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ORIGINAL ARTICLE

Cystathionine γ-lyase, an Enzyme Related to the Reverse Transsulfuration Pathway, is Functional in *Leishmania* spp.

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ABSTRACT

Leishmania parasites seem capable of producing cysteine by de novo biosynthesis, similarly to bacteria, some pathogenic protists, and plants. In *Leishmania* spp., cysteine synthase (CS) and cystathionine β-synthase (CBS) are expected to participate in this metabolic process. Moreover, the reverse transsulfuration pathway (RTP) is also predicted to be operative in this trypanosomatid because CBS also catalyzes the condensation of serine with homocysteine, and a gene encoding a putative cystathionine γ-lyase (CGL) is present in all the sequenced genomes. Our results show that indeed, *Leishmania major* CGL is able to rescue the wild-type phenotype of a *Saccharomyces cerevisiae* CGL-null mutant and is susceptible to inhibition by an irreversible CGL inhibitor, DL-propargylglycine (PAG). In *Leishmania* promastigotes, CGL and CS are cytosolic enzymes. The coexistence of de novo synthesis with the RTP is extremely rare in most living organisms; however, despite this potentially high redundancy in cysteine production, PAG arrests the proliferation of *L. major* promastigotes with an IC_{50} of approximately 65 μM. These findings raise new questions regarding the biological role of CGL in these pathogens and indicate the need for understanding the molecular mechanism of PAG action in vivo to identify the potential targets affected by this drug.

LEISHMANIA parasites cause a broad spectrum of clinical manifestations in man, all of which are referred to as leishmaniases. Phlebotomine sandflies bite and infect several million humans every year, and approximately 350 million people are at risk of infection in tropical and subtropical countries worldwide (WHO 2010). Currently, the clinical treatments against these neglected tropical diseases are far from satisfactory due to the high toxicity and low efficacy of the available drugs (Croft and Olliaro 2011). In pathogenic protists, the metabolic pathways related to sulfur-containing amino acids are divergent from those of their mammalian hosts and the enzymes involved in these processes are considered as potential targets for drug development (for review see, Ali and Nozaki 2007; Nozaki et al. 2005).

In most organisms, cysteine plays a wide range of relevant biological roles. Moreover, particularly in *Leishmania* spp. and trypanosomes, cysteine is an essential building block for trypanothione biosynthesis. This notably abundant (in the mM range) low-molecular-mass dithiol provides the reducing equivalents required for neutralizing free radicals and, therefore, maintains the redox homeostasis in all of the developmental stages of these pathogenic protists (Krauth-Siegel and Comini 2008). Both *Trypanosoma cruzi* and *Leishmania* parasites are believed to display redundant biosynthetic routes leading to cysteine production: (i) de novo synthesis, a two-step pathway catalyzed by serine acetyltransferase (SAT) and CS and (ii) the reactions involving the broad-specificity cystathionine β-synthase (CBS), which can function as a serine sulfhydrylase and a CS, in addition to operating as a catalyst for the condensation of serine with homocysteine to produce cystathionine, an intermediate metabolite of reverse transsulfuration pathway (RTP; for clarity, see Fig. 1) (Marciano et al. 2010, 2012; Nozaki et al. 2001; Williams et al. 2009). Unlike trypanosomes, *Leishmania* promastig-

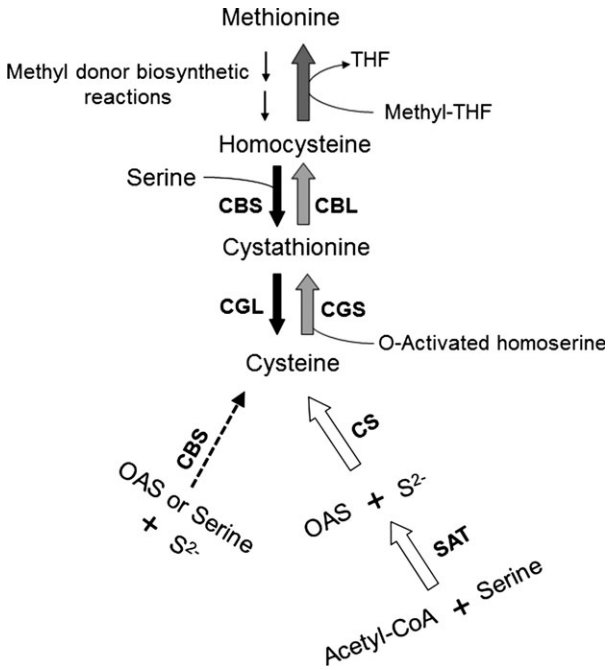

Figure 1 Scheme showing methionine/cysteine interconversion pathways in different living organisms. Typically, plants and bacteria synthetize de novo L-cysteine and convert this amino acid into methionine *via* the forward transsulfuration pathway (Mozzarelli et al. 2011; Wirtz and Hell 2006). These routes are indicated by open and filled light gray arrows, respectively. L-homocysteine is methylated by methionine synthase (reaction indicated by filled dark gray arrow). By contrast, animals and a few microorganisms are auxotroph for methionine. Hence, the L-homocysteine derived from methionine catabolism is partially utilized for cysteine production through the reverse transsulfuration pathway (indicated by filled black arrows). *Leishmania* parasites and *Trypanosoma cruzi* might exhibit alternative routes for de novo synthesis of cysteine (Marciano et al. 2010, 2012; Nozaki et al. 2001; Williams et al. 2009), the reactions are indicated by open and dashed arrows. SAT = serine acetyltransferase, OAS = O-acetylserine, CS = cysteine synthase, CGS = cystathionine γ synthase, CBL = cystathionine β lyase, CBS = cystathionine β synthase, CGL = cystathionine γ lyase.

otes are expected to depend exclusively on intracellular processes for cysteine generation because these parasites are unable to uptake cysteine from the extracellular environment (Williams et al. 2009).

Serine acetyltransferases and CSs are functional almost exclusively in microorganisms and plants (Mozzarelli et al. 2011; Wirtz and Hell 2006). In these organisms, cysteine represents the organic sulfur source for methionine biosynthesis. By contrast, mammals lack SAT and CS; instead, cysteine is endogenously produced via the RTP. Therefore, in mammals, cysteine production depends on the nutritional availability of methionine and on the operability of CBS and cystathionine γ-lyase (CGL) (Fig. 1). Interestingly, *T. cruzi* is the first protist to exhibit the uncommon feature of functional enzymes that are expected to participate in both the de novo synthesis of cysteine and the RTP. This condition reflects the particularly unusual redundancy in metabolic pathways plausibly leading to cysteine production in this pathogen. The *T. cruzi* CGL exhibits kinetic parameters that are similar, but not identical, to those reported for its eukaryotic counterparts, such as those of mammals and yeast (Marciano et al. 2012; Steegborn et al. 1999; Yamagata et al. 1993). Moreover, in the presence of DL-propargylglycine (PAG), a well-known inhibitor of CGLs, this pathogen's CGL activity decreases at a remarkably lower concentration than that reported to inhibit the mammalian homolog (half maximal inhibitory concentration, IC_{50} 12 μM vs. 2 mM) (Marciano et al. 2012; Sun et al. 2009). The functionality of CGL in *Leishmania* parasites has not yet been assessed, and even though the putative CGLs reveal remarkable sequence identities (> 80%) with the functional *T. cruzi* homolog (see Fig. 1), unlike the *T. cruzi* enzyme, the recombinant leishmanial CGLs could not be functionally expressed in *Escherichia coli* cultures (our unpubl. results). This fact prompted us to undertake the approach of functional complementation of a *S. cerevisiae* CGL-null mutant to assess the activity of this enzyme in *Leishmania* parasites.

Our results show that *L. major* indeed exhibits a functional CGL; this enzyme appears to be quite susceptible to inhibition by PAG and, in parallel to CS, localizes in the cytosol of *Leishmania* promastigotes. Moreover, despite the redundancy in the potential metabolic routes on which *Leishmania* parasites might rely for cysteine production, the proliferation of *L. major* promastigotes was arrested in the presence of PAG (IC_{50} of approximately 65 μM), a well-known inhibitor of CGLs.

MATERIALS AND METHODS

Parasite culture

Leishmania mexicana promastigotes were grown as previously described (Cazzulo et al. 1985), whereas, promastigotes from the *L. major* Friedlin strain were maintained in vitro by successive passage in medium 199 (Gibco®, Life Technologies, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum, at 25 °C every 48 h.

PCR and cloning

Total DNA from *L. major* promastigotes was isolated (Medina-Acosta and Cross 1993). *Leishmania major CGL* (*LmjF35.3230,* Lmj_*CGL*) was amplified by PCR using genomic DNA as template, *Pfu*-Turbo DNA-polymerase (Agilent, Genetimes Technology Hong Kong Gmall, Shatin, Hong Kong) and specific primers designed on the basis of the predicted ORF in the genome projects database (http://www.genedb.org): *Lmj_cgl-fw-Hind*III: 5′-AAGCTTATGTCCTCGCAGCAGC-3′ and *Lmj_cgl-rev-Xho*I: 5′-CTCGAGCTAGACGAGGGCATCCATG-3′. The PCR settings were as follows: 5 min at 95 °C and 25 cycles under the following conditions: (i) denaturation at 95 °C for 45 s, (ii) annealing at 58 °C during 45 s, (iii) extension at 72 °C for 1 min, in addition to a final extension step performed for 10 min. DNA fragments were fully sequenced to

confirm the predicted sequence. For functional complementation in *S. cerevisiae*, Lmj_*CGL* was cloned in a pYES2 shuttle vector (Invitrogen®, Carlsbad, CA, USA) by using the *Hind*III and *Xho*I restriction sites. A pYES2-Lmj_*CGL* was generated; this plasmid contained a selectable marker (*URA3*) and a *GAL1* promoter for the inducible expression of the inserted gene (Invitrogen®).

Yeast strain manipulation

A double-mutant yeast derived from the BY4743 strain (YAL012w, Δ*CYS3*) was used. In this yeast variant, both the CGL alleles are replaced by a kanamycin cassette (YAL012w::kanmx4, EUROSCARF collection, Germany). Moreover, in the BY4743 strain, the *MET25* gene is disrupted; therefore, these yeast cells depend on an organic sulfur source for cell growth (Fig. 2). Additionally, the *S. cerevisiae* parental S288c strain was used as the wild-type (WT) (for clarity, see legend in Fig. 2). The ΔCYS3 yeast cells were transformed with an empty pYES2 (control) and pYES2-Lmj_*CGL* construct, using the polyethylene glycol–lithium acetate method (Burke et al. 2000; Gietz et al. 1992). Following transformation, yeast cells were grown in rich solid medium (YPD-2X) containing 2% agar-base (w/v), 2% yeast extract (w/v), 4% peptone (w/v), 4% D-glucose (v/v), and 300 μg/ml of geneticin (G418). To select yeast transformants and confirm the auxotrophic phenotypes, cells were plated in synthetic defined medium (SD) containing 1.5% agar-base (w/v), 0.5% urea (v/v), 2% galactose (v/v), and 0.17% yeast nitrogen base deprived from amino acids and ammonium sulfate (w/v), but supplemented with complete minimal dropout medium without methionine and uracil nucleotide base (hereafter referred as the SD-Gal$^+$/Ura$^-$/Met$^-$ medium). The ΔCYS3 mutants harboring pYES2-Lmj_*CGL* were phenotypically recognized by the capability of the transformed yeast cells to grow on SD-Gal$^+$/Ura$^-$/Met$^-$ medium supplemented with cystathionine (40 μg/ml) as the sole sulfur source. By contrast, ΔCYS3-pYES2 transformants were grown on SD-Gal$^+$/Ura$^-$/Met$^-$ medium containing 40 μg/ml of cysteine as the sole sulfur source. Plates were incubated at 30 °C, and colonies appeared after 3–5 d. Positive clones were screened by standard colony-PCR using Lmj-*CGL* specific primers. The ΔCys3, ΔCys3-pYES2 and ΔCys3-Lmj_CGL clones were maintained in the presence of 300 μg/ml of G418 and 15 μg/ml of kanamycin. Furthermore, the expression of *L. major* CGL in ΔCys3-Lmj_CGL clones was confirmed by Western blots, and the transformed and WT yeast were grown in SD-Gal$^+$/Ura$^-$/Met$^-$ medium supplemented with the corresponding sulfur source (as described above) until the mid-log phase was reached (OD_{600} = 2). Yeast cells were harvested, resuspended in 50 mM Tris–HCl buffer, pH 8.0, containing protease inhibitors, and mechanically disrupted using standard acid-washed glass beads (Sigma-Aldrich®, St. Luis, MO). Equal amounts of protein extracts were resolved on a 10% polyacrylamide SDS-gel and electrotransferred onto nitrocellulose membranes. The latter were probed with a polyclonal mouse antiserum raised against the recombinant *T. cruzi* CGL (Marciano et al. 2012) and a secondary antibody coupled to peroxidase (Sigma-Aldrich®). ECL Plus Western Blotting Detection Kit was used, and membranes were exposed to ECL Hyperfilm (GE Healthcare Bio-Sciences, Pittsburgh, PA).

Enzyme assays

Cystathionine γ-lyase activity was measured in 100 mM Tris–HCl buffer, pH 8.0, supplemented with 150 mM NaCl and containing 5 mM cystathionine (Sigma-Aldrich®) (Marciano et al. 2012). CS activity was determined in 150 mM triethanolamine buffer, pH 7.4, in the presence of 10 mM O-acetyl-serine and 12.5 mM Na_2S. Both reactions

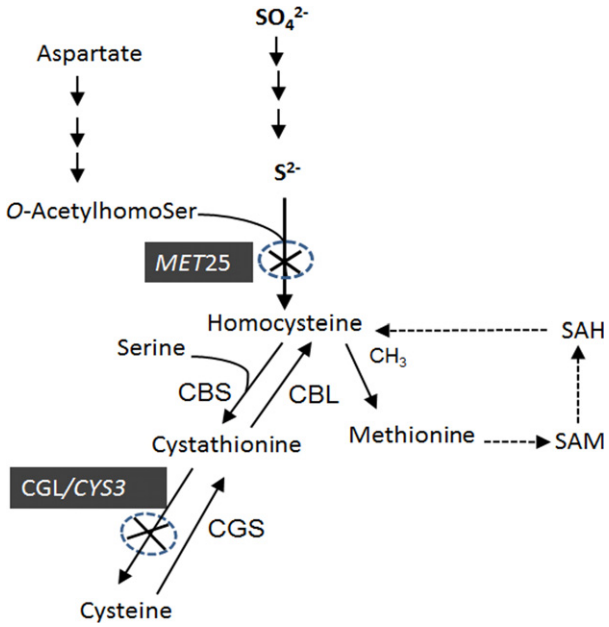

Figure 2 Schematic representation of the sulfur-containing amino acid metabolism in yeast. *Saccharomyces cerevisiae* reduces sulfate into sulfide by means of the reductive sulfate assimilation pathway. Sulfide is incorporated into a four-carbon chain, O-acetylhomoserine, by O-acetylhomoserine sulfhydrylase (encoded by *MET25*), leading to L-homocysteine formation. *Saccharomyces cerevisiae* exhibits two active transsulfuration pathways; in the reverse route, homocysteine is converted into cysteine by CBS and CGL, the latter is encoded by *CYS3*. By contrast, in the forward direction, cysteine is converted into L-homocysteine, which is subsequently methylated by homocysteine methyltransferase to form methionine. Moreover, sulfur-containing intermediates such as S-adenosyl-homocysteine (SAH) could be reutilized in methionine regeneration (methionine cycle) (Revised in Thomas and Surdin-Kerjan 1997). The yeast strains and mutants used to assess the functionality of the putative *L. major* CGL were S288c (MATα; gal2; wild-type), BY4743 (MATa/α; met25Δ0/MET25; ura3Δ0/ura3Δ0; cysteine or methionine auxotroph) and YAL012w (MATa/α; met25/MET25Δ0, ura3Δ0/ura3Δ0; YAL012w::kanMX4/YAL012w::kanMX4; deletion of *CYS3* in BY4743, cysteine and methionine auxotroph). The BY4743 strain is only able to grow on an organic sulfur source due to the disruption of the *MET*25 gene. YAL012w is a double mutant in which the *MET25* and *CYS3* genes have been disrupted.

were started by adding promastigotes cell-free extract as enzyme source. After incubation for 5 or 10 min at 37 ºC, trichloroacetic acid was added to a final concentration of 6%. Samples were centrifuged at 12,000 *g* and cysteine was determined as previously described (Gaitonde 1967). A calibration curve was constructed with cysteine. Enzyme units are expressed as μmoles/min/mg of proteins.

Effect of DL-propargylglycine on the growth of the *S. cerevisiae* transformed mutant and *L. major* promastigotes

The effect of PAG on the proliferation of WT yeast and the Δcys3-Lmj_CGL clone was evaluated by growing the yeast cells in SD-Gal$^+$/Ura$^-$/Met$^-$ medium supplemented with the corresponding organic sulfur source (as described above), until the initial log phase was reached (OD_{600nn} = 0.6). Subsequently, all the cultures were diluted 1:10 in the same medium and grown for further 8 h at 28 °C and 15 g. Next, the yeast cell cultures were divided into 25 ml aliquots and grown in the presence of PAG (0, 5, 10, and 50 μM). The effect of PAG on the proliferation of WT cells and the ΔCys3-pYES2 and ΔCys3-Lmj_*CGL* clones was monitored by measuring the OD_{600nn} during the next 16 h.

To test whether PAG might exhibit any effect on the proliferation of promastigotes, mid-log phase *L. major* parasites (1×10^6 cells/ml) were collected, washed with phosphate-buffered saline (PBS), and resuspended in medium 199 containing PAG (0–300 μM). In addition, promastigotes were grown in the absence of PAG but in the presence of 20 μM rotenone plus 0.5 μM antimycin-A, as a positive control for complete growth inhibition. Cell growth was monitored by measuring the OD_{600nn}, every day for 6 d, in 200 μl aliquots of cell suspensions grown in 96-well plates. The concentration of PAG that inhibited 50% of parasite growth (IC_{50}) was determined at the exponential phase of growth. As previously described, the values of growth inhibition were plotted as a function of PAG concentration by using the classical sigmoidal equation. The obtained curves represent the mean values of three different experiments (n = 4). Moreover, to examine the potential effect of PAG on promastigote viability, cells were grown in medium 199 without PAG or with two different concentrations of PAG (6.5 and 65 μM). Following a 24 h incubation, 5 mM L-serine or L-cysteine was added to the cultures grown in the presence of 65 μM PAG, and promastigotes were grown for additional 48 h. Cells were then collected and washed with PBS, and a MTT assay was conducted as previously described (Magdaleno et al. 2009). The percentage of viability was determined using the values obtained from those cultures that were grown in medium 199 without PAG (100% viability) and those assayed in the presence of the rotenone-antimycin-A cocktail (100% growth inhibition).

Subcellular distribution of CGL and CS

To assess the co-expression of CGL and CS in *L. major* promastigotes, parasites were grown in medium 199 for 5 d; 2×10^7 cells were withdrawn daily, washed twice with cold PBS and resuspended in lysis buffer (50 mM Tris–HCl buffer at pH 7.4, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton v/v, 20% glycerol v/v, 1 mM PMSF and protease inhibitor cocktail from Sigma-Aldrich® diluted 1:100). Equal amounts of total proteins (30 μg) were subjected to 12% SDS-PAGE and electroblotted onto nitrocellulose membranes. CS and CGL were immunodetected by mouse polyclonal serum raised against *L. major* CS that was produced in *E. coli* (our unpubl. data) and *T. cruzi* CGL, respectively. In parallel, GAPDH was used as the total protein loading control and was immunodetected using specific mouse antiserum raised against *T. cruzi* GAPDH.

The subcellular localization of CGL and CS was examined by two complementary approaches, indirect immunofluorescence microscopy and promastigotes permeabilization with digitonin. For immunomicroscopy analysis, *L. major* promastigotes were fixed, permeabilized, and layered on poly-lysine-coated glass slides (Field et al. 2004). Glass coverslips were treated with polyclonal antisera raised against *T. cruzi* CGL (1:50) and *L. major* CS (1:20), which were diluted in PBS supplemented with 20% FCS (v/v). Rabbit antiserum raised against the *L. major* cytosolic malate dehydrogenase (MDH) isozyme (1:20) was used as the cytosolic marker in the co-localization assays (Leroux et al. 2006). After 1 h incubation at room temperature, slides were washed three times with PBS. CGL and CS were immunodetected with secondary antibodies raised against mouse anti-IgG (H + L) conjugated to Alexa Fluor-488 (Invitrogen®) (1:400) and against rabbit anti-IgG conjugated (H + L) to Rhodamine™ Red-X (Invitrogen®) (1:600). DNA was stained by incubation (2 min) with Hoechst 33258 probe (1:2,000) (Invitrogen®). The coverslips were subsequently mounted by addition of Fluoromount-G (SouthernBiotech, Birmingham, AL), and the cells were visualized in an Axio Imager motorized M.2 (Carl Zeiss Microimaging GmbH, Göttingen, Germany) fluorescence microscope. Merged images were obtained using the ImageJ v1.45s software (NIH, Bethesda, MD, USA) for iOS-64-bits.

For the permeabilization with digitonin, *L. mexicana* promastigotes (40 mg wet weight, corresponding to 6.4×10^8 cells) were resuspended in TSEB buffer: 25 mM HCl–Tris buffer, pH 7.6, 0.25 M sucrose, 1 mM EDTA, 10 μM E-64 supplemented with digitonin (0–5 mg), in a final volume of 1 ml. Cells were permeabilized with increasing concentrations of detergent, the soluble (S) and pellet fractions (P) at each concentration were obtained and activities of pyruvate kinase (PK), alanine aminotransferase (ALAT), and hexokinase were measured and used as cytosolic, glycosomal and mitochondrial markers, respectively (Marciano et al. 2009). Due to the low CGL activity in the soluble and pellet fractions, the subcellular localization of this enzyme was assessed by Western blot analysis. Selected soluble and insoluble fractions obtained at different digitonin concentrations were subjected to Western blot analysis and probed with specific antisera raised against CS, CGL, and the mitochondrial malate dehydrogenase (mMDH).

RESULTS

Functional characterization of *L. major* cystathionine γ-lyase

The survey of the sequenced genomes of the *Leishmania* genus (*L. major*, *L. infantum*, *L. braziliensis*, *L. donovani*, etc.) provided evidence for the presence of syntenic and highly identical sequences (> 95%) that were predicted to encode putative CGLs (http://www.genedb.org, Fig. 1). CGLs are PLP-dependent enzymes that are comprised within the subgroup of the Cys-Met metabolism related proteins. The members of this protein subfamily exhibit a high overall structural similarity, but relatively low sequence relatedness and different catalytic capabilities (Messerschmidt et al. 2003). Therefore, the substrate specificities and functionality of these enzymes need to be experimentally examined. As a preliminary approach, we tested the functionality of a putative CGL by measuring the enzyme activity in cell-free extracts of *L. major* promastigotes. CGL specific activity rendered values of 0.2 ± 0.006 mU/mg of total soluble proteins. Hence, to further confirm that the putative leishmanial CGLs exhibited the predicted activity, we examined the capability of the protein encoded by the *L. major* gene (*LmjF35.3230*) to rescue the lethal phenotype of the *S. cerevisiae* CGL-null mutant. The selected yeast strain was auxotrophic for cysteine as a result of the deletion of the two alleles of the endogenous *CGL* gene (*CYS3* or *STR1*), in addition to the *MET25* gene (Fig. 2). Therefore, SD-Gal$^+$/Ura$^-$/Met$^-$ solid medium supplemented with cystathionine was utilized for the phenotypic selection. Positive ΔCYS3-Lmj_CGL clones were identified by colony-PCRs, which rendered DNA bands with the expected molecular sizes for Lmj_*CGL* (not shown). The capability of the ΔCYS3-Lmj_CGL mutant to decompose cystathionine into cyste-

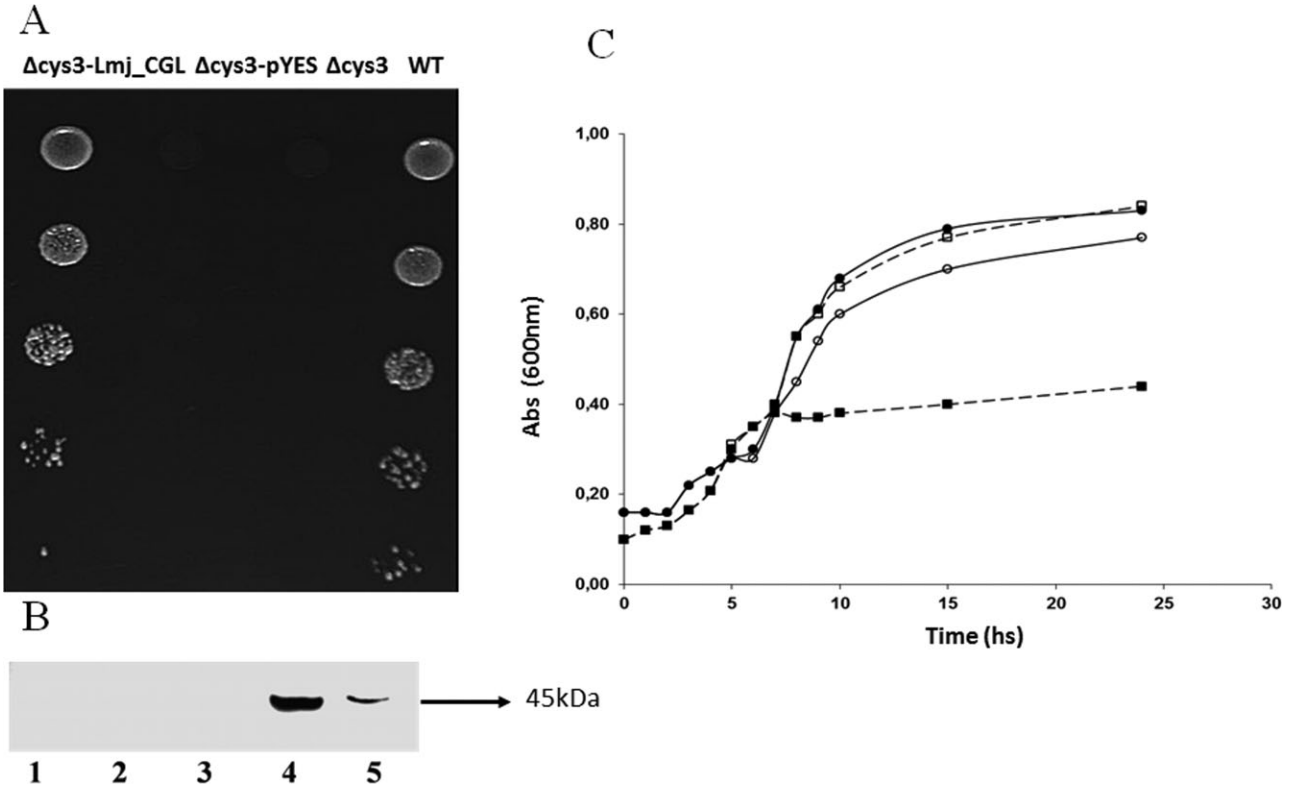

Figure 3 Functional complementation of a *Saccharomyces cerevisiae* null mutant (ΔCYS3) by *Leishmania major CGL*. **A**. ΔCYS3-Lmj_CGL and ΔCYS3_pYES2 clones in addition to the ΔCYS3 yeast mutant and the WT yeast were grown in liquid SD-Gal$^+$/Ura$^-$/Met$^-$ medium, supplemented with cystathionine or cysteine as described in the Materials and Methods. Upon overnight incubation at 30 °C, cultures were serially diluted 1:10 from OD_{600} = 1; subsequently, 5 μl of each dilution was spotted on SD-Gal$^+$/Ura$^-$/Met$^-$ agar-plates containing cystathionine as the sole sulfur source and incubated at 30 °C for 3–4 d. **B.** Identical numbers of cells corresponding to each of the yeast variants were lysed and subjected to SDS-PAGE and Western blot. As described in the Materials and Methods, polyclonal antiserum raised against *Trypanosoma cruzi* CGL was used. Lane 1, wild-type yeast strain; lane 2, ΔCYS3 mutant; lane 3, ΔCYS3_pYES2 clone; lane 4, ΔCYS3-Lmj_CGL clone and lane 5, 30 ng of the recombinant *T. cruzi* CGL (positive control). **C.** A wild-type yeast strain and the ΔCYS3-Lmj_CGL clone were grown in liquid SD-Gal$^+$/Ura$^-$/Met$^-$ medium, supplemented with cystathionine or cysteine as described in the Materials and Methods in the absence and presence of PAG. Wild-type cells without PAG (o) and in the presence of 5 μM PAG (●); ΔCYS3-Lmj_CGL without PAG (□) and in the presence of 5 μM PAG (■). The arrow indicates PAG addition.

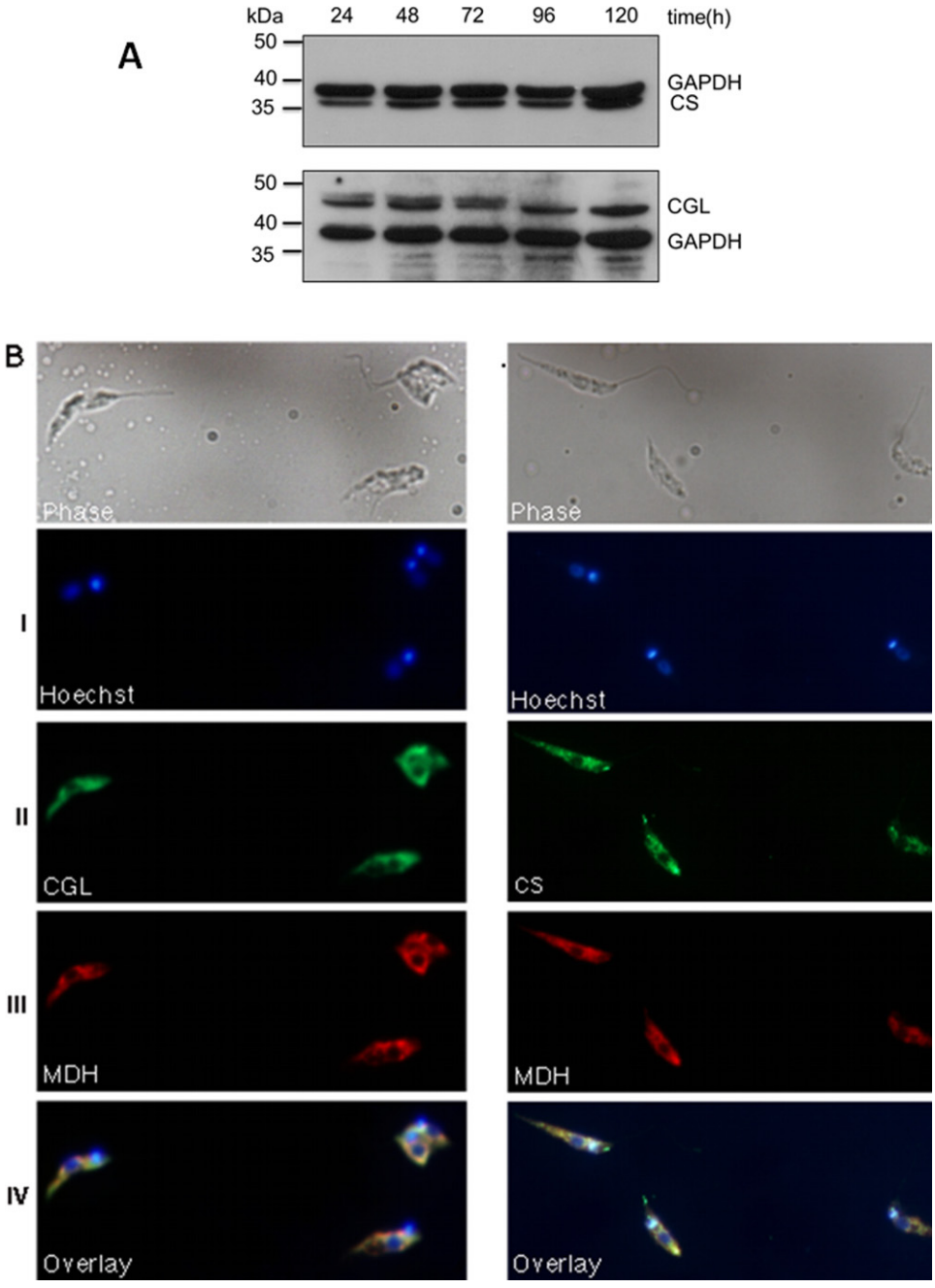

Figure 4 Western blot analyses and subcellular localization of CGL and CS in *Leishmania* parasites. **A.** The relative abundances of CS and CGL were evaluated in promastigotes grown along 5 d in medium 199. Cells were withdrawn daily and resuspended in lysis buffer; equal amounts of total proteins (30 μg) were subjected to 12% SDS-PAGE and electroblotted onto nitrocellulose membranes as indicated in the Materials and Methods. CS (35 kDa) and CGL (45 kDa) were immunodetected by mouse polyclonal sera raised against *Leishmania major* CS and *Trypanosoma cruzi* CGL, respectively. GAPDH (39 kDa) was used as a protein mass loading control and detected by a specific mouse polyclonal antiserum raised against *T. cruzi* GAPDH. **B.** Images were obtained by indirect immunofluorescence microscopy of *L. major* promastigotes, which were manipulated and stained as indicated in the Materials and Methods. Upper panel, phase contrast; panel I, nuclear and kinetoplastic DNA evidenced by the blue fluorescence resulting from Hoechst 33258 staining; panel II, green fluorescence corresponding to the primary antibodies specifically raised against the *T. cruzi* CGL and *L. major* CS, respectively; panel III, the red fluorescence outcomes from the primary antibodies specifically raised against the cytosolic *L. major* MDH; panel IV, merge of the images obtained in panels II and III.

ine was tested on SD plates supplemented with 40 μg/ml of cystathionine. In parallel, the WT strain, the Δ*CYS3*_pYES2 clone and the Δ*CYS3*-null mutant were grown in equally supplemented SD plates. As expected for a functional CGL, in the presence of cystathionine as the sole sulfur source, the ΔCYS3-Lmj_CGL clone alone grew similarly to the WT yeast cells (Fig. 3A). The expression of Lmj_CGL in yeast cells was further confirmed by Western blot analyses of cell-free extracts corresponding to ΔCYS3-Lmj_CGL grown in SD-Gal$^+$/Ura$^-$/Met$^-$ liquid medium supplemented with cystathionine. A band with the expected apparent molecular mass was only immunodetected in ΔCYS3-Lmj_CGL cell-free extract; however, no signals were immunodetected in the negative control clones (ΔCYS3_pYES2 and ΔCYS3 null mutant) or in the WT strain (Fig. 3B). Subsequently, when the effect of PAG on *L. major* CGL was examined at different concentrations of this inhibitor, the growth of the ΔCYS3-Lmj_CGL clone was clearly arrested at the lowest concentration of PAG assayed (5 μM), whereas the proliferation of the WT strain was not affected (Fig. 3C).

CS and CGL coexpression and subcellular distribution in *Leishmania* promastigotes

When CS activity was measured in cell-free extracts of promastigotes from *Leishmania* species such as *L. mexicana* and *L. major*, values of approximately 490 ± 50 nmol/min/mg were determined in both parasites. However, because of CS and CBS partially overlapped substrate specificities (CBS also condensates O-acetylserine with sulfide, Fig. 1), the expression of CS needed to be experimentally confirmed. Hence, Western blot analyses of cell-free extracts from promastigotes of *L. major* grown for 5 d were performed; these assays provided clear evidence that this pathogen expresses CGL in parallel with CS (Fig. 4A). Given that the coexistence of enzymes related to de novo synthesis and the RTP is not commonly observed in most living organisms, we addressed the issue of whether CS and CGL might be associated with cysteine production in different subcellular compartments. To answer this question, indirect immunofluorescence microscopy analysis and digitonin extraction assays were performed in *L. major* and *L. mexicana* promastigotes, respectively. For the immunofluorescence microscopy analysis, the nuclear and kinetoplastic DNA of *L. major* promastigotes was stained in blue (Fig. 4B, line I). When the antisera raised against CS and CGL were individually tested, a diffuse but intense green staining was observed. The resulting pattern provided evidence for a cytosolic localization of both CS and CGL (Fig. 4B, line II). To confirm this subcellular distribution, the cytosolic MDH was immunodetected with specific rabbit antibodies (Fig. 4B, line III), and an identical fluorescence pattern (red signal) was observed. Finally, when the images obtained with anti-CS and anti-CGL serum were overlapped with those obtained with the anti-MDH serum, a yellow signal was visualized due to the overlaid red signal corresponding to the cytosolic MDH with the green fluorescence resultant of the immunoreactivity of CGL and CS with their specific antibodies (Fig. 4B, line IV). Additionally, when *L. mexicana* promastigotes were permeabilized with increasing concentrations of digitonin, the release of CS activity strictly paralleled the extraction pattern of the cytosolic marker, PK (Fig 5A). Because of the low CGL activity in the soluble and pellet fractions, the subcellular distribution of this enzyme was assessed by Western blot analysis. To achieve this aim, selected soluble and insoluble fractions at different digitonin concentrations were probed with specific antisera raised against each of these enzymes. As a mitochondrial marker, the organelle-specific malate dehydrogenase (mMDH) isozyme was used (Fig. 5B). The activity extraction pattern of CS in addition to the immunodetection patterns of CS and CGL in the soluble and insoluble fractions clearly revealed that CGL and CS co-localized in the cytosol of *Leishmania* promastigotes.

Exploring the potential biological role of CGL in *L. major* promastigotes

To gain an insight into the possible biological relevance of CGL in *Leishmania* promastigotes, we first tested the sensitivity of the enzyme to PAG inhibition in cell-free extracts of *L. major* promastigotes. Figure 6A shows that when PAG concentrations reached values of approximately 100 μM, the specific activity determined in the cell-free extracts decreased nearly 50%. Next, to examine the effect of this inhibitor on the proliferation of *L. major* promastigotes, this protist (1×10^6 cells/ml) was cultivated in medium 199 supplemented with PAG (0–300 μM). In parallel, promastigotes were also grown in the absence of PAG, but in the presence of 60 μM rotenone plus 0.5 μM antimycin-A. Figure 6B shows that PAG exhibited a dose-dependent inhibition of the growth of *L. major* promastigotes, which was completely arrested in the presence of 300 μM PAG. The percentage of growth inhibition was determined at 72 h, and an IC_{50} value of 65.5 ± 2 μM was calculated (Fig. 6B, inset). Analysis of the potential biological effect of PAG by the MTT assay provided evidence that this inhibitor interfered with the cell viability of the protist. (Fig. 6C). To identify the potential target(s) of PAG in vivo and considering the pathway redundancy for cysteine production, L-Ser (a precursor for cysteine production) and L-Cys (the end product of the RTP) were independently added at a final concentration of 5 mM to the culture medium containing 65 μM PAG. None of the assayed amino acids could reverse the growth inhibition by PAG (Fig. 6C), especially the inability of re-establishing growth upon addition of cysteine fits in well with the extremely low uptake of cysteine from extracellular environments by *Leishmania* parasites (Williams et al. 2009) and with the well-known cytotoxicity of this amino acid. In addition, to assess the possibility that combined targets might have a synergistic effect on parasite proliferation, the inhibitory effect of PAG on the activity of other leishmanial PLP-dependent enzymes previously characterized in our laboratory, such as aspartate and ALAT as well as cysteine desulfhydrase, was investigated; however, no change in the activity of any of these recombinant enzymes

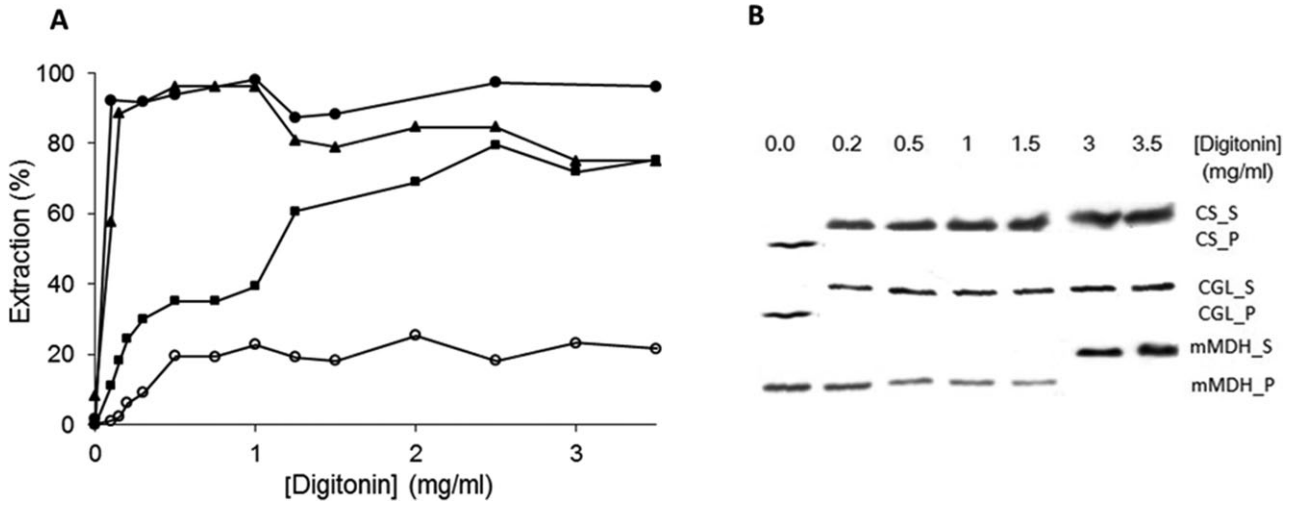

Figure 5 Validation of the subcellular localization of *Leishmania mexicana* cysteine synthase and cystathionine γ lyase by means of digitonin extractions. **A.** Intact promastigotes were treated with increasing concentrations of digitonin, pyruvate kinase (▲ = cytosolic marker), hexokinase (○, glycosomal marker), alanine aminotransferase (■ cytosolic and mitochondrial marker) and cysteine synthase (●) were measured. **B.** Selected soluble and insoluble fractions at different digitonin concentrations were subjected to Western blot analysis and probed with specific antisera raised against each of the enzymes. The soluble and insoluble fractions are indicated by (S) and (P), respectively. Line CS_S, anti *Leishmania major* CS; line CS_P, anti *L. major* CS; line CGL_S, anti *Trypanosoma cruzi* CGL, line CGL_P, anti *T. cruzi* CGL; line mMDH_S, anti *L. major* mMDH; line mMDH_P, anti *L. major* mMDH.

was observed in the presence of PAG (not shown). Based on these data, it is tempting to argue that CGL might play an essential role in the survival of the *Leishmania* parasite; it is also worth noting that further studies are required for understanding the biological role of CGL and the in vivo molecular mechanism of growth inhibition by PAG.

DISCUSSION

In most living organisms, the coexistence of enzymes that regulate the de novo synthesis of cysteine with those belonging to the RTP is remarkably infrequent. Interestingly, the RTP is absent in the majority of pathogenic protists. The results presented in our current study show that *Leishmania* parasites exhibit a functional CGL, an enzyme comprised within the RTP. The putative *L. major* CGL was indeed able to functionally complement the phenotype of the *S. cerevisiae* Δ*CGL*-null mutant. Moreover, our findings provided evidence that in the presence of PAG, a canonical, even though not mono-specific, irreversible inhibitor of CGLs, the growth of the ΔCYS3-Lmj_CGL yeast mutant was clearly arrested when compared with that of the WT strain. These findings fit in well with our previous observations, which indicated that the activity of the recombinant *T. cruzi* CGL was inhibited at a significantly lower concentration of PAG than that reported to inhibit eukaryotic counterparts such as the human enzyme (Marciano et al. 2012; Steegborn et al. 1999). Also, relatively low concentrations of PAG (IC_{50} of approximately 65 μM) arrested the proliferation of *L. major* promastigotes, and similarly, analogous concentrations of PAG also inhibited the proliferation of *L. amazonensis* promastigotes as well as diminished to the same extent the CGL activity in the cell-free extracts of these parasites (not shown). Jointly, these observations suggest an essential, although unknown, biological role for CGL in *Leishmania* parasites. These pathogens appear to display an unexpectedly high redundancy in cysteine production, presumably due to (i) their inability to uptake cysteine from the extracellular environments (Williams et al. 2009), (ii) the need to convert the highly toxic L-homocysteine, resulting from methionine metabolism into cystathionine, a valuable precursor of cysteine generation, (iii) the necessity of cysteine for the synthesis of iron–sulfur clusters comprised in numerous enzymes as well as to maintain the continuous production of trypanothione, an abundant low-molecular-mass dithiol, which is essential for the intracellular redox balance (iv) a compensatory mechanism to fulfill the requirement of cysteine in those environments where methionine is of low abundance, or precursors for the *de novo* synthesis of cysteine (for instance acetyl-CoA or serine) are scarce.

Notably, cell-free extracts of *L. mexicana* promastigotes exhibit a relatively high SAT activity (Marciano et al. 2010); also CS is enzymatically active (present study) and particularly proteomic analysis of *Leishmania panamensis* has shown a remarkable higher abundance of this enzyme in amastigotes than in the insect stage (Walker et al. 2006). However, in contrast to plants and bacteria, *Leishmania* parasites lack the genes coding for the enzymes required for sulfate assimilation and sulfide production (http://www.genedb.org). Thus, the source of the sulfide needed to accomplish the biosynthetic pathway (cysteine production by means of CS and CBS) is still enigmatic. Our studies showed that CGL and CS are expressed in the cytosol of

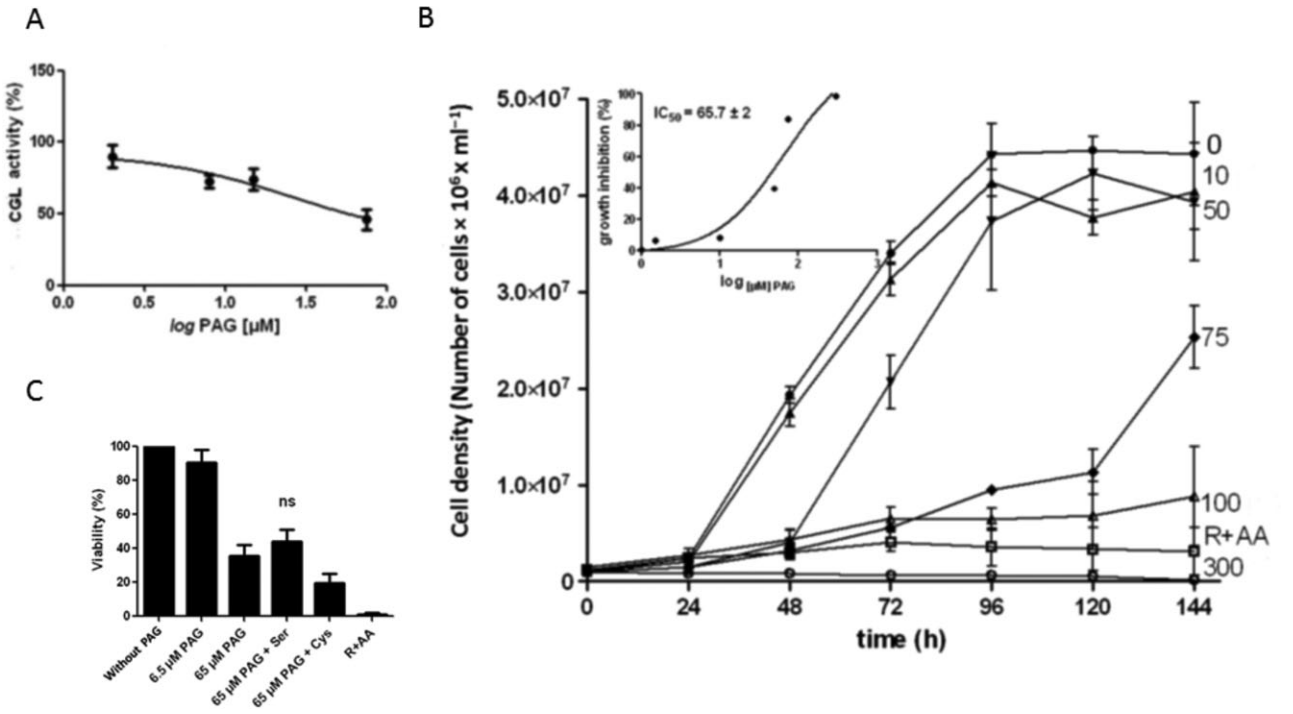

Figure 6 Effect of DL-propargylglycine (PAG) on CGL activity and *Leishmania major* promastigote proliferation. **A**. CGL activity in cell-free extracts of promastigotes was assayed in the presence of PAG; final concentrations in the μM range (0, 2, 8, 15 and 75) were used. The percentage of residual CGL activity was plotted vs log (μM) of PAG. **B.** The effect of PAG on *L. major* promastigote proliferation was tested by growing this protozoon in medium 199 supplemented with varied concentrations of PAG (0–300 μM). For complete cell growth inhibition (non-proliferation control), 200 μM rotenone and 0.5 μM antimycin-A were added to medium 199. The values on the right side of the panel indicate the μM concentration of PAG utilized in each condition of growth, whereas the proliferation pattern obtained in the presence of rotenone plus antimycin-A is labeled by (R + AA). Curves represent the mean values of three independent experiments ($n = 4$). **C.** The effect of PAG on promastigote viability was assayed by incubating this protozoon in medium 199 without PAG as well as in the presence of two different concentrations of PAG (6.5 and 65 μM), and with 65 μM PAG plus 5 mM of L-serine or L-cysteine. The percentage of viability was determined by the MTT assay, as indicated in the Materials and Methods. ns = non-significant.

promastigotes from two *Leishmania* species, which indicates that the de novo synthesis of cysteine and the RTP (CBS also localizes in the cytosol, our unpubl. results) will take place in the same subcellular compartment.

In most living cells, cysteine is the accepted primary target of ROS (Mustafa et al. 2009; Pastore and Piemonte 2012); also, it is likely that leishmanial CGL might play unrelated roles to metabolic processes. For instance, *T. cruzi* CGL has been demonstrated to establish interactions with proteins belonging to the complex system of enzymes involved in maintaining the cellular redox status, including tryparedoxin 1 (Piñeyro et al. 2011). Moreover, proteomic analyses of *L. mexicana* intracellular amastigotes provide evidence that CGL and CS, in addition to 67 other proteins, might be potentially secreted into the phagosome lumen despite the absence of a secretion signal (Paape et al. 2010).

In addition, it is worth noting that *Leishmania* spp. are methionine auxotrophs and that L-homocysteine is included among the by-products of this amino acid catabolism. The fate of this highly toxic intermediate has not been addressed in detail yet; however, L-homocysteine can be consumed by the promiscuous CBS, which is able to catalyze the production of cystathionine (Williams et al. 2009). On the other hand, in *Leishmania* parasites, but not in trypanosomes, L-homocysteine might be re-methylated by means of a putative homocysteine methyltransferase to regenerate methionine (Vickers et al. 2006). To date, there are few data regarding the in vivo fate of L-homocysteine and its potential contribution to cysteine production. However, the inhibitory effect of PAG on promastigote proliferation poses a challenge to understanding the metabolism of sulfur-containing derivatives in these pathogenic protists, as well as shows the high need of metabolomic studies related to sulfur-containing amino acids.

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