THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 20, Issue of May 14, pp. 20850–20857, 2004

Printed in U.S.A.

Identification of the Mitochondrial GTP/GDP Transporter in *Saccharomyces cerevisiae**

Received for publication, December 12, 2003, and in revised form, February 27, 2004
Published, JBC Papers in Press, March 3, 2004, DOI 10.1074/jbc.M313610200

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The genome of *Saccharomyces cerevisiae* contains 35 members of a family of transport proteins that, with a single exception, are found in the inner membranes of mitochondria. The transport functions of the 16 biochemically identified mitochondrial carriers are concerned with shuttling substrates, biosynthetic intermediates, and cofactors across the inner membrane. Here the identification and functional characterization of the mitochondrial GTP/GDP carrier (Ggc1p) is described. The *ggc1* gene was overexpressed in bacteria. The purified protein was reconstituted into liposomes, and its transport properties and kinetic parameters were characterized. It transported GTP and GDP and, to a lesser extent, the corresponding deoxynucleotides and the structurally related ITP and IDP by a counter-exchange mechanism. Transport was saturable with an apparent K_m of 1 μM for GTP and 5 μM for GDP. It was strongly inhibited by pyridoxal 5′-phosphate, bathophenanthroline, tannic acid, and bromcresol purple but little affected by the inhibitors of the ADP/ATP carrier carboxyatractyloside and bongkrekate. Furthermore, in contrast to the ADP/ATP carrier, the Ggc1p-mediated GTP/GDP heteroexchange is H^+-compensated and thus electroneutral. Cells lacking the *ggc1* gene had reduced levels of GTP and increased levels of GDP in their mitochondria. Furthermore, the knock-out of *ggc1* results in lack of growth on nonfermentable carbon sources and complete loss of mitochondrial DNA. The physiological role of Ggc1p in *S. cerevisiae* is probably to transport GTP into mitochondria, where it is required for important processes such as nucleic acid and protein synthesis, in exchange for intramitochondrially generated GDP.

In the mitochondrial matrix, GTP is required as an energy source for protein synthesis; as a substrate for the synthesis of tRNA, mRNA, rRNA, and RNA primers; and as a phosphate group donor for the activity of GTP-AMP phosphotransferase (1) and G proteins (2, 3). In several organisms, GTP is synthesized in the mitochondria by succinyl-CoA ligase, which catalyzes the conversion of succinyl-CoA to succinate with the generation of GTP, and by nucleoside diphosphate kinase, which catalyzes the transfer of the γ phosphate from ATP to a nucleoside diphosphate to yield nucleotide triphosphates. In *Saccharomyces cerevisiae*, however, succinyl-CoA ligase produces ATP instead of GTP (4), and the mitochondrial nucleoside diphosphate kinase is localized in the intermembrane space and absent in the matrix (5). These observations imply that in *S. cerevisiae* GTP has to be imported into the mitochondria probably via a carrier system embedded in the inner mitochondrial membrane.

Despite the importance of GTP in mitochondrial metabolism, the transport of guanine nucleotides has not been characterized in yeast mitochondria, nor has any mitochondrial protein responsible for this transport been identified. There are only two indirect observations that suggest that GTP is transported across the inner mitochondrial membrane of *S. cerevisiae*. First, mitochondrial protein synthesis is stimulated by the addition of external GTP (6), and second, on incubating yeast mitochondria with labeled GTP, the amount of radioactivity associated with mitochondria is time-dependent, inhibited by GDP and insensitive to carboxyatractyloside (7).

The inner membranes of mitochondria contain a family of proteins that transport various substrates and products into and out of the matrix (for a review see Ref. 8). These proteins are characterized by three tandem sequence repeats, each being approximately 100 amino acids in length and folded into two transmembrane α-helices joined by an extensive hydrophilic loop. The nuclear genome of *S. cerevisiae* encodes 35 members of this family. The functions of many members are unknown because the substrates transported have not yet been discovered. One of these, Ggc1p, *i.e.* the GTP/GDP carrier (encoded by YDL198c and previously known as Yhm1p) has been shown to be localized to mitochondria and to be a multicopy suppressor (by an unknown mechanism) of the ability of the *abf2* null mutant to grow at 37 °C on glycerol (9).

Here we report the identification and functional characterization of Ggc1p. This protein has been overexpressed in *Escherichia coli,* reconstituted into phospholipid vesicles, and identified from its transport properties as a carrier for GTP and GDP. Ggc1p operates in yeast mitochondria with transport properties similar to those observed with the recombinant protein. In addition, *ggc1*Δ cells exhibit lower levels of GTP and increased levels of GDP in their mitochondria, are unable to grow on nonfermentable substrates, and have lost their mtDNA. The physiological role of Ggc1p in *S. cerevisiae* is probably to catalyze the exchange between external GTP and internal GDP to satisfy the need for GTP in the mitochondrial matrix, where this compound cannot be synthesized. This report presents the first information on the molecular properties of the mitochondrial GTP/GDP carrier and a definitive identification of its gene in *S. cerevisiae*.

* This work was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Tecnologica, University's Local Funds, the Italian National Research Council, National Research Council-Ministero dell'Università e della Ricerca Scientifica e Tecnologica project "Functional Genomics," the Centro di Eccellenza Geni in campo Biosanitario e Agroalimentare, and by the European Social Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES

Sequence Search and Analysis—Data bases were screened with the sequence of Ggc1p (encoded by YDL198C) with BLASTP and TBLASTN. The amino acid sequences were aligned with ClustalW (version 1.7).

Yeast Strains, Media, and Preparation of Mitochondria—BY4741 (wild-type) and *ggc1*Δ yeast strains were provided by the EUROFAN resource center EUROSCARF (Frankfurt, Germany). In the *ggc1*Δ mutant the *ggc1* (YDL198c) locus of *S. cerevisiae* strain BY4741 (MATa; *his*3Δ1; *leu*2Δ0; met15Δ0; *ura*3Δ0) (10) was replaced by kanMX4. Wild-type cells and the deletion strain were grown in rich medium containing 2% bactopeptone and 1% yeast extract (YP), supplemented with either fermentable (2% glucose or 2% galactose) or nonfermentable (2% ethanol, 3% acetate, 10 mM oxaloacetate, 2% pyruvate, 2% lactate, or 3% glycerol) carbon sources. The final pH was adjusted to 4.5 or, with pyruvate or acetate, to 6.5. The mitochondria were isolated by standard procedures. The amount of Ggc1p in wild-type mitochondria was determined by quantitative immunoblotting (11).

Bacterial Expression and Purification of Ggc1p—The coding sequence of *ggc1* (open reading frame YDL198c) was amplified from *S. cerevisiae* genomic DNA by PCR. The oligonucleotide primers were synthesized corresponding to the extremities of the coding sequence, with additional BamHI and HindIII sites. The product was cloned into the pMW7 expression vector, and the construct was transformed into *E. coli* DH5α cells. Transformants were selected on 2× TY plates containing ampicillin (100 μg/ml) and screened by direct colony PCR and restriction digestion of plasmids. The overproduction of Ggc1p as inclusion bodies in the cytosol of *E. coli* was accomplished as described before (12), except that the host cells were *E. coli* C0214(DE3) (13, 14). Control cultures with the empty vector were processed in parallel. Inclusion bodies were isolated, and Ggc1p was purified by centrifugation and washing steps as described previously (13, 15). The proteins were separated by SDS-PAGE in 17.5% gels and either stained with Coomassie Blue dye or transferred to nitrocellulose membranes for immunodetection with a rabbit antiserum raised against bacterially expressed Ggc1p. The N terminus was sequenced, and the yield of purified Ggc1p was estimated by laser densitometry of stained samples (11).

Reconstitution into Liposomes and Transport Assays—The recombinant protein in sarkosyl was reconstituted into liposomes in the presence of substrates, as described before (16). External substrate was removed from proteoliposomes on Sephadex G-75 columns, pre-equilibrated with 50 mM NaCl and 10 mM PIPES-NaOH[1] at pH 7.0 (buffer A) or 1 mM PIPES-NaOH at pH 7.0 in the experiments reported in Table II. Transport at 25 °C was started by adding [8-^{3}H]GTP (Amersham Biosciences), [8,5′-^{3}H]GDP, or [α-^{33}P]dGTP (PerkinElmer Life Sciences) to proteoliposomes and terminated by the addition of 15 mM bathophenanthroline and 30 mM pyridoxal 5′-phosphate (the "inhibitor stop" method (16)). In controls, the inhibitors were added at the beginning together with the radioactive substrate. All of the transport measurements were carried out in the presence of 10 mM PIPES at pH 7.0 in the internal and external compartments, except in the experiments reported in Table II, where 1 mM PIPES at pH 7.0 was used. The external substrate was removed, and the radioactivity in the liposomes was measured (16). The experimental values were corrected by subtracting control values. The initial transport rate was calculated from the radioactivity taken up by proteoliposomes after 20 s (in the initial linear range of substrate uptake). For efflux measurements, proteoliposomes containing 1 mM substrate were labeled with carrier free [8-^{3}H]GTP by carrier-mediated exchange equilibration (16). After 40 min, the external radioactivity was removed by passing the proteoliposomes through Sephadex G-75. Efflux was started by adding unlabeled external substrate or buffer A alone and terminated by adding the inhibitors indicated above.

Other Methods—GTP and GDP were determined in mitochondrial extracts by enzymatic assays (17). K^+ diffusion potentials were generated by adding valinomycin (1.5 μg/mg phospholipid) to proteoliposomes in the presence of KCl gradients. For the formation of an artificial ΔpH (acidic outside), nigericin (50 ng/mg phospholipid) was added to proteoliposomes in the presence of an inwardly directed potassium gradient. The membrane potential of isolated mitochondria was assessed by recording the fluorescence changes of the voltage-sensitive dye 3,3′-dipropylthiadicarbocyanine iodide DiSC (3, 5) (Molecular Probes) as previously described (18). For DNA detection, the BY4741 and the isogenic *ggc1*Δ strains were fixed with formaldehyde following growth on galactose to an A_{600} of 2.0. Then the DNA was stained by incubation with 1 μg/ml DAPI at 4 °C overnight. DAPI fluorescence was detected using an inverted Zeiss Axiovert 200 epifluorescence microscope equipped with a CoolSNAP HQ CCD camera (Roper Scientific, Trenton, NJ) and the Metamorph software (Universal Imaging Corporation, Downington, PA).

[1] The abbreviations used are: PIPES, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); mtDNA, mitochondrial DNA; DAPI, 4′,6-diamidino-2′-phenylindole-dihydrochrolide.

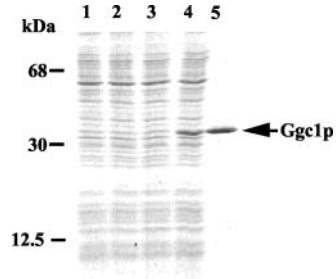

FIG. 1. **Expression of yeast Ggc1p in *E. coli* and its purification.** The proteins were separated by SDS-PAGE and stained with Coomassie Blue dye. The positions of the markers (bovine serum albumin, carbonic anhydrase, and cytochrome *c*) are shown on the *left*. *Lanes 1–4*, *E. coli* CO214(DE3) containing the expression vector with (*lanes 2* and *4*) and without the coding sequence of Ggc1p (*lanes 1* and *3*). The samples were taken at the time of induction (*lanes 1* and *2*) and 5 h later (*lanes 3* and *4*). The same number of bacteria was analyzed in each sample. *Lane 5*, purified Ggc1p (6 μg) originating from bacteria shown in *lane 4*.

RESULTS

Bacterial Expression of Ggc1p—Ggc1p was expressed at high levels in *E. coli* C0214(DE3) (Fig. 1, *lane 4*). It accumulated as inclusion bodies and was purified by centrifugation and washing (Fig. 1, *lane 5*). The apparent molecular mass of the recombinant protein was 33.5 kDa (the calculated value with initiator methionine was 33,215 Da). The identity of the purified protein was confirmed by N-terminal sequencing. Approximately 80–90 mg of purified protein were obtained per liter of culture. The protein was not detected in bacteria harvested immediately before induction of expression (Fig. 1, *lane 2*) nor in cells harvested after induction but lacking the coding sequence in the expression vector (Fig. 1, *lane 3*).

Functional Characterization of Recombinant Ggc1p—Ggc1p was reconstituted into liposomes, and its transport activities for a variety of potential substrates were tested in homoexchange experiments (*i.e.* with the same substrate inside and outside). Using external and internal substrate concentrations of 1 and 10 mM, respectively, the reconstituted protein catalyzed an active [8-^{3}H]GTP/GTP exchange, inhibitable by a mixture of bathophenanthroline and pyridoxal 5′-phosphate. It did not catalyze homoexchanges for phosphate, ATP, ADP, AMP, pyruvate, malate, oxoglutarate, citrate, glutamate, aspartate, proline, histidine, lysine, arginine, serine, threonine, tryptophan, glutathione, carnitine, and choline. No [8-^{3}H]GTP/GTP exchange activity was observed with Ggc1p that had been boiled before incorporation into liposomes nor by reconstitution of sarkosyl-solubilized material from bacterial cells either lacking the expression vector for Ggc1p or harvested immediately before the induction of expression.

The substrate specificity of Ggc1p was examined in greater detail by measuring the uptake of [α-^{33}P]dGTP into proteoliposomes that had been preloaded with various potential substrates (Fig. 2*A*). High rates of [α-^{33}P]dGTP uptake into proteoliposomes were observed with internal GDP, GTP, dGDP, dGTP, IDP, and ITP. Much smaller activities were found with internal guanosine 5′-tetraphosphate and (deoxy)nucleoside di- and triphosphates of U and T. No activity was detected with internal (d)NDP and (d)NTP of A and C, with GMP and all the

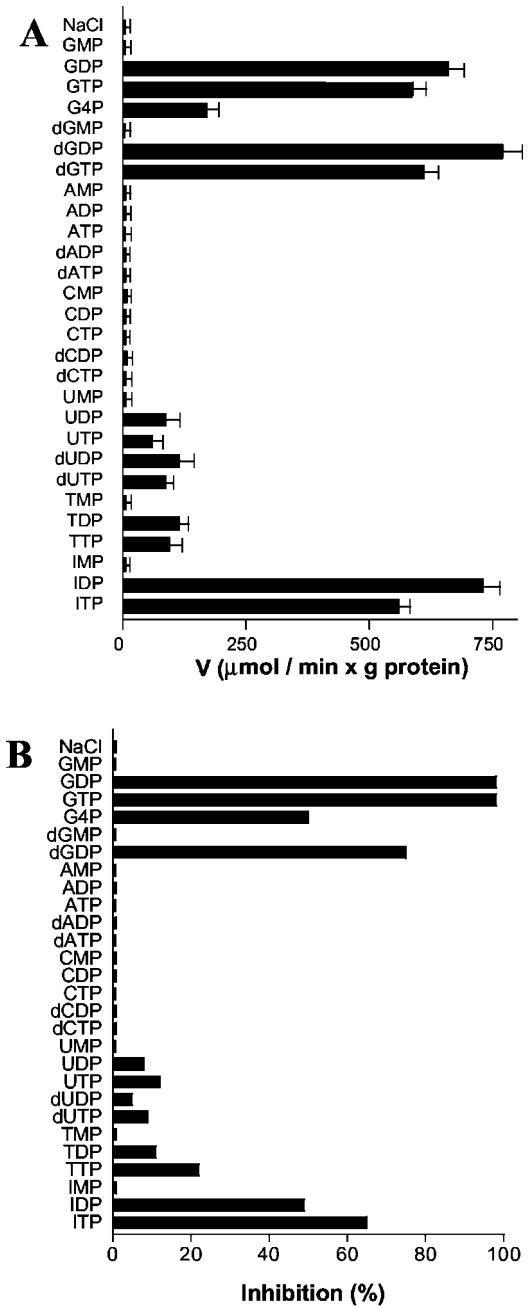

FIG. 2. **Substrate specificity of Ggc1p.** *A*, dependence of Ggc1p activity on internal substrate. Proteoliposomes were preloaded internally with various substrates (concentration 5 mM). Transport was started by the addition of 15 μM [8-^{3}H]dGTP and stopped after 20 s. The values are the means ± S.D. of at least three experiments. *B*, inhibition of the rate of [α-^{33}P]dGTP uptake by external substrates. The proteoliposomes were preloaded internally with 5 mM dGTP. Transport was started by adding 15 μM [8-^{3}H]dGTP and stopped after 20 s. External substrates (concentration 150 μM) were added together with [8-^{3}H]dGTP. The extents of inhibition (%) from a representative experiment are reported. The control value for uninhibited exchange was 0.65 mmol/min/g of protein. *G4P*, guanosine 5′-tetraphosphate.

other (deoxy)nucleoside monophosphates tested, with NaCl and (not shown) with adenosine, adenine, NMN, FMN, thiamine pyrophosphate, NADH, NADPH, FAD, phosphate, pyrophosphate, and malate.

External GDP and GTP and, to a lesser extent, dGDP, guanosine 5′-tetraphosphate, IDP, and ITP inhibited the [α-^{33}P]dGTP/dGTP exchange (Fig. 2*B*). A low inhibition was found with UDP, UTP, dUDP, dUTP, TDP, and TTP, and virtually no effect was detected with (deoxy)nucleoside di- and

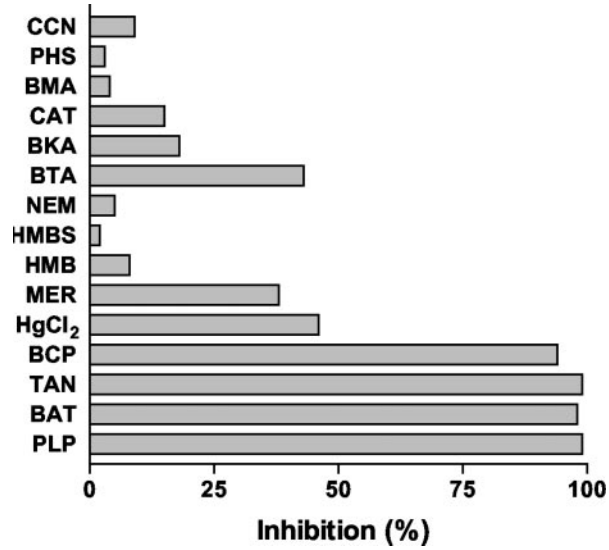

FIG. 3. **Effect of inhibitors on the [8-^{3}H]GTP/GTP exchange by Ggc1p.** The proteoliposomes were preloaded internally with 5 mM GTP, and transport was initiated by adding 1 μM [8-^{3}H]GTP. The incubation time was 20 s. Thiol reagents and α-cyanocinnamate were added 2 min before the labeled substrate; the other inhibitors were added together with [8-^{3}H]GTP. The final concentrations of the inhibitors were 0.1 mM for *p*-hydroxymercuribenzoate (*HMB*), *p*-hydroxymercuribenzene sulfonate (*HMBS*), and mercuric chloride ($HgCl_2$); 2 mM for pyridoxal 5′-phosphate (*PLP*), bathophenanthroline (*BAT*), *N*-ethylmaleimide (*NEM*), benzene-1,2,3-tricarboxylate (*BTA*), butylmalonate (*BMA*), and phenylsuccinate (*PHS*); 0.3 mM for bromcresol purple (*BCP*); 0.05% for tannic acid (*TAN*); 100 μM for carboxyatractyloside (*CAT*) and bongkrekic acid (*BKA*), and 1 mM for α-cyanocinnamate (*CCN*). The extents of inhibition (%) from a representative experiment are given.

triphosphates of A and C, GMP, the other nucleoside monophosphates and (not shown) phosphate, malate, succinate, ornithine, carnitine, oxaloacetate, sulfate, 2-oxoaminoadipate, and thiamine mono- and diphosphate.

The [8-^{3}H]GTP/GTP exchange reaction catalyzed by reconstituted Ggc1p was inhibited strongly by pyridoxal 5′-phosphate and bathophenanthroline (inhibitors of many mitochondrial carriers), tannic acid, and bromcresol purple (inhibitors of the mitochondrial glutamate carrier) and only partially by the sulfydryl reagents mercuric chloride and mersalyl and by 1,2,3-benzenetricarboxylate (inhibitor of the mitochondrial citrate carrier) (Fig. 3). Carboxyatractyloside and bongkrekate (powerful inhibitors of the ADP/ATP carrier) had little effect at much higher concentrations than those that completely inhibit the ADP/ATP carrier. Very little inhibition was observed with *p*-hydroxymercuribenzoate, *p*-hydroxymercuribenzene sulfonate, *N*-ethylmaleimide, butylmalonate, phenylsuccinate, and α-cyano-4-hydroxycinnamate (inhibitors of other mitochondrial carriers).

Kinetic Characteristics of Recombinant Ggc1p—In Fig. 4, the kinetics are compared for the uptake of 0.5 mM [8-^{3}H]GTP into proteoliposomes either in the presence or in the absence of internal 5 mM GTP. The uptake of GTP by exchange followed a first order kinetics (rate constant, 22.6 min^{-1}; initial rate, 1.9 mmol/min/g protein), isotopic equilibrium being approached exponentially (Fig. 4*A*). In contrast, no [8-^{3}H]GTP uptake was observed without internal substrate, suggesting that Ggc1p does not catalyze a unidirectional transport (uniport) of GTP. The uniport mode of transport was further investigated by measuring the efflux of [8-^{3}H]GTP from prelabeled active proteoliposomes because it provides a more convenient assay for unidirectional transport (16). In the absence of external substrate no efflux was observed even after incubation for 20 min (Fig. 4*B*). However, upon the addition of external GTP or GDP, an extensive efflux of radioactivity occurred, and this efflux

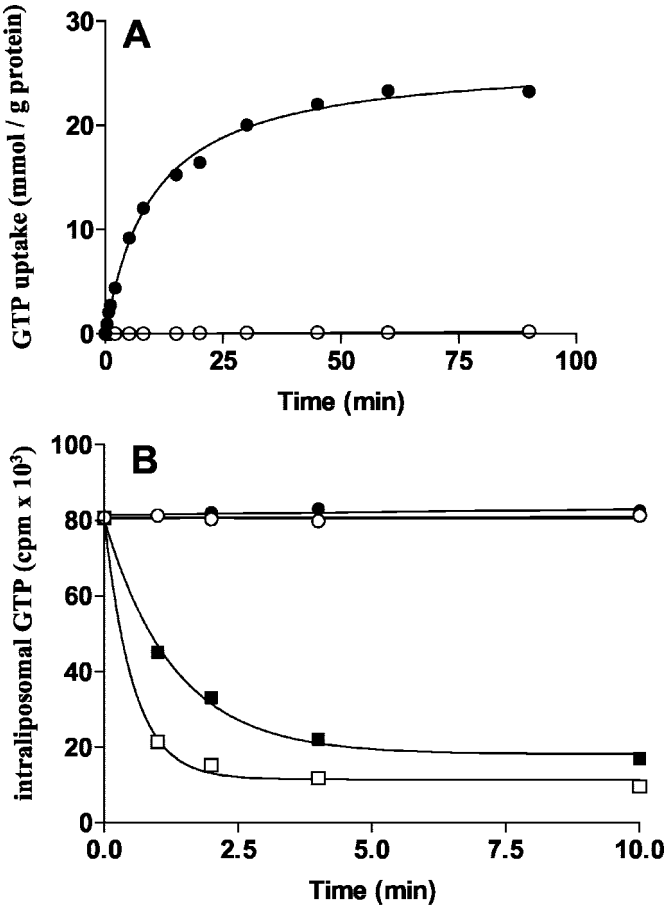

FIG. 4. **Kinetics of [8-^{3}H]GTP transport in proteoliposomes reconstituted with Ggc1p.** *A*, uptake of GTP. 0.5 mM [8-^{3}H]GTP was added to proteoliposomes containing 5 mM GTP (exchange, ●) or 5 mM NaCl and no substrate (uniport, ○). Similar results were obtained in three independent experiments. *B*, efflux of [8-^{3}H]GTP from proteoliposomes reconstituted in the presence of 1 mM GTP. The internal substrate pool was labeled with [8-^{3}H]GTP by carrier-mediated exchange equilibration. Then the proteoliposomes were passed through Sephadex G-75. The efflux of [8-^{3}H]GTP was started by adding buffer A alone (○), 5 mM GTP in buffer A (□), 5 mM GDP in buffer A (■), or 5 mM GTP, 15 mM bathophenanthroline and 45 mM pyridoxal 5′-phosphate in buffer A (●). Similar results were obtained in three independent experiments.

was prevented completely by the presence of the inhibitors pyridoxal 5′-phosphate and bathophenanthroline (Fig. 4*B*). These results indicate that, at least under the experimental conditions used, the reconstituted Ggc1p catalyzes an obligatory exchange reaction of substrates.

The exchange rate of internal GTP, GDP, or dGTP (5 mM) depended on the external concentration of [8-^{3}H]GTP (0.2–20 μM), [8,5′-^{3}H]GDP (1–100 μM), or [α-^{33}P]dGTP (1–100 μM). With all three external substrates, the linear functions were obtained in double-reciprocal plots. They were independent of the internal substrate and intersected the ordinate close to a common point (not shown). For GTP, GDP, and dGTP, the transport affinities (K_m) were 1.2 ± 0.1, 4.5 ± 0.7, and 15.9 ± 1.8 μM (mean values of 20, six and seven experiments, respectively). The average value of V_{max} was 2.0 ± 0.4 mmol/min/g of protein. Several external substrates were competitive inhibitors of [8-^{3}H]GTP uptake (Table I) because they increased the apparent K_m without changing the V_{max} (not shown). These results confirm that GTP is the highest affinity external substrate (K_i, 0.9 μM). The K_i values of all of the NTPs are lower than those of their corresponding NDPs. Furthermore, the affinity of Ggc1p for GTP and GDP is approximately 1 order of magnitude higher than for dGTP and dGDP and approximately 2 orders of magnitude higher than for ITP and IDP.

TABLE I
Competitive inhibition by various substrates of [8-^{3}H]GTP uptake in proteoliposomes containing recombinant Ggc1p

The values were calculated from Lineweaver-Burk plots of the rate of [8-^{3}H]GTP *versus* substrate concentrations. The competing substrates at appropriate constant concentrations were added together with 0.2–20 μM [8-^{3}H]GTP to proteoliposomes containing 5 mM GTP and reconstituted with recombinant Ggc1p. The data represent the means ± S.D. of at least three different experiments. G4P, guanosine 5′-tetraphosphate.

Substrate	K_i
	μM
GTP	0.9 ± 0.2
GDP	5.0 ± 0.6
dGDP	53 ± 8
dGTP	24 ± 4
G4P	235 ± 36
IDP	208 ± 27
ITP	145 ± 30
TDP	1358 ± 182
TTP	356 ± 47
UDP	2036 ± 323
UTP	1243 ± 190
dUDP	3259 ± 444
dUTP	1856 ± 295

Influence of Membrane Potential and pH Gradient on the Ggc1p-mediated GTP/GDP Exchange—In view of the different charges carried by GTP and GDP at physiological pH levels, we investigated the influence of the membrane potential on the [8,5′-^{3}H]GDP/GTP or [8-^{3}H]GTP/GDP heteroexchanges catalyzed by the recombinant Ggc1p. A K^+ diffusion potential was generated across the proteoliposomal membranes with valinomycin/KCl (calculated value was approximately 100 mV, positive inside) (Table II). The rates of the GDP_{out}/GTP_{in} and GTP_{out}/GDP_{in} heteroexchanges as well as of the GDP/GDP and GTP/GTP homoexchanges were unaffected by valinomycin in the presence or absence of the K^+ gradient. In contrast, the $aspartate_{out}/glutamate_{in}$ exchange, mediated by the recombinant and reconstituted aspartate/glutamate carrier, which is known to catalyze an electrophoretic exchange between $aspartate^-$ and $glutamate^- + H^+$ (13, 19), was stimulated by valinomycin in the presence of a K^+ gradient of 1/50 (mM/mM, in/out) (not shown). These results indicate that the GTP/GDP heteroexchange catalyzed by Ggc1p is not electrophoretic. We therefore became interested in the question as to whether the charge imbalance of the GTP/GDP heteroexchange is compensated by proton movement in the same direction as GTP. A pH difference across the liposomal membranes (basic inside the vesicles) was created by the addition of the K^+/H^+ exchanger nigericin to proteoliposomes in the presence of a potassium gradient of 1/50 (mM/mM, in/out) (Table II). Under these conditions the uptake of [8,5′-^{3}H]GDP in exchange for internal GTP decreased, and the uptake of [8-^{3}H]GTP in exchange for internal GDP increased, whereas no effect on the GDP/GDP and GTP/GTP homoexchanges was observed. Therefore, the GTP/GDP heteroexchange in either direction is driven by the ΔpH, indicating that the charge imbalance of the exchanged substrates is compensated by the movement of protons. Furthermore, no uptake of [8,5′-^{3}H]GDP or [8-^{3}H]GTP by unloaded liposomes was observed even in the presence of an energy input (either membrane potential or pH gradient). In other experiments it was found that the rate of 1 μM [8-^{3}H]GTP uptake by proteoliposomes containing 2 mM GDP increased approximately three times on decreasing the external pH from 8.0 to 6.5 at a fixed internal pH of 8.0 (see Ref. 20 for the experimental conditions), whereas the rate of GTP/GTP exchange was

TABLE II

Influence of membrane potential and ΔpH on the activity of reconstituted Ggc1p

The exchanges were started by the addition of 5 μM [8,5′-^{3}H]GDP or 1 μM [8-^{3}H]GTP to proteoliposomes containing 2 mM GDP or GTP. K^+_{in} was included as KCl in the reconstitution mixture, whereas K^+_{out} was added as KCl together with the labeled substrate. The differences in osmolarity were compensated for by the addition of NaCl. 1 mM PIPES-NaOH at pH 7.0 was present in the internal and external compartments. Valinomycin or nigericin was added in 10 μl ethanol/ml proteoliposomes. In the control samples the solvent alone was added. The exchange reactions were stopped after 20 s. Similar results were obtained in four independent experiments.

Uptake of	Internal substrate	K^+_{in}/ K^+_{out}	Transport activity		
			Control	+Valinomycin	+Nigericin
	mM/mM		*μmol/min/g of protein*		
[8,5′-^{3}H]GDP	GDP	1/1	872	856	885
	GDP	1/50	895	834	862
	GTP	1/1	780	795	746
	GTP	1/50	810	783	497
	None	1/50	0	2	0
[8-^{3}H]GTP	GDP	1/50	708	694	871
	GTP	1/50	685	712	694
	None	1/50	3	0	2

virtually unaffected (not shown). Taken together, these results indicate that the reconstituted Ggc1p catalyzes an electroneutral H^+-compensated GTP/GDP heteroexchange.

Mitochondria Lacking ggc1 Are Impaired in GTP Uptake and Contain Reduced Levels of GTP—Having established the transport function of Ggc1p by *in vitro* assays, the effect of deleting its gene on yeast cells was investigated. We first measured the contents of GTP and GDP in the mitochondria of wild-type and mutant cells. The amount of GTP was approximately 7-fold lower in *ggc1*Δ mitochondria than in the organelles from wild-type cells (Fig. 5*A*). Vice versa the amount of GDP was approximately 5.5-fold higher in *ggc1*Δ than in wild-type mitochondria (Fig. 5*A*). These results are consistent with Ggc1p controlling the entry of GTP and the exit of GDP.

In the next step, the [8-^{3}H]GTP/GTP exchange was measured in proteoliposomes that were reconstituted with Triton X-100 extracts of wild-type and *ggc1*Δ mitochondria. No GTP/GTP exchange activity was detected upon reconstitution of the mitochondrial extracts from the knock-out strain (Fig. 5*B*). In contrast, an active GTP/GTP exchange was observed using parental mitochondrial extracts (Fig. 5*B*). The reconstituted GTP/GTP exchange was inhibited markedly by 2 mM pyridoxal 5′-phosphate, 2 mM bathophenanthroline, or 0.05% tannic acid and by the Ggc1p substrates GDP and dGTP (but not by ADP, ATP, CDP, and CTP) added at a concentration of 20 μM together with 1 μM [8-^{3}H]GTP (data not shown). Similar results were obtained by measuring the [8,5′-^{3}H]GDP/GDP and the [α-^{33}P]dGTP/dGTP exchanges in proteoliposomes reconstituted with mitochondrial extracts from wild-type and *ggc1*Δ cells. Therefore, the Ggc1p present in the mitochondria exhibits the same specificity and inhibitor sensitivity than the reconstituted protein. As a control, Fig. 5*B* shows that, compared with the proteoliposomes reconstituted with wild-type extracts, in those reconstituted with *ggc1*Δ extracts the phosphate/phosphate exchange and (not shown), the ADP/ADP exchange fell by approximately 60% but was not abolished. When analyzing the levels of several mitochondrial proteins, we found that Ggc1p was completely absent in the mutant mitochondria (Fig. 5*C*). However, the amounts of the phosphate and the ADP/ATP carriers and (not shown) the oxaloacetate-sulfate and the thiamine pyrophosphate carriers and of cytochrome c_1 and subunit 9 of complex III but not of porin were 50–60% lower in *ggc1*Δ mitochondria than in the organelles from wild-type cells (Fig. 5*C*). Therefore, in the mutant mitochondria, Ggc1p and GTP transport are completely absent, whereas the activities of other transporters are diminished because the transporters are present in smaller amounts in *ggc1*Δ than in wild-type mitochondria.

Because the presence of a membrane potential (Δψ) across the inner membrane is a prerequisite for any protein transport into or across this membrane ((import) (21), we assessed the membrane potential of *ggc1*Δ mitochondria by using the fluorescent dye DiSC (3, 5, 22). The difference between the fluorescence after the addition of mitochondria and substrates and that after the subsequent addition of the potassium ionophore valinomycin (in the presence of external K^+, leading to a complete dissipation of Δψ) is taken as an assessment of the mitochondrial membrane potential (18). The decrease in valinomycin-sensitive fluorescence observed with *ggc1*Δ mitochondria was only approximately 4% of that observed with wild-type mitochondria (Fig. 5*D*), demonstrating that *in vitro* the membrane potential of *ggc1*Δ mitochondria was very low.

*Ggc1*Δ *Yeast Cells Are Not Able to Grow on Nonfermentable Carbon Sources and Have Lost Their DNA*—The *ggc1*Δ mutant was also tested for its ability to utilize different carbon sources. Yeast cells lacking *ggc1* showed substantial growth on YP medium containing fermentable carbon sources (glucose or galactose), similarly to the wild-type strain. However, they did not grow on the same medium containing nonfermentable substrates (glycerol, lactate, ethanol, acetate, pyruvate, or oxaloacetate) (data not shown). It should be noted that, in wild-type yeast cells, we found by quantitative immunoblotting that Ggc1p was expressed at similar levels on fermentable (glucose and galoctose) and nonfermentable (lactate, glycerol, ethanol, and acetate) carbon sources (data not shown). The abundance of Ggc1p in mitochondria from yeast cells fed on galactose was 210 ± 47 pmol/mg of protein, in four determinations. The lack of growth on lactate was observed previously upon YDL198c deletion in the YPH499 strain (23), whereas no substantial defect on glycerol was found upon disruption of YDL198c in the W303 strain (9).

The inability of *ggc1*Δ mutant cells to grow on nonfermentable media led us to check whether these cells had lost their mitochondrial genome. To address this problem, mutant cells were stained with the DNA-specific dye DAPI and examined by fluorescence microscopy. The major fluorescence source in the central region of the cells, corresponding to DAPI-stained nuclear DNA, was observed both in wild-type and in *ggc1*Δ cells (Fig. 6). In contrast, the small and weak fluorescent spots in the cell periphery, corresponding to mtDNA, were observed only in wild-type cells, indicating that *ggc1*Δ cells were devoid of mitochondrial DNA (Fig. 6).

DISCUSSION

In this work, overexpression in *E. coli* of a hitherto unidentified mitochondrial carrier, reconstitution of the recombinant protein in liposomes, and phenotype analysis of yeast knock-out cells have been employed to investigate the function of the *S. cerevisiae* Ggc1p (encoded by YDL198c). The results ob-

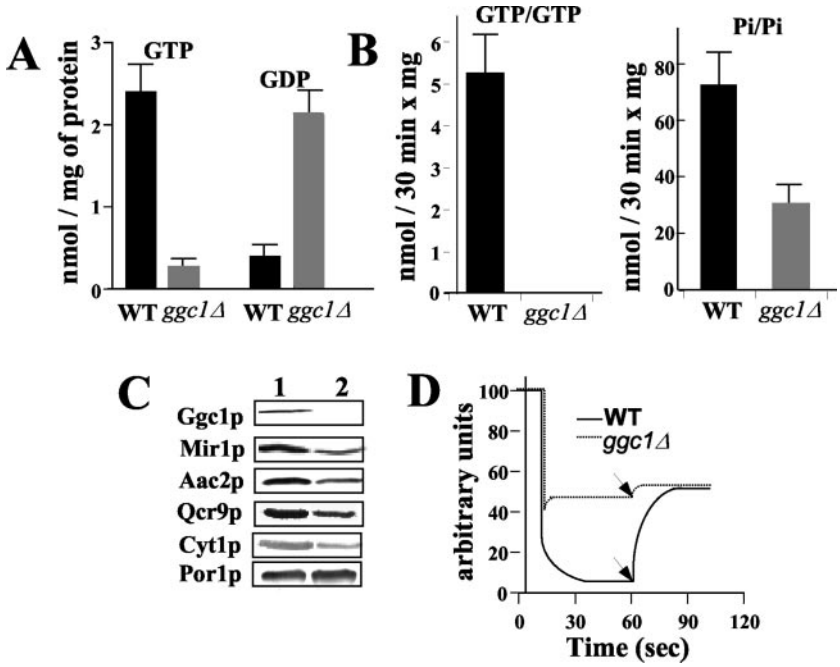

FIG. 5. **Characterization of mitochondria isolated from *ggc1*Δ yeast cells grown on galactose.** *A*, mitochondria isolated from *ggc1*Δ cells contain lower levels of GTP and higher levels of GDP. Mitochondrial perchloric extracts (5 mg of protein) from wild-type and *ggc1*Δ cells were assayed for GTP and GDP contents. The values are the means ± S.D. of four experiments. *B*, GTP/GTP and phosphate/phosphate exchange activities in liposomes reconstituted with yeast mitochondrial extracts. *ggc1*Δ (*gray columns*) and wild-type (*black columns*) mitochondria were solubilized (0.8 mg of protein/ml) in 2% Triton X-100, 50 mM NaCl, 1 mM EDTA, and 10 mM PIPES (pH 7.0) for 20 min at 0 °C and centrifuged (138,000 × *g* for 10 min). The supernatants (approximately 10 μg of protein) were reconstituted into liposomes, and the indicated exchanges were tested. Transport was started by adding 1 μM [8-^{3}H]GTP or 100 μM [α-^{33}P]phosphate to proteoliposomes preloaded with 5 mM GTP or 20 mM phosphate, respectively. The values are the means ± S.D. of four experiments. *C*, immunoblotting analyses of yeast mitochondrial proteins. Wild-type (*lane 1*) and *ggc1*Δ (*lane 2*) mitochondria (5 μg of protein) were separated by SDS-PAGE, transferred to nitrocellulose, and detected with specific antibodies for the indicated proteins. *Ggc1p*, GTP/GDP carrier; *Mir1p*, phosphate carrier; *Aac2p*, ATP/ADP carrier; *Qcr9p*, subunit 9 of the ubiquinol-cytochrome *c* reductase; *Cyt1p*, cytochrome c_1; *Por1p*, porin. *D*, assessment of the membrane potential of *ggc1*Δ mitochondria. Wild-type and *ggc1*Δ mitochondria (25 μg of protein) were incubated with the membrane potential-sensitive dye DiSC (3) (5), and the fluorescence changes were recorded. At the *arrows*, 1 μM valinomycin was added. *WT*, wild type.

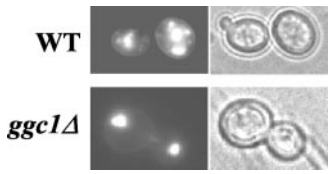

FIG. 6. **mtDNA detection in wild-type and *ggc1*Δ cells.** The cells were grown to stationary phase on YP medium containing 2% galactose. After fixation with formaldehyde, the cells were stained with DAPI and visualized by fluorescence microscopy (*left panel*) and phase contrast microscopy (*right panel*). *WT*, wild type.

tained here, together with the targeting of Ggc1p to mitochondria reported before (9), demonstrate that this protein is the mitochondrial transporter for GTP and GDP. This is the first time that a mitochondrial carrier for guanine nucleotides has been identified from any organism. Ggc1p does not show significant sequence homology with any other mitochondrial carrier functionally identified until now in yeast, mammals, and plants (Refs. 8, 20, and 24–27 and references therein). In a phylogenetic tree of the *S. cerevisiae* members of the mitochondrial carrier family (28, 29), Ggc1p clusters together with transporters for nucleotides or nucleotide analogs (the three isoforms of the ADP/ATP carrier (30–32) and the carriers for coenzyme A (33) and for thiamine pyrophosphate (20) and with YDL119c (20% identity), which has not yet been identified). Some proteins encoded by the genomes of lower eukaryotes, such as AL031525 from *Schizosaccharomyces pombe* (67% of identical amino acids), q8wzw8 from *N. crassa* (70%), AN5132.1 from *Aspergillus nidulans* (63%), and AE017168 from *Trypanosoma brucei* (43%), are likely orthologs of Ggc1p. It is doubtful, however, that there is an orthologous carrier in higher eukaryotes as the closest sequences to Ggc1p in *Caenorhabditis elegans* (F55C1, 26% of identical amino acids), *Drosophila melanogaster* (AE003693, 25%), *Arabidopsis thaliana* (AT2G26360, 20%), and *Homo sapiens* (the uncoupling protein 2, UCP2, 20%) exhibit a low degree of similarity with Ggc1p as compared with the basic homology existing between the different members of the mitochondrial carrier family. Furthermore, in these organisms the presence of the GTP/GDP carrier is not strictly necessary, because with the exception of *A. thaliana*, they possess a mitochondrial GTP-producing succinyl-CoA ligase.

Besides transporting GTP and GDP with high efficiency, reconstituted Ggc1p also transports the corresponding deoxynucleotides, the structurally related ITP and IDP, and, to a much lesser extent, the (deoxy)nucleoside di- and triphosphates of U and T, but none of the many other compounds tested. The substrate specificity of Ggc1p is distinct from that of any other previously characterized member of the mitochondrial carrier family. In particular, Ggc1p differs markedly from the well known ADP/ATP carrier (34, 35), because both the yeast and the human ADP/ATP carrier isoforms transport-only (deoxy)adenine nucleotides are strongly inhibited by carboxyatractyloside and bongkrekic acid and share only 9–14 and 16–18% of identical amino acids, respectively, with the Ggc1p. Ggc1p is also quite different from the human deoxynucleotide carrier (36) and its most closely related protein in *S. cerevisiae* (the thiamine pyrophosphate carrier (Tpc1p (20)), because deoxynucleotide carrier transports all (deoxy)NDPs and less efficiently the corresponding (deoxy)NTPs, whereas Tpc1p transports all of the (deoxy)nucleotides with the following order of efficiency: NMPs > NDPs > NTPs. Further-

more, at variance with Ggc1p, Tpc1p catalyzes both the uniport and the exchange modes of transport.

In *ggc1*Δ yeast cells the mitochondrial content of GTP is drastically decreased, indicating that Ggc1p catalyzes the uptake of GTP into the mitochondrial matrix. In the mitochondria, GTP is converted to GDP (in protein synthesis for the formation of the initiation complex and for the elongation of the polypeptide chain, by GTP-AMP phosphotransferase and by GTPases) or incorporated into the various types of RNA present in the mitochondria, including the RNA primers, which are required for the initiation of DNA replication and repair. Therefore, because in *S. cerevisiae* GTP is not synthesized in the mitochondrial matrix, Ggc1p appears to be essential for a number of major processes occurring in the mitochondria that depend on the availability of intramitochondrial GTP, such as the initiation of DNA replication and repair, protein synthesis, and recovery of AMP. Because Ggc1p functions by a strict exchange mechanism, the carrier-mediated uptake of GTP requires the efflux of a counter-substrate. On the basis of our transport measurements, GDP, that is produced from GTP intramitochondrially and is phosphorylated by nucleoside diphosphate kinase in the intermembrane space, may serve as the counter-substrate of Ggc1p for GTP. Therefore, the main physiological role of Ggc1p is most likely to catalyze the uptake of GTP into the mitochondrial matrix in exchange for internal GDP. The results obtained by imposing an external energy gradient on our simple liposomal system show that the charge imbalance of the GTP/GDP heteroexchange is compensated by H^+ carried by Ggc1p in the same direction as GTP. The physiological consequence of this finding is that in mitochondria the GTP_{out}/GDP_{in} exchange catalyzed by Ggc1p is driven by the ΔpH component of the proton motive force generated by electron transport and is unaffected by its electrical component. The H^+-compensated electroneutral type of mechanism of the newly identified GTP/GDP carrier is consistent with its main function (to import GTP in exchange for matrix GDP) and is different from that of the well studied ADP/ATP carrier, which is electrophoretic and ΔpH-insensitive (37). Another function of Ggc1p may be to catalyze the uptake of dGTP into the mitochondria where it is required for DNA replication and repair. However, the affinity of dGTP for Ggc1p is more than 1 order of magnitude lower than that of GTP. Furthermore, deoxynucleotides can be imported by Tpc1p (see Ref. 20), which is the protein encoded by the yeast genome with the highest sequence similarity to the human deoxynucleotide carrier.

The importance of Ggc1p is highlighted by the observation that the *ggc1* null mutant does not grow on any nonfermentable carbon source and has no mitochondrial DNA. The complete loss of mtDNA in *ggc1*Δ cells shows that the *ggc1* gene product is essential for the maintenance of mtDNA. The involvement of Ggc1p in mtDNA maintenance is in agreement with the previous observation that Ggc1p is a multicopy suppressor of the temperature-sensitive defect of the *abf2* null mutant (9). Abf2p is a histone-like mitochondrial DNA-binding protein that is required for maintenance of the yeast mitochondrial genome at 37 °C. Our results suggest that Abf2p plays an accessory role to Ggc1p in a GTP-dependent reaction involved in mtDNA maintenance, and hence its inactivation is rescued by the presence of Ggc1p at 25–28 °C and by Ggc1p overexpression at 37 °C (9). One possibility is that Ggc1p promotes the replication of the mtDNA by stimulating the synthesis of the RNA primers by the enzymes mtRNA polymerase and primase. Interestingly, the mammalian counterpart of Abf2p has been shown to be a limiting factor for mtDNA replication (38). The identification of the mitochondrial GTP/GDP carrier reported here provides a new tool for gaining further insight into the molecular mechanisms underlying the regulation of mtDNA maintenance and metabolism in yeast.

During the revision of this work an accumulation of iron in the mitochondria of yeast cells lacking the *yhm1* gene, *i.e.* the *ggc1* gene, was published (39). There are no data available on the role of intramitochondrial guanine nucleotides on iron metabolism in yeast and higher eukaryotes. However, it may be speculated that the Ggc1p-mediated import of GTP into the mitochondrial matrix is required for or regulates a reaction involved in entry/exit of iron into/from the mitochondria or in the synthesis of heme and of iron-sulfur clusters. It is interesting that the bacterial membrane protein FeoB, which is essential for Fe(II) uptake in bacteria, contains a G protein similar to small regulatory G proteins found in eukaryotes and that the function of the G protein is required for Fe(II) uptake through the FeoB-dependent system (40). It is also possible that iron accumulation in mitochondria of *yhm1*Δ yeast cells is a secondary effect of the mitochondrial lesions mentioned above caused by the shortage of GTP in the mitochondria. Further studies are necessary to clarify how the Ggc1p-catalyzed transport of guanine nucleotides across the mitochondrial membrane influences iron metabolism.

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Identification of the Mitochondrial GTP/GDP Transporter in *Saccharomyces cerevisiae*

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J. Biol. Chem. 2004, 279:20850-20857.
doi: 10.1074/jbc.M313610200 originally published online March 3, 2004
