sporulated, and the resulting ascospores were screened for Trp^+ and Leu^+ phenotypes, yielding MC1057 (*MATa ura3-52 leu2-3,-112 trp1Δ63 gcd1::LEU2* ⟨*HIS4–lacZ*⟩ pBM31-

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Abbreviations: uORF, short upstream open reading frame; HA, hemagglutinin; mAb, monoclonal antibody; $tRNA_i^{Met}$, initiator $tRNA^{Met}$.

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[*GCD1,TRP1*]) and MC1061 (*MATa ura3-52 leu2-3,-112 trp1Δ63 gcd1::LEU2* ⟨*HIS4–lacZ*⟩ pBM10[*GCD1,TRP1*]).

Purification of eIF-2 and the GCD·eIF-2 Complex. Fifteen liters of strain TD28 (*MATa ura3-52 ino1-13*) was grown in yeast extract/peptone/dextrose medium (14) to an OD_{600} of 8–10, harvested, and broken as described (8). After centrifugation at 22,000 × *g*, the crude extract was adjusted to 500 mM KCl while being stirred on ice and centrifuged for 2 hr at 200,000 × *g*. The supernatant was dialyzed at 4°C against 3 liters of buffer A [20 mM Tris·HCl, pH 7.5/10% (vol/vol) glycerol] containing 100 mM KCl (buffer A-100) and protease inhibitors as described (8). The dialysate was applied to a heparin-Sepharose column (2.6 × 18 cm; Pharmacia), washed sequentially with buffer A-100 and buffer A containing 300 mM KCl (buffer A-300), and eluted with buffer A containing 550 mM KCl (buffer A-550). The buffer A-550 eluate was dialyzed against 2 liters of buffer A-100 containing all protease inhibitors, applied to a DEAE-CL6B column (2.6 × 10 cm; Pharmacia) and washed with buffer A-100. The proteins were eluted with buffer A-325, dialyzed against 1 liter of buffer A-100, and precipitated by sequential addition of ammonium sulfate to 40%, 50%, and 70% saturation, with centrifugation at 25,000 × *g* after each addition. The 50–70% ammonium sulfate precipitate was resuspended in ≈5 ml of buffer A-100, dialyzed against 1 liter of buffer A-100, and applied to a phosphocellulose column (1.6 × 4 cm; Whatman). This column was washed sequentially with buffer A-100 and buffer A-450, and eluted with buffer A-750. The column fractions containing eIF-2 activity were identified by assaying eIF-2·GTP·Met-tRNA$_i^{Met}$ ternary-complex formation. Peak fractions were dialyzed against 1 liter of 100 mM potassium phosphate, pH 7.5, containing 10% glycerol (buffer B-100) and applied to a hydroxylapatite column (1.6 × 3 cm) equilibrated with buffer B-100. The column was washed with buffer B-100, and the eIF-2 activity was eluted with buffer B-250 and dialyzed against buffer A-100. This fraction was applied to a Mono Q column (5 × 5 cm; Pharmacia), washed with buffer A-100, and eluted with a 20-ml linear gradient of 100–500 mM KCl in buffer A. Fractions containing eIF-2 activity were concentrated and adjusted to 100 mM KCl by centrifugation in a Centricon 30 (Amicon) filtration apparatus. Typically, 150 μg of eIF-2 was isolated from 60 liters (≈500 g) of cells.

The GCD·eIF-2 complex was isolated following the above procedure for purifying eIF-2, with the following modifications. The heparin-Sepharose fraction was dialyzed against buffer A-100 and applied to a DEAE-Sepharose CL-6B column (2.6 × 10 cm; Pharmacia) and washed sequentially with buffer A-100 and buffer A-175. The buffer A-325 eluate was dialyzed against 1 liter of buffer A-100, applied to a phosphocellulose column, and washed with buffer A-100. Proteins were eluted with a 20-ml linear gradient of 100–820 mM KCl in buffer A. Fractions containing GCD1 and GCD2 were identified by immunoblot analysis using antisera against these proteins, as described (8). GCD1 and GCD2 coeluted precisely from the phosphocellulose column. Peak fractions containing GCD1 and GCD2 were dialyzed against 1 liter of buffer B-100, applied to the hydroxylapatite column, and washed with buffer B-100. Proteins were eluted with a 20-ml linear gradient of 100–600 mM KPO_4 in buffer B. Peak fractions containing GCD1 and GCD2 were identified by immunoblotting, concentrated, adjusted to 100 mM KCl in a Centricon C-30 filtration apparatus, and subjected to velocity-sedimentation in an SW41 rotor for 25 hr at 39,000 rpm on a 15–40% glycerol gradient prepared in buffer A-100. Peak fractions containing GCD1 and GCD2 were identified by immunoblot analysis and pooled. Typically, 50 μg of GCD·eIF-2 complex was obtained (at ≈50% purity) from 175 g of wild-type yeast cells. For immunoprecipitation experiments, peak fractions containing GCD1 from the DEAE-Sepharose CL-6B column were separated on a Sephacryl S-300 column, and the fractions containing GCD1 and GCD2 were identified by immunoblotting, all as described (8).

Immunoprecipitations. Monoclonal antibody (mAb) 12CA5 was purified from 1 ml of mouse ascites fluid as described (15). Protein A-agarose beads (Boehringer Mannheim) were resuspended in an equal volume of nondenaturing binding buffer (20 mM Tris·HCl, pH 7.5/100 mM KCl/0.1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/0.7 μg of pepstatin A per ml). Twenty microliters (≈20 μg) of mAb 12CA5 was incubated with 20 μl of the protein A–agarose suspension and 200 μl of binding buffer for 2 hr at room temperature. The beads were pelleted in a microcentrifuge for 5–10 sec and washed three times with 300 μl of binding buffer and once in binding buffer adjusted to 50 mM KCl (binding buffer-50). Protein fractions were added, and the volume of each sample was brought to 300 μl with binding buffer-50. Reaction mixtures were rocked at 4°C for 4 hr, after which the beads were pelleted and washed by resuspension in 500 μl of binding buffer-50 by rocking for 10 min at 4°C. This washing procedure was repeated three times with binding buffer-50 and twice with exchange buffer (20 mM Tris·HCl, pH 7.5/100 mM KCl/1 mM $MgCl_2$/2 mM phenylmethylsulfonyl fluoride/1 mM dithiothreitol). To elute proteins, 10 μg of the peptide YPYDVPDYA in 50 μl of binding buffer-50 was added to the beads and rocked overnight at 4°C. After the beads were pelleted in a microcentrifuge for 10 min, the supernatant was removed and centrifuged a second time for 10 min. The final supernatant was mixed with 150 μl of Laemmli sample buffer (16), boiled for 5 min, and separated by SDS/10% PAGE. Gels were silver-stained or subjected to immunoblot analysis as described (8).

Assays for eIF-2 and eIF-2B Biochemical Activities. Ternary-complex formation by eIF-2 was assayed as GTP-dependent binding of [^{3}H]Met-tRNA$_i^{Met}$ as described (17), except that GTP was used at 900 μM. [^{3}H]Met-tRNA$_i^{Met}$ was formed from total yeast tRNA and crude *Escherichia coli* methionyl-tRNA synthetase as described (18). Displacement of ^{3}H-labeled GDP from eIF-2 was assayed as described (19), with the modifications listed below. [^{3}H]GDP·eIF-2 was prepared by mixing 100 μg of purified eIF-2 (≈0.8 nmol, assuming 100% purity) in ≈250 μl of buffer A-100 with 50 μCi (5 nmol; 1 Ci = 37 GBq) of lyophilized [^{3}H]GDP (10 Ci/mmol) and incubating at 30°C for 10 min. The reaction was adjusted to 10 mM $MgCl_2$ and incubated at 30°C for 2 min. An equal volume of guanine nucleotide binding buffer (GBB; 20 mM Tris·HCl, pH 7.5/100 mM KCl/0.1 mM EDTA/10% glycerol) containing 200 μg of creatine phosphokinase was added, and the mixture was desalted on a PD-10 column equilibrated with GBB at 4°C, collecting 250-μl fractions. Peak fractions containing the eIF-2·[^{3}H]GDP binary complex were used for assays. Typically, the specific activity of the eIF-2·[^{3}H]GDP complex was 27,000 dpm per μg (≈3400 dpm per pmol) eIF-2. To measure GDP displacement, 5 pmol of eIF-2·[^{3}H]GDP, 100 μg of creatine phosphokinase, and 40 μM GDP were incubated at 15°C in 100 μl of GBB containing 1 mM $MgCl_2$ (GBM buffer) in the presence or absence of additional factors. Twenty-microliter aliquots were removed at 2-min intervals, added to 1 ml of ice-cold GBM buffer, filtered through nitrocellulose filters (Schleicher & Schuell BA85, 2.5 cm) and washed with 9 ml of cold GBM buffer. The filters were dried and mixed with 5 ml of Econofluor (Beckman); radioactivity was then counted by liquid scintillation.

To measure stimulation of ternary-complex formation, 5.6 pmol of yeast eIF-2 (calculated assuming 100% purity) was incubated at 25°C in 12 μl of GBB in the presence or absence of 20 μM GDP for 5 min. After adding 1 μl of 25 mM $MgCl_2$ the reaction mixture was incubated at 15°C for 5 min. The reaction was brought to a final volume of 25 μl and 10 μM GDP with the addition of GTP to 48 μM, plus the GCD·eIF-2

complex and ≈5 pmol of [^{3}H]Met-tRNA$_i^{Met}$ in GBB and incubated at 15°C for 8 min. The amount of [^{3}H]Met-tRNA$_i^{Met}$ used in this reaction was shown to be saturating for ternary-complex formation in the absence of GDP. The reaction was stopped by the addition of 1 ml of cold GBM buffer, applied to nitrocellulose filters, and washed with 5 ml of cold GBM buffer. The filters were dried and counted as above.

RESULTS AND DISCUSSION

An Activity for Guanine Nucleotide Exchange on eIF-2 Copurifies with the GCD1-Containing Complex. The genetic and physical properties of the high-molecular-weight complex containing GCD1, GCD2, and GCN3 (8, 20, 21) suggested to us that this GCD complex is the yeast equivalent of mammalian eIF-2B. To test this hypothesis, we purified yeast eIF-2 and the GCD complex and assayed the latter for the guanine nucleotide-exchange activity for eIF-2, ascribed to mammalian eIF-2B. Antibodies against GCD1, GCD2, and the α and β subunits of eIF-2 were used to identify these proteins when yeast extracts were fractionated by ion-exchange column chromatography, as described in *Materials and Methods*. In agreement with our previous findings (8), velocity-sedimentation analysis of a fraction enriched for GCD1 and GCD2 revealed that these two proteins were present in a high-molecular-weight complex of ≈15S that also contained GCN3 and a portion of the eIF-2 present in the starting fraction (Fig. 1*B*, fractions 12 and 13, and data not shown). Contrary to previous results (9), we found that highly purified yeast eIF-2 that was devoid of detectable GCD1 and GCD2 would form a stable binary complex with GDP. Therefore, we tested the gradient fractions described in Fig. 1 for the ability to stimulate the time-dependent dissociation of radiolabeled GDP from eIF-2·GDP binary complexes in the presence of excess unlabeled GDP. As shown in Fig. 1*C*, the nucleotide-exchange activity for eIF-2 cosedimented precisely with the GCD·eIF-2 complex.

We reasoned that if the GCD complex were eIF-2B, its ability to stimulate displacement of GDP from eIF-2 should be specific for guanine nucleotides. As shown in Fig. 2 *A* and *B*, release of the labeled GDP from eIF-2 was stimulated by the purified GCD·eIF-2 complex in the presence of 40 μM unlabeled GDP and, to a lesser extent, with an equivalent amount of unlabeled GTP, whereas no dissociation of the

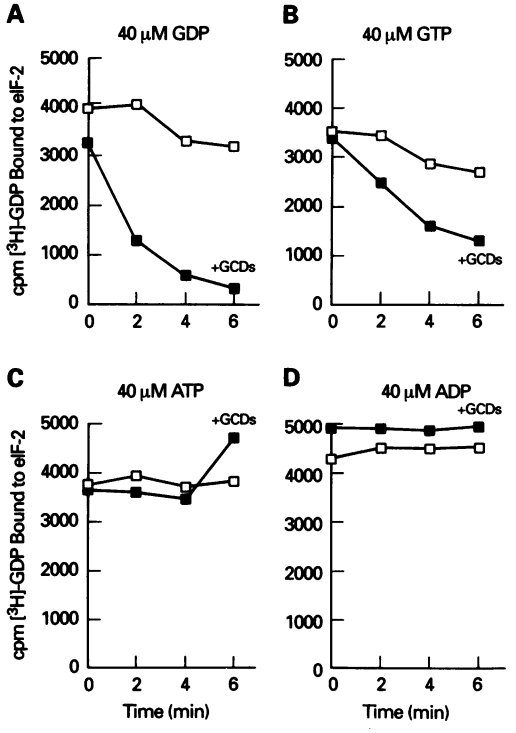

FIG. 2. Nucleotide exchange on [^{3}H]GDP·eIF-2 binary complexes catalyzed by the GCD·eIF-2 complex shows specificity for guanine nucleotides. Preformed [^{3}H]GDP·eIF-2 binary complexes were incubated at 15°C in the presence of 40 μM concentrations of GDP (*A*), GTP (*B*), ATP (*C*), or ADP (*D*) in GDP-displacement assays containing 3 μg of protein from fractions 12 and 13 of the glycerol gradient in Fig. 1 and 1 pmol of preformed [^{3}H]GDP·eIF-2 binary complexes per time point. Aliquots (20 μl) were removed at 2-min intervals to measure the [^{3}H]GDP that remained bound to eIF-2. ■, Reactions containing protein from fractions 12 and 13 of the glycerol gradient; □, assays with no added protein.

eIF-2·[^{3}H]GDP complex occurred in the presence of 40 μM unlabeled ADP or ATP (Fig. 2 *C* and *D*), or when nucleotide was omitted (data not shown). Thus, the GCD·eIF-2 complex exhibited the specificity for guanine nucleotides expected for the exchange reaction catalyzed by eIF-2B. The more effi-

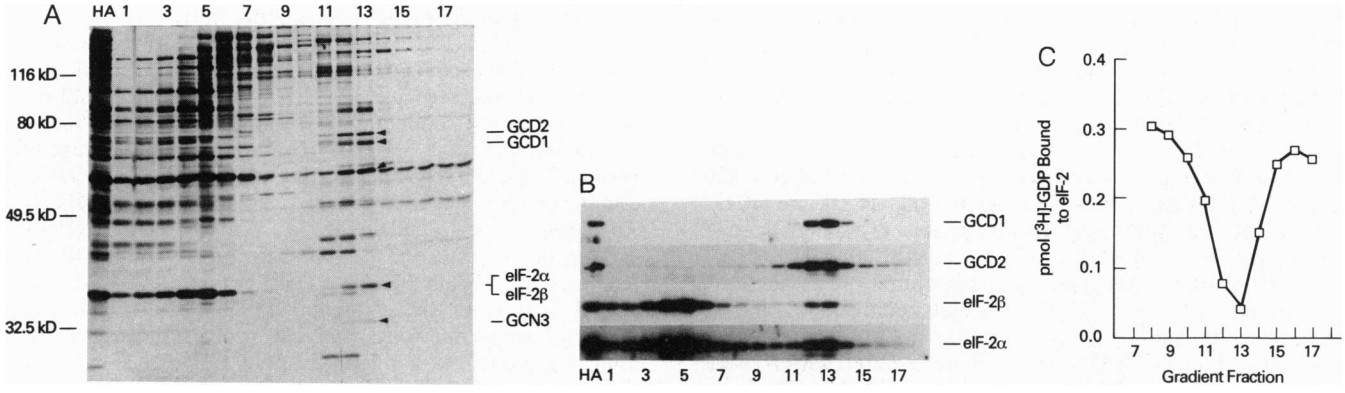

FIG. 1. Cosedimentation of GCD·eIF-2 complex with guanine nucleotide-exchange activity for eIF-2. The hydroxylapatite fraction enriched for the GCD·eIF-2 complex was isolated from strain MC1057 as described, and ≈700 μg was centrifuged on a 15–40% glycerol gradient. Then 600-μl fractions were collected from the top of the gradient, and 25 μl of each was analyzed by SDS/10% PAGE. The fraction number from the top of the gradient is given in *A* and *B*; lane HA (for hydroxyapatite) contains 20 μg of the sample applied to the gradient. Proteins were visualized by silver-staining (*A*) or immunoblot analysis with antibodies against GCD1, GCD2, eIF-2β, or eIF-2α, as described (8) (*B*). Two identical immunoblots were divided in half, and each of four strips was probed with a different antibody. Bands shown were the only immunoreactive species visible on the blots and had the expected electrophoretic mobilities. In *A*, molecular size markers are indicated at left in kDa, and components of the GCD·eIF-2 complex are indicated at right, as deduced from the immunoblot analysis in *B* and similar immunoblots conducted with GCN3 antibodies. (*C*) The ability of 20-μl aliquots from each gradient fraction to displace [^{3}H]GDP from preformed eIF-2·[^{3}H]GDP binary complexes in 100-μl reactions containing 0.3 pmol (≈0.04 μg) of [^{3}H]GDP·eIF-2 binary complexes and 40 μM unlabeled GDP after 8 min at 15°C.

cient nucleotide displacement stimulated by GDP compared with GTP (compare Fig. 2 *A* and *B*) has also been seen in mammalian systems, reflecting the greater affinity of eIF-2 for GDP versus GTP (19, 22). The fact that labeled GDP was not released from eIF-2 in the presence of adenine nucleotides supports the idea that guanine nucleotide exchange is responsible for the dissociation of the eIF-2·[^{3}H]GDP complex, as opposed to nonspecific mechanisms, such as proteolysis of eIF-2 or degradation of the GDP.

Because of the higher affinity of mammalian eIF-2 for GDP versus GTP, a GDP–GTP exchange factor is crucial for the formation of eIF-2·GTP·Met-tRNA$_i^{Met}$ ternary complexes in the presence of GDP (23). If the GCD complex catalyzes GDP–GTP exchange, it should stimulate formation of eIF-2·GTP·Met-tRNA$_i^{Met}$ ternary complexes when incubated with preformed eIF-2·GDP binary complexes. As expected if yeast eIF-2 has a higher affinity for GDP versus GTP, preincubation of purified eIF-2 with GDP inhibited subsequent formation of ternary complexes upon addition of GTP and [^{3}H]Met-tRNA$_i^{Met}$. As shown in Fig. 3, ternary complex formation was reduced by a factor of five relative to the amount of product formed in the absence of GDP (from 0.34 pmol to 0.07 pmol). Addition of the purified GCD·eIF-2 complex stimulated ternary-complex formation by eIF-2, which had been preincubated with GDP, restoring the yield of ternary complexes to a level similar to that seen in the absence of GDP (0.31 pmol, Fig. 3). This stimulation could not be accounted for by the eIF-2 present in the GCD·eIF-2 complex because, when incubated alone, the latter supported a much smaller amount of ternary-complex formation (0.08 pmol, Fig. 3). The fact that the GCD·eIF-2 complex can overcome the inhibitory effect of GDP on ternary-complex formation provides strong support for the idea that the GCD·eIF-2 complex catalyzes exchange of GDP for GTP on eIF-2.

Immunoprecipitation of Guanine Nucleotide-Exchange Activity with the GCD·eIF-2 Complex. To provide independent evidence that the GCD complex contains guanine nucleotide-exchange activity, we sought to demonstrate that this activity could be specifically immunoprecipitated with subunits of the GCD complex. The *GCD1* gene was modified to introduce the HA epitope (24) at the C terminus of the coding region and introduced into a yeast strain disrupted for the chromosomal *GCD1* gene. The growth rate of the resulting transformant was indistinguishable from that of the isogenic wild-type *GCD1* strain. A mAb against the HA epitope was used under nondenaturing conditions to coimmunoprecipitate GCD1 with its associated proteins from Sephacryl-300 fractions containing the GCD·eIF-2 complex from strains expressing the HA-tagged version of GCD1. Control fractions prepared from strains expressing wild-type GCD1 were treated identically with the HA mAb. As expected, the GCD·eIF-2 complex was immunoprecipitated only from the extract containing HA-tagged GCD1 (see below). Before immunoprecipitation, the two preparations containing epitope-tagged or wild-type GCD1 had comparable levels of GCD1, GCD2, eIF-2α, and eIF-2β proteins and similar specific activities for GDP–GTP exchange on eIF-2 (data not shown). The immune complexes formed in each case were bound to protein A-agarose beads, washed, and tested for the ability to catalyze GDP–GTP exchange on eIF-2, using a modification of the assay described above. As shown in Fig. 4, immune complexes prepared from extracts containing the tagged form of GCD1 stimulated guanine nucleotide exchange on eIF-2, whereas complexes prepared from extracts containing wild-type GCD1 did not. These results provide strong evidence that the guanine nucleotide-exchange activity for eIF-2 detected in our purified preparations of the GCD·eIF-2 complex is attributable to GCD1 or its associated proteins.

To determine the polypeptide composition of the complex that coimmunoprecipitated with the epitope-tagged form of GCD1, proteins were eluted from the immune complexes by incubation with the 9-amino acid peptide corresponding to the HA epitope. In the experiment shown in Fig. 5, six distinct electrophoretic species were identified by PAGE and silver staining of the eluate obtained from beads containing the epitope-tagged version of GCD1 (lane 5). None of these polypeptides was present in the corresponding eluate obtained

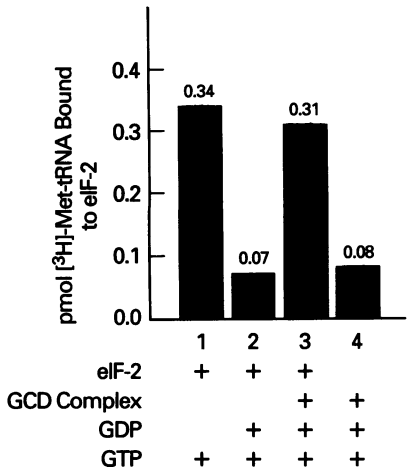

FIG. 3. GCD·eIF-2 complex overcomes the inhibitory effect of GDP on ternary-complex formation by eIF-2. Purified yeast eIF-2 (0.7 μg, ≈5.6 pmol) was preincubated in a 12-μl reaction containing 20 μM GDP and 0.5 mM $MgCl_2$ and combined with 2 μg of fractions 12 and 13 of the glycerol gradient in Fig. 1, 2.7×10^5 dpm of [^{3}H]Met-tRNA$_i^{Met}$, 0.8 μg of total tRNA, and 48 μM GTP in a 25-μl vol, yielding a final GDP concentration of 10 μM. After incubation for 8 min, the total reaction was applied to a nitrocellulose filter, washed, and dried; radioactivity was counted by liquid scintillation. The amount of [^{3}H]Met-tRNA$_i^{Met}$ used in these reactions was saturating for the formation of ternary complexes in the absence of GDP.

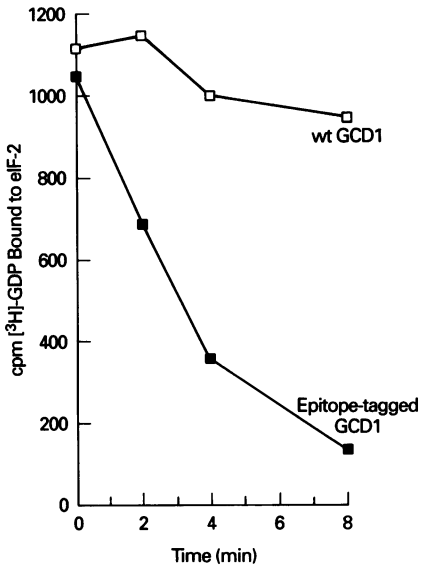

FIG. 4. Immunoprecipitation of guanine nucleotide-exchange activity for eIF-2 with GCD·eIF-2 complexes containing epitope-tagged GCD1. Samples containing 180 μg of protein enriched in the GCD·eIF-2 complex were obtained by Sephacryl-300 chromatography of partially purified preparations isolated from strain MC1061 containing wild-type (wt) GCD1 or MC1057 containing epitope-tagged GCD1 and incubated with antibodies against the HA epitope attached to protein A–agarose beads. The beads were washed and assayed for displacement of [^{3}H]GDP from [^{3}H]GDP·eIF-2 binary complexes, as described, except that 1.0 pmol of [^{3}H]GDP·eIF-2 was used, and the reaction mix was centrifuged for 5 sec to pellet the beads before removing aliquots at the indicated times.

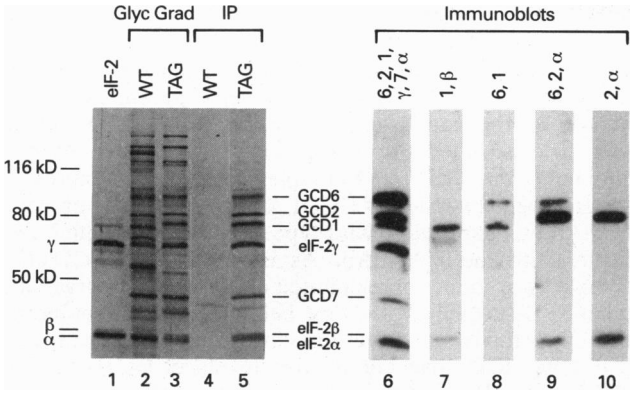

FIG. 5. Silver-staining and immunoblot analysis of immunoaffinity-purified GCD·eIF-2 complex from a yeast strain expressing epitope-tagged GCD1. Immune complexes were formed by using antibodies against the HA epitope immobilized on protein A-agarose beads from Sephacryl-300 fractions enriched for the GCD·eIF-2 complex from strains containing wild-type GCD1 (WT) or epitope-tagged GCD1 (TAG). One-third of each sample eluted from these complexes by HA peptide was analyzed by SDS/10% PAGE (lanes labeled IP for immunoprecipitated), in parallel with 1 μg of glycerol-gradient (lanes Glyc Grad) fractions containing the GCD·eIF-2 complex isolated from the same two yeast strains and 0.2 μg of purified yeast eIF-2. In lanes 1–5, proteins were visualized by silver-staining. The positions of molecular size markers (in kDa) and the α, β, and γ subunits of eIF-2 are indicated at left. In lanes 6–10, the remaining two-thirds of the proteins eluted from the immune complexes prepared from the epitope-tagged strain (silver-stained in lane 5) was fractionated by SDS/PAGE and analyzed by immunoblotting using the mixtures of antibodies designated at the top of each lane as follows: 1, GCD1; 2, GCD2; 6, GCD6; 7, GCD7; α, eIF-2α; β, eIF-2β; γ, eIF-2γ. The identities of subunits of the GCD·eIF-2 complex deduced from these and other immunoblots are indicated at left. GCD6 and GCD7 antibodies were raised against trpE fusion proteins expressed in *E. coli* (25); eIF-2γ antibodies were provided by E. Hannig (University of Texas, Dallas).

from control beads prepared with the corresponding fractions containing wild-type GCD1 (lane 4), indicating that each of the eluted polypeptides was specifically associated with the tagged GCD1 protein. Two of these species comigrated exactly with the γ subunit and a doublet containing the α and β subunits of purified yeast eIF-2, respectively (Fig. 5). Two of the remaining polypeptides had estimated molecular sizes in agreement with the deduced amino acid sequences of GCD1 (8, 12) and GCD2 (26). The predicted sizes of the final two species are very similar to the deduced molecular weights of GCD6 and GCD7, translational regulators of *GCN4* mRNA with genetic properties (27) very similar to those described for *GCD1* and *GCD2* (2). All of these assignments were confirmed by immunoblot analysis using antisera specific for each of the six proteins (Fig. 5). GCN3 protein was present at relatively low levels in the epitope-tagged complex used for the experiment shown in Fig. 5; however, in other experiments GCN3 was present as a major constituent of the complex (25) and was specifically eluted by excess HA peptide from immune complexes prepared with epitope-tagged GCD·eIF-2 complexes (data not shown). The fact that activity could be detected in immune complexes depleted of GCN3 is not surprising because GCN3 is dispensable *in vivo* (28) and is thought to function as a regulatory subunit of eIF-2B (2, 20, 21).

The number and size of the polypeptides in the GCD·eIF-2 complex is similar to that reported for eIF-2B purified from mammalian cells. The approximate molecular weights reported for the five subunits of eIF-2B from mammalian sources (85,000, 67,000, 52,000, 37,000, and 27,000) (23, 29) correspond reasonably well to the predicted molecular weights of GCD6 (81,000; ref. 27); GCD2 (71,000; ref. 26), GCD1 (68,000; refs. 8 and 12), GCD7 (43,000; ref. 27) and GCN3 (34,000; ref. 28), respectively. In addition, GCD6 has 30% amino acid sequence identity with the largest subunit of mammalian eIF-2B cloned recently from rabbit reticulocytes (27). These similarities provide additional support for the idea that the GCD complex represents the eIF-2B of *S. cerevisiae* and suggest that the structure and function of eIF-2B has been conserved throughout eukaryotic evolution.

Our identification of the GCD complex as the eIF-2B of yeast explains why reduced-function mutations in its *GCD*-encoded subunits lead to high-level *GCN4* expression in the absence of GCN2 kinase function, as these mutations should mimic the inhibitory effect of phosphorylated eIF-2 on the function of eIF-2B. The fact that inactivation of the GCN3 subunit prevents derepression of *GCN4* in the presence of GCN2 and high-level phosphorylated eIF-2α (2, 20, 21) strongly suggests that phosphorylation of eIF-2 stimulates GCN4 translation by antagonizing the recycling function of eIF-2B. The availability of the yeast genes for each of the subunits of eIF-2B should greatly facilitate molecular studies to determine which subunits are required for guanine nucleotide exchange on eIF-2 and for other postulated activities of eIF-2B required late in the initiation pathway (1). In addition, we hope to investigate the molecular basis for the inhibition of eIF-2B function(s) by the phosphorylated form of eIF-2 and the role played by the GCN3 subunit in this important regulatory interaction.

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