

# pLG72 Modulates Intracellular D-Serine Levels through Its Interaction with D-Amino Acid Oxidase

## EFFECT ON SCHIZOPHRENIA SUSCEPTIBILITY\*<sup>§</sup>

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Human genes coding for pLG72 and D-amino acid oxidase have recently been linked to the onset of schizophrenia. pLG72 was proposed as an activator of the human FAD-containing flavoprotein D-amino acid oxidase (hDAAO). In the brain this oxidizes D-serine, a potent activator of N-methyl-D-aspartate receptor. We have investigated the mechanistic regulation of hDAAO by pLG72. Immunohistochemical analyses revealed that hDAAO and pLG72 are both expressed in astrocytes of the human cortex, where they most likely interact, considering their partial overlapping subcellular distribution and their coimmunoprecipitation. We demonstrated that the specific *in vitro* interaction of the two proteins yields a complex composed of 2 hDAAO homodimers and 2 pLG72 molecules. Binding of pLG72 did not affect the kinetic properties and FAD binding ability of hDAAO; instead, a time-dependent loss of hDAAO activity in the presence of an excess of pLG72 was found. The binding affects the tertiary structure of hDAAO, altering the amount of the active form. We finally demonstrated that overexpression of hDAAO in glioblastoma cells decreases the levels of D-serine, an effect that is null when pLG72 is coexpressed. These data indicate that pLG72 acts as a negative effector of hDAAO. Therefore, a decrease in the synaptic concentration of D-serine as the result of an anomalous increase in hDAAO activity related to hypoexpression of pLG72 may represent a molecular mechanism by which hDAAO and pLG72 are involved in schizophrenia susceptibility.

Schizophrenia is one of the most widely spread psychiatric disorders; it is a complex disease or, more likely, a group of related illnesses to which an individual has a strong genetic

predisposition (1). Among the identified schizophrenia susceptibility genes (2), the gene *G72* encodes for several splicing isoforms; pLG72 represents the longest open reading frame (153 amino acids), which is mainly expressed in brain (3). *G72* is present only in primates: there are no homologues of this gene in databases nor has sequence analysis of the putative open reading frame revealed any likely function (2, 3). Yeast two-hybrid experiments using pLG72 as bait identified D-amino acid oxidase (EC 1.4.3.3, DAAO<sup>4</sup>) on 12q24 as a putative interacting partner, and preliminary functional measurements showed that pLG72 should function as an *in vitro* activator of pig kidney DAAO (pkDAAO) (3). DAAO is a FAD-containing flavoenzyme that catalyzes the oxidative deamination of D-amino acids to the corresponding  $\alpha$ -keto acids, hydrogen peroxide and ammonia (4, 5).

Based on current findings we can hypothesize that in brain, the physiological role of DAAO is to modulate the levels of D-serine, an important glial-derived messenger that acts as the endogenous allosteric modulator of the glutamatergic NMDA receptor subtype (6–8). D- and L-serine can be reversibly isomerized in astrocytic glia, which unsheathes synapses, by serine racemase. Compelling evidence has indicated that glutamate neurotransmission hypofunction is associated with symptoms of schizophrenia. D-Serine levels are inversely related to the regional expression of DAAO during development (6) and application of DAAO to brain slices or cell cultures has a considerable effect on NMDA receptor-dependent synaptic transmission and long term plasticity (9–12) or protects neurons from the NMDA receptor-related excitotoxicity (13–15). Adult DAAO-deficient mice display an increase in D-serine levels, especially in areas where levels are normally low (16). The association between alterations in D-serine metabolism and schizophrenia is further supported by studies demonstrating decreased D-serine levels in the cerebrospinal fluid and serum of schizophrenia patients. It is noteworthy that clinical trials have demonstrated a benefit of adding D-serine to the antipsy-

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<sup>4</sup> The abbreviations used are: DAAO, D-amino acid oxidase; hDAAO, human DAAO; pkDAAO, pig kidney DAAO; RgDAAO, *Rhodotorula gracilis* DAAO; NLS, N-lauroylsarcosine; CPZ, chlorpromazine; NGS, normal goat serum; GFAP, glial fibrillary acidic protein; GS, glutamine synthase; NMDA, N-methyl-D-aspartate; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescence protein.

chotic regimen for treating schizophrenia and bipolar disorders (17).

The physiopathologic hypothesis proposed by Chumakov *et al.* (3) suggests that increasing the endogenous level of pLG72 could lower NMDA-type glutamate receptor activity through DAAO activation and subsequent D-serine depletion, resulting in hypofunction of glutamatergic synapses and, thus, predisposing these individuals to schizophrenia (see "Discussion"). To provide biochemical evidence supporting the involvement of the identified genes in this pathologic process, we recently reported on the overexpression of pLG72 and human DAAO (hDAAO) in *Escherichia coli* and biochemically characterized hDAAO (18, 19). In the present study we have investigated the interaction of pLG72 with hDAAO *in vitro* and the effect of pLG72 binding on the functional and structural properties of the flavoenzyme as well as their effect on the cellular concentration of D-serine. We also investigated the cellular and subcellular distribution of pLG72 and hDAAO by immunofluorescence in human cortex and in pure human astrocyte cultures.

## EXPERIMENTAL PROCEDURES

**hDAAO and pLG72 Purification**—The recombinant hDAAO was expressed in *E. coli* cells and purified as reported in Molla *et al.* (19); see Fig. 1A. The final enzyme preparation was stored in 20 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 5% glycerol, 5 mM 2-mercaptoethanol, and 40  $\mu$ M FAD. The G72 gene was also expressed in *E. coli* cells; the protein was expressed in the insoluble fraction and then solubilized and purified according to the procedure reported in Molla *et al.* (18); see Fig. 1A. This final protein preparation was stored in 20 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5% glycerol, and 5 mM 2-mercaptoethanol and contained  $\sim$ 0.1% *N*-lauroylsarcosine (NLS). The absorbance spectrum of the refolded and purified pLG72 shows a maximum at 271 nm, whereas the absorbance maximum for the unfolded form is centered at  $\sim$ 267 nm (18). Near- and far-UV CD spectra were also used to demonstrate that the refolded protein acquired the secondary and tertiary structure; see below and Ref. 18. hDAAO and pLG72 protein concentrations were determined according to their extinction coefficients (18, 19). Recombinant yeast DAAO (RgDAAO) was purified from *E. coli* cells as reported in (20). The critical micelle concentration of detergent NLS (2.8 mM) was not significantly affected by the composition of the buffer used (the amount of NLS used in most experiments, 0.06%, corresponds to 2 mM concentration). pkDAAO was purchased from Fluka and used after extensive dialysis against 20 mM sodium pyrophosphate buffer, pH 8.0, 2% glycerol, and 10  $\mu$ M FAD.

**Kinetic and Spectral Analysis**—The apparent kinetic parameters of hDAAO on D-serine (in the absence or in the presence of pLG72) were determined by measuring the oxygen consumption by means of a Hansatech oxygen electrode in 20 mM Tris-HCl, pH 8.5, 150 mM NaCl, 5% glycerol, 5 mM 2-mercaptoethanol (buffer A) with or without 0.06% NLS and 40  $\mu$ M FAD at 25 °C and air saturation. One DAAO unit is defined as the amount of enzyme that converts 1  $\mu$ mol of D-amino acid per min at 25 °C (20). The amount of catalytically active hDAAO after the binding of pLG72 was evaluated on the basis of the amount of FAD cofactor that can be reduced by the substrate

under anaerobic conditions. This value was determined from the change in absorbance at 455 nm after the anaerobic addition of 1 mM D-serine to hDAAO solutions (15  $\mu$ M) containing different amounts of pLG72 and free FAD. A similar assay was used to assess the effect of different amounts of pLG72:FAD complex on the rate of flavin binding to the apoprotein form of hDAAO (8 nmol); the binding was followed by means of the substrate-induced reduction of the flavin at 455 nm under anaerobic conditions.

Circular dichroism spectra were recorded with a J-815 Jasco spectropolarimeter at 15 °C and were further analyzed with the Jasco software; samples contained 0.4 mg/ml (10  $\mu$ M) of protein in buffer A plus 0.06% NLS. The binding constants for FAD and chlorpromazine (CPZ) were determined by titrating the apoprotein form of hDAAO (1  $\mu$ M) or pLG72 (4  $\mu$ M) with increasing amounts of ligands and by monitoring the reconstitution by following the quenching of protein fluorescence at 342 nm. 8-Anilino-naphthalene-1-sulfonate binding experiments were carried out at 15 °C and at 0.1 mg/ml protein concentration by measuring emission spectra in the 450–600-nm range with excitation at 370 nm. Binding of this probe to solvent-accessible clusters of nonpolar side chains in proteins results in a marked increase in its fluorescence, and the emission fluorescence maximum shows a blue shift.

**Determination of the hDAAO·pLG72 Complex Oligomeric State**—The oligomeric state of free and complexed hDAAO was determined by gel permeation chromatography on a Superdex 200 column (GE Healthcare) using buffer A to which 40  $\mu$ M FAD and 0.06% NLS was added as the elution buffer. The detergent was required because the solubility and the oligomeric state of pLG72 strongly depends on the presence of NLS; the recovery of refolded pLG72 protein in the absence of detergent in the elution buffer was low (<20%), and the protein largely eluted with the void volume, whereas at a NLS concentration  $\geq$ 0.06% pLG72 is quantitatively recovered in a single peak with an elution volume of  $\sim$ 15 ml. Concerning hDAAO, we previously demonstrated that the holoenzyme and apoprotein (apohDAAO) forms are present as a 80-kDa homodimer (19). The addition of up to 0.06% NLS to hDAAO does not affect its elution volume, and more than 70% of the initial enzymatic activity is recovered after chromatographic separation; this amount of NLS was, thus, used in all the experiments. The area of each peak was estimated by nonlinear curve-fitting of the elution profile using PeakFit software (Systat Software) (Fig. 1B); the error was <10% as estimated using known amounts of DAAO as standard protein (5–100 nmol/run). The amount of pLG72 and hDAAO present in the peak corresponding to the complex was estimated by means of the intensity of their bands after SDS-PAGE, as obtained using the program Quantity One (Bio-Rad) and a known amount of purified hDAAO and pLG72 as standard (in the 0.2–20  $\mu$ g/lane range).

**Western Blot Experiments**—For Far Western blot experiments, 2–10  $\mu$ g of pLG72 separated by SDS-PAGE electrophoresis were blotted onto nitrocellulose membrane, which was then blocked with 1% bovine serum albumin (BSA) and incubated with a 2–20-fold molar excess of hDAAO in binding buffer (20 mM potassium phosphate buffer, pH 7.0, 150 mM NaCl, and 1% BSA) for 60 min at room temperature, with gentle

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stirring. Alternatively, purified pLG72 was adsorbed on a nitrocellulose membrane, blocked with binding buffer, and then overlaid with DAAO from different sources (dot Far Western blot). In both cases and after the unbound protein was removed, the membrane was incubated with anti-DAAO antibodies and followed by secondary antibodies conjugated to alkaline phosphatase, as detailed in Molla *et al.* (20).

For quantitative pulldown experiments, 8  $\mu\text{g}$  of commercial affinity-purified polyclonal goat antibodies raised against a peptide mapping at the N terminus end of pLG72 ((G72(N-15); Santa Cruz Biotechnology) were covalently cross-linked to 50  $\mu\text{l}$  of Dynabeads protein G (Invitrogen) and used to immunoprecipitate protein samples containing 0.1 nmol of recombinant pLG72 (1.8  $\mu\text{g}$ ) and increasing amounts of recombinant hDAAO (from 0 to 0.8 nmol, corresponding to 32  $\mu\text{g}$ ). Proteins were diluted in cell homogenization buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 100  $\mu\text{g}/\text{ml}$  phenylmethylsulfonyl fluoride, 0.7  $\mu\text{g}/\text{ml}$  pepstatin, 1  $\mu\text{g}/\text{ml}$  leupeptin, 0.06% NLS, 10  $\mu\text{M}$  FAD) and incubated for 45 min at room temperature before precipitation. The amount of hDAAO precipitated by Dynabeads-bound pLG72 was revealed by SDS-PAGE and Western blot analysis using anti-hDAAO antibodies (affinity-purified polyclonal rabbit antibodies raised against the recombinant hDAAO; Davids Biotechnologie) and quantified by densitometric analysis.

**Surface Plasmon Resonance Analysis**—For the surface plasmon resonance analysis, a BIAcore X system was used as described in Ruzzene *et al.* (21). hDAAO was covalently coupled to a CM5 sensor chip (carboxymethylated dextran surface) by amine-coupling chemistry to a final density of 700 resonance units; a flow cell with no immobilized protein was used as a control. The pLG72 solutions were injected under the conditions specified in the figure legend by using HBS-EP buffer (10 mM Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% P20 (GE Healthcare)) or 20 mM Tris-HCl buffer, pH 8.5, containing 150 mM NaCl, 0.06% NLS, and 40  $\mu\text{M}$  FAD; each sensorgram (time course of the surface plasmon resonance signal) was corrected for the response obtained in the control flow cell and normalized to base line. Experiments were also carried out using immobilized pLG72 and circulating hDAAO. After each injection the surface was regenerated by injecting 100 mM NaOH for 1 min; this treatment restored the base line to the initial resonance unit value. The kinetic data were analyzed using the BIAevaluation 3.0 surface plasmon resonance kinetic software (BIAcore).

**Human Tissues and Cell Cultures**—Frozen human cortex tissues and primary astrocyte cultures derived from human fetal thalamus were a kind gift of Vincent Prevot (INSERM, Lille, France). Frozen human cortex was kept at  $-80^\circ\text{C}$  until use. Primary astrocyte cultures and U87 human glioblastoma cells (ATCC) were grown to near confluence in flasks at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 6 g/liter glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin-streptomycin, and Fungizone.

**Immunoblot and Coimmunoprecipitation Analyses**—Tissues were homogenized in ice-cold cell homogenization buffer (see above), sonicated (twice for 10 s with 30 s incubation in ice),

clarified by centrifugation (13,000 rpm, 30 min,  $4^\circ\text{C}$ ), and stored at  $-80^\circ\text{C}$ . Protein extracts were subjected to 8–15% acrylamide SDS-PAGE analysis (200  $\mu\text{g}$  protein/lane) and electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membranes were incubated for 2 h in blocking solution containing 4% dried milk in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 8.0, 500 mM NaCl) and 0.1% Tween (TTBS) and then incubated for 1.5 h at room temperature with anti-DAAO (diluted 1/3000; Davids Biotechnologie) or anti-pLG72 antibodies (G72(N15), diluted 1/1500; Santa Cruz Biotechnology). Membranes were washed extensively in TTBS containing 2% dried milk and then incubated for 1 h at room temperature with peroxidase-conjugated donkey anti-rabbit or anti-goat immunoglobulins (1/15000; Jackson ImmunoResearch); immunoreactivity was then determined by enhanced chemiluminescence (GE Healthcare). Protein content was measured by the Lowry method using the Bradford reagent (Sigma). Controls included incubation with antibodies preabsorbed with hDAAO or pLG72.

For coimmunoprecipitation experiments, TrueBlot anti-rabbit Ig immunoprecipitation beads (a suspension of activated agarose beads coupled with goat anti-rabbit IgG; eBioscience) were used; 500  $\mu\text{l}$  of human cortex crude extract (20 mg protein/ml) were subjected to preclearing by the addition of 50  $\mu\text{l}$  of anti-rabbit IgG beads. Then 5  $\mu\text{g}$  of primary antibodies (affinity-purified rabbit anti-hDAAO or anti-pLG72 antibodies raised against the C-terminal end of the protein; Davids Biotechnologie) were added to the precleared crude extract and incubated overnight at  $4^\circ\text{C}$ ; the antigen-antibody complex was then precipitated by centrifugation at 10,000 rpm for 1 min. The beads were washed 3 times with 500  $\mu\text{l}$  of homogenization buffer and subsequently resuspended in 100  $\mu\text{l}$  of 25 mM Tris-HCl, pH 6.7, 6% SDS, 10% glycerol, and 50 mM dithiothreitol. The samples were heat-denatured and centrifuged at 10,000 rpm for 3 min; then 40  $\mu\text{l}$  of the supernatant was separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was developed as detailed in the TrueBlot protocol using goat anti-pLG72 G72(N15) antibodies and, after stripping, using anti-hDAAO antibodies (dilution as above). The membrane was blocked overnight at  $4^\circ\text{C}$  with 5% dried milk in TrueBlot buffer (25 mM Tris-HCl, pH 7.3, 0.15 M NaCl, 0.1% Tween 20) and subsequently incubated with primary antibodies diluted in 5% dried milk in TrueBlot buffer for 2 h at room temperature. After extensive washing, the membrane was incubated for 1 h at room temperature with rabbit IgG TrueBlot (1:1000 dilution in 5% dried milk in TrueBlot buffer); finally, a horseradish peroxidase conjugated anti-goat or anti-rabbit IgG immunoblotting reagent that preferentially detects the nonreduced rabbit IgG form (thereby minimizing interference by the heavy and the light chain of the immunoprecipitating antibodies) was used to visualize the complex. Immunoreactivity was determined by enhanced chemiluminescence. Controls included incubation without primary antibodies as well as without crude extracts.

**Immunostaining on Slices from Human Cortex**—Sections from human cortex were cut serially to a 50- $\mu\text{m}$  thickness using a cryostat and then mounted on Superfrost Plus glass slides. The brain sections were then used for immunofluorescence

analysis as previously described (22). Briefly, after extensive washing and quenching of autofluorescence, nonspecific binding sites were blocked, and tissues were permeabilized by incubation with 4% normal goat serum (NGS) and 0.2% Triton X-100 in PBS for 1 h at room temperature. For immunofluorescence staining, slides were incubated for 24–48 h at 4 °C with the following antibodies diluted in PBS plus 4% NGS and 0.1% Triton X-100: rabbit polyclonal anti-DAAO (diluted 1/200; Davids Biotechnologie), rabbit polyclonal anti-pLG72 (diluted 1/50; Davids Biotechnologie), rabbit polyclonal anti-gial fibrillary acidic protein (GFAP; diluted 1/500; Sigma), and mouse monoclonal anti-glutamine synthase (GS; diluted 1/200; Chemicon) or anti-S100 $\beta$  (diluted 1/1000; Sigma) antibodies. After washing in PBS plus 1% NGS, the sections were incubated with goat anti-rabbit Alexa 546-conjugated antibodies and/or donkey anti-mouse Alexa 488-conjugated antibodies (Molecular Probes) for 1 h at room temperature. When paired rabbit polyclonal antibodies were used in double immunofluorescent staining, the Zenon Rabbit IgG Labeling kit (Molecular Probes) was employed according to the company's protocol using the Alexa 594- and 488-labeled Fab fragments complexed with the primary antibody. Finally, the sections were washed 3 times in PBS supplemented with 1% NGS and three times with PBS alone before being mounted in Vectashield mounting medium (Vector Laboratories).

**Immunostaining on Secondary Human Astrocyte Cultures**—Primary human astrocytes grown in flasks were detached with trypsin and were plated onto poly-L-lysine-coated glass coverslips at a density of  $0.5 \times 10^5$  cells/ml. After 1 week, secondary astrocyte cultures were ready for staining. Cells were extensively washed with PBS and fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 30 min at room temperature. Immunofluorescence on cells was performed as previously described for brain sections and according to Mothet *et al.* (23) with the only exception that horse serum was used instead of NGS. Immunostaining was performed by incubating the coverslips with paired antibodies for 24 h at 4 °C; both the rabbit anti-DAAO (diluted 1/50) and the goat anti-pLG72 (diluted 1/20) antibodies were simultaneously analyzed with commercial antibodies against specific markers of subcellular compartments, such as PMP70 for peroxisomes (rabbit polyclonal antibody, diluted 1/400; Sigma), 58K Golgi protein and GM130 for Golgi apparatus, and lysosome-associated membrane protein 2 for lysosomes (mouse monoclonal antibodies, diluted 1/500, 1/200, and 1/100, respectively; Abcam). Immunoreactivity was demonstrated with donkey anti-rabbit Alexa 647-, chicken anti-goat Alexa 488-, donkey anti-mouse Alexa 488-, and donkey anti-goat Alexa 647-conjugated antibodies (diluted 1/1000; Molecular Probes). The specific stain of mitochondria was obtained by using MitoTracker Green FM (Molecular Probes), a green fluorescent dye that localizes to this compartment regardless of the mitochondrial membrane potential; after incubation with the secondary antibody, the coverslips were incubated for 20 min at room temperature in 30 nM MitoTracker Green FM and extensively washed with PBS. The double immunostaining experiments were performed using the rabbit anti-PMP70 and anti-hDAAO antibodies (1  $\mu$ g) labeled with Alexa 594- and

488-tagged Fab fragments, respectively (Zenon Rabbit IgG Labeling kit; Invitrogen). After extensive washes the coverslips were mounted with Vectashield mounting medium (Vector Laboratories).

Immunofluorescence from brain sections and astrocyte cultures was visualized using an upright or inverted laser-scanning confocal microscope (TCS SP2, Leica Microsystems, Mannheim, Germany) equipped with a 63.0 $\times$ /1.25 NA plan apochromate oil immersion objective. The confocal images were acquired using the Leica TCS software with a sequential mode (if necessary) to avoid interference between each channel and without saturating any pixel. Controls included omission of primary antibodies and incubation in immune serum preadsorbed on the antigen. These control sections did not display any staining. Quantitative colocalization was determined using the colocalization plug-in of ImageJ software (rsb.info.nih.gov/ij). Measured colocalization was corrected for random colocalization by superimposing a mirror image of the second channel onto the image of the first channel (24).

**Transient Transfection Experiments**—The cDNA fragments coding for hDAAO and pLG72 were inserted into the pEGFP-C3 (Clontech Laboratories) vector between the HindIII and EcoRI restriction sites in-frame with the gene coding for EGFP. The HindIII and EcoRI restriction sites were inserted at the ends of hDAAO and pLG72 cDNA by mutagenic PCR using the pET11-hDAAO (19) and pET11-G72 (18), respectively, as templates. The U87 human glioblastoma cells (ATCC) were transfected by using the ExGen 500 Transfection reagent (Fermentas Life Sciences) as suggested by the supplier. Three micrograms of pEGFP-C3, pEGFP-C3-hDAAO, and pEGFP-C3-pLG72 eukaryotic expression constructs were transfected into  $2 \times 10^5$  cells/well. The expression of the fusion proteins was monitored by detecting green fluorescence protein emission; after 19 h of incubation, the cells were collected, counted, washed once in PBS, and stored at  $-80$  °C for further analysis.

To determine cellular D- and L-serine levels, about  $5 \times 10^5$  transfected cells were resuspended in 1 ml of ice-cold 5% trichloroacetic acid, sonicated, and centrifuged for 30 min at 13,000 rpm. The soluble fraction was extracted with water-saturated ether and neutralized with NaOH before precolumn derivatization with *o*-phthalaldehyde/*N*-acetyl-L-cysteine in borate buffer (12). D- and L-serine were resolved by HPLC chromatography on a 5- $\mu$ m Waters C8 (4.6  $\times$  250 mm) reverse-phase column eluted under isocratic conditions using 100 mM sodium acetate buffer, pH 6.2, 1% tetrahydrofuran at 1 ml/min. The compounds were identified and quantified on the basis of retention times and peak areas compared with those associated with D- and L-serine as standards. Linearity was determined by injecting increasing concentrations of amino acids (20–100 pmol); the calibration curves were used to determine the amount of amino acids in the experimental samples. The analyses were replicated five times for each condition, and statistical analyses were performed using Kaleidagraph software (Synergy Software). Variation between groups was evaluated by one-way analysis of variance, and post-hoc significance tests were performed using Student's *t* test. Significance was assessed at  $p < 0.05$ .

DAAO activity measurements on control and transfected U87 cell crude extracts were performed by the Amplex UltraRed assay kit (Invitrogen) based on the detection of H<sub>2</sub>O<sub>2</sub> by the peroxidase-mediated oxidation of the fluorogenic Amplex UltraRed dye. Cells were suspended in ice-cold 50 mM sodium phosphate buffer, pH 7.4, containing 1 μM pepstatin, 2 μM leupeptin, and 10 μM FAD, sonicated, and centrifuged at 13,000 rpm for 10 min (4 °C); 300 μl of these solutions (corresponding to 2 × 10<sup>4</sup> cells) were diluted 1:2 in the activity assay solution containing 50 μM Amplex UltraRed, 0.2 units/ml horseradish peroxidase, 10 mM NaN<sub>3</sub>, 10 μM FAD, 50 mM D-serine and incubated for 30 min at room temperature in the dark. The reactions were blocked by adding 120 μl of Amplex UltraRed stop reagent, and fluorescence emission at 590 nm was measured. For each sample a control without the substrate D-serine was prepared; DAAO activity was expressed as the difference in fluorescence emission between sample and control assay mixtures. Furthermore, 1 mM sodium benzoate (a well known DAAO inhibitor) (4, 5) was added to determine whether the fluorescence changes were effectively due to DAAO activity. A calibration curve was obtained by adding known amounts of recombinant hDAAO to U87 crude extracts (0.02–0.4-milliu-nit range). Statistical analyses were performed as reported above.

## RESULTS

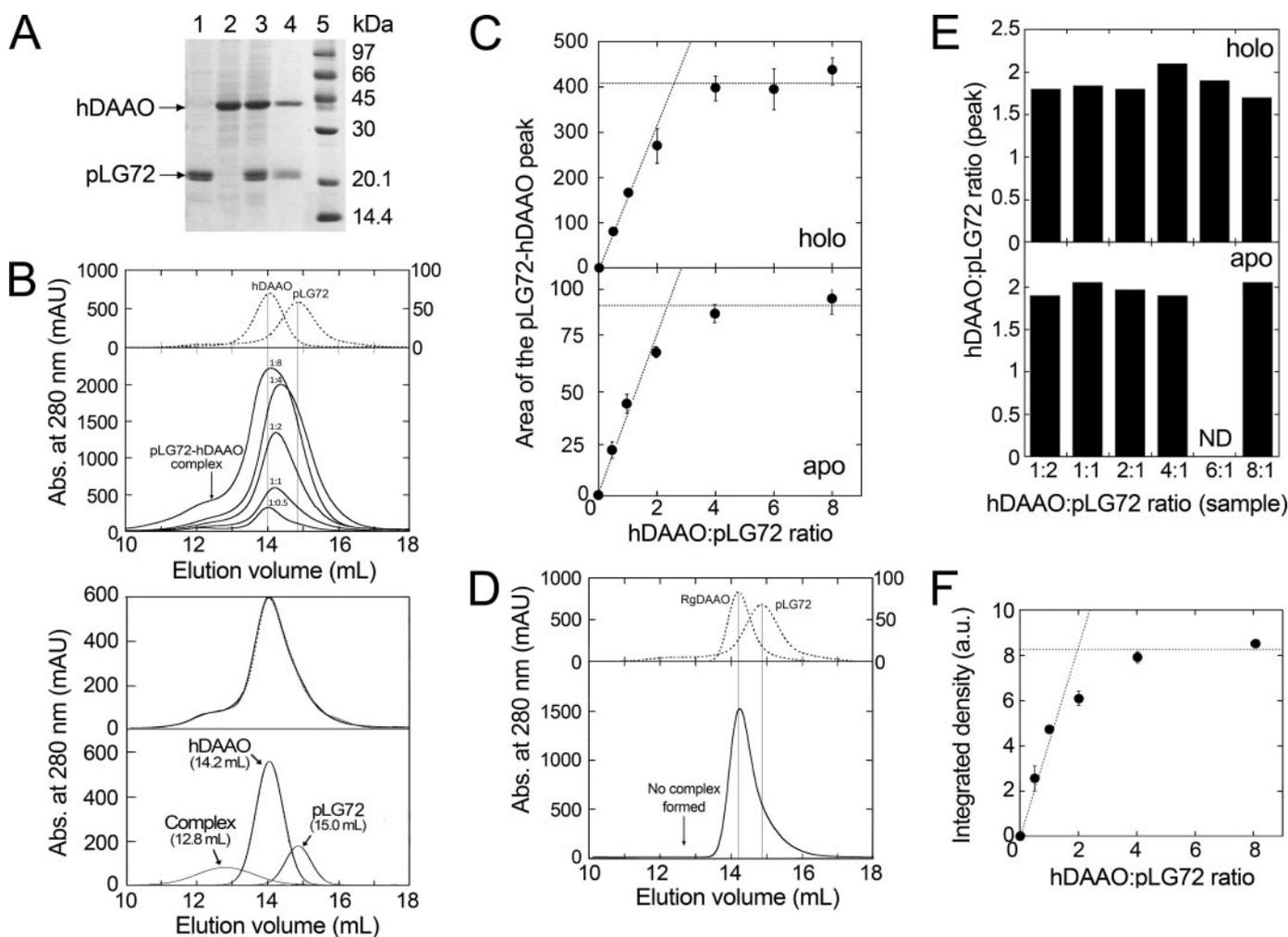
**Effect of pLG72 on DAAO from Pig Kidney**—By using yeast two-hybrid experiments, DAAO was previously identified as a protein that interacts with pLG72 (3). This interaction was confirmed *in vitro*, but these experiments were performed essentially on porcine DAAO, *e.g.* by binding to a column with immobilized pkDAAO. Similarly, by measuring the enzymatic activity of pkDAAO in the presence of increasing amounts of pLG72, the latter was suggested to be an activator of DAAO. The only experiment performed on hDAAO was a glutathione S-transferase pull down of pLG72 using immobilized hDAAO, which demonstrates the interaction between the two proteins (3). The amino acid sequence for the hDAAO protein is more than 80% identical to that of pkDAAO. However, hDAAO can be distinguished from pkDAAO because of the weaker FAD binding and the stable homodimeric state even in the apoprotein form (19). First, we repeated the experiments from (3) using commercial pkDAAO and our recombinant pLG72 protein. A signal corresponding to the interaction of pkDAAO to pLG72 was evident in Far Western blot experiments (supplemental Fig. 1A, *left*). As shown in supplemental Fig. 2A and using the same experimental conditions reported by Chumakov *et al.* (3), the activity of pkDAAO (45 μg/ml) decreased in the presence of pLG72 (270 μg/ml), whereas adding BSA as a control to pkDAAO did not affect the activity of the flavoprotein. The inactivation of DAAO by pLG72 was even faster at increasing concentrations of NLS, a detergent required to solubilize pLG72 (18). Furthermore, the addition of pLG72 directly into the DAAO activity assay solutions (*i.e.* without any previous incubation between the two proteins) did not modify pkDAAO activity (0.51 ± 0.06 and 0.53 ± 0.09 units/ml in the absence and in the presence of pLG72, respectively). In all cases, the pkDAAO activity in the presence of pLG72 was higher if

there was an excess of free FAD in the assay solution, although the presence of free FAD did not alter the time course of enzyme inactivation. These results confirm the previously reported *in vitro* interaction between pkDAAO and pLG72 (3) but fail to show any enzymatic activation by pLG72. In contrast, we showed that pLG72 inactivates pkDAAO.

**Determination of pLG72 Binding Affinity to hDAAO**—To verify the inferred interaction of pLG72 to hDAAO *in vitro* (3), we first used Far Western blot analysis using purified recombinant proteins (*lanes 1 and 2* in Fig. 1A). A signal corresponding to the interaction of hDAAO to pLG72 was evident under denaturing conditions when nitrocellulose membranes were overlaid with hDAAO and probed with monospecific polyclonal anti-hDAAO antibodies after SDS-PAGE separation and transblotting of pLG72. The signal was even more apparent under native conditions as revealed by dot Far Western blot (wherein pLG72 was adsorbed onto a nitrocellulose membrane) (supplemental Fig. 1A, *central panel*). The immuno-recognition is strictly specific for the mammalian DAAO as no signal was observed using DAAO from yeast (RgDAAO) (supplemental Fig. 1A, *right panel*).

The pLG72 binding to hDAAO was then followed by gel permeation chromatography on a Superdex 200 column; the separations were performed using a fixed amount of pLG72 (25 nmol) and increasing amounts of hDAAO (from 5 to 200 nmol). The elution volume of hDAAO was shifted from 14.2 to 12.8 ml, *i.e.* from the homodimeric state of 80 kDa to a form of ~200 ± 20 kDa (Fig. 1B). The presence of both pLG72 and hDAAO in the 12.8-ml peak was demonstrated by SDS-PAGE electrophoresis (*lane 4* in Fig. 1A). The saturation of the area of the peak at 12.8 ml was obtained at an ~2 hDAAO monomers:1 pLG72 monomer ratio (see the *intercept* of the *straight lines* in Fig. 1C, *top panel*). As a control of the specificity of this interaction, the same experiment was performed using RgDAAO; its elution volume (14.5 ml) was not affected by the addition of pLG72 (Fig. 1D). Next, we performed the same experiments using the apoprotein form of hDAAO; the elution profiles resemble the ones obtained for the holoenzyme. The area of the 12.3-ml peak corresponding to the protein complex increased with the amount of added apohDAAO; from the plot reported in Fig. 1C, *bottom panel*, an ~2:1 apohDAAO:pLG72 stoichiometry is evident. The stoichiometry of hDAAO:pLG72 complex formation was further confirmed using densitometric analysis to quantifying the amount of the two proteins in the fraction eluted at the volume corresponding to the complex; for both holo- and apoprotein forms, a hDAAO:pLG72 molar ratio of 1.93 ± 0.15 is evident (Fig. 1E). These results demonstrate that pLG72 interacts with both the holo- and the apoprotein form of hDAAO to yield a similar complex.

Analogously, quantitative pulldown experiments performed using anti-pLG72 antibodies on protein samples containing a fixed amount of pLG72 (0.1 nmol) and increasing concentrations of hDAAO (0–0.8 nmol) showed a saturation of the amount of immunoprecipitated hDAAO at a hDAAO:pLG72 ratio of 2:1 (see Fig. 1F). Finally, similar results were obtained using spectroscopic methods, *e.g.* by following the changes in fluorescence of the hydrophobic probe 8-anilino-naphthalene-1-sulfonate during titration of a fixed amount of pLG72 with



**FIGURE 1.** Analysis of pLG72 binding to hDAAO by gel permeation chromatography (A–E) and quantitative pull-down experiments (F). **A**, SDS-PAGE analysis of purified pLG72 (5  $\mu$ g, lane 1) and hDAAO (5  $\mu$ g, lane 2), of a mixture of hDAAO and pLG72 (lane 3), and of the hDAAO-pLG72 complex isolated by gel-permeation chromatography (lane 4). Lane 5, standard proteins. **B**, top panel, elution profiles of gel permeation chromatography of purified hDAAO (left, y axis) and pLG72 (right, y axis) proteins (upper) and of mixtures containing 25 nmol of pLG72 and different amounts of hDAAO (from 12.5 to 150 nmol, lower). Bottom panel, example of analysis of peaks from gel permeation chromatography of 25 nmol of hDAAO and 25 nmol of pLG72 by nonlinear curve fitting (using PeakFit software); shown is deconvolution of the peaks corresponding to pLG72 (15.0 ml), hDAAO (14.2 ml), and the complex (12.8 ml). **C**, effect of hDAAO concentration on the area of the 12.8-ml peak corresponding to the hDAAO-pLG72 complex for holo-hDAAO (upper) and at 12.3 ml for apohDAAO (lower). Data from the elution profiles reported in panel B were analyzed as reported under “Experimental Procedures.” **D**, control experiment showing that yeast DAAO does not form a complex with pLG72; (upper) elution profiles of purified RgDAAO (left, y axis) and pLG72 (right, y axis); lower elution profile of a mixture containing 25 nmol of pLG72 and 50 nmol of RgDAAO. **E**, results from densitometric analysis of the bands corresponding to hDAAO and pLG72 after SDS-PAGE separation of the proteins eluted in the peak corresponding to the hDAAO-pLG72 complex (see lane 4 in panel A), expressed as hDAAO:pLG72 molar ratio for holo- (upper) and apohDAAO (lower). Data reported in panels C and E are the average of three determinations. Noteworthy, in the absence of NLS in the elution buffer, the addition of pLG72 shifted the elution volume of hDAAO from 14.1 to 8.1 ml (close to the void volume of the column), i.e. from a dimeric to a polymeric state (data not shown), reaching a maximum at a hDAAO:pLG72 ratio of between a 2:1 and 4:1 ratio. **F**, results from densitometric analysis of the band corresponding to hDAAO precipitated by quantitative pull-down experiments performed using anti-pLG72 antibodies on protein samples containing 0.1 nmol of pLG72 and increasing concentrations of hDAAO (0–0.8 nmol).

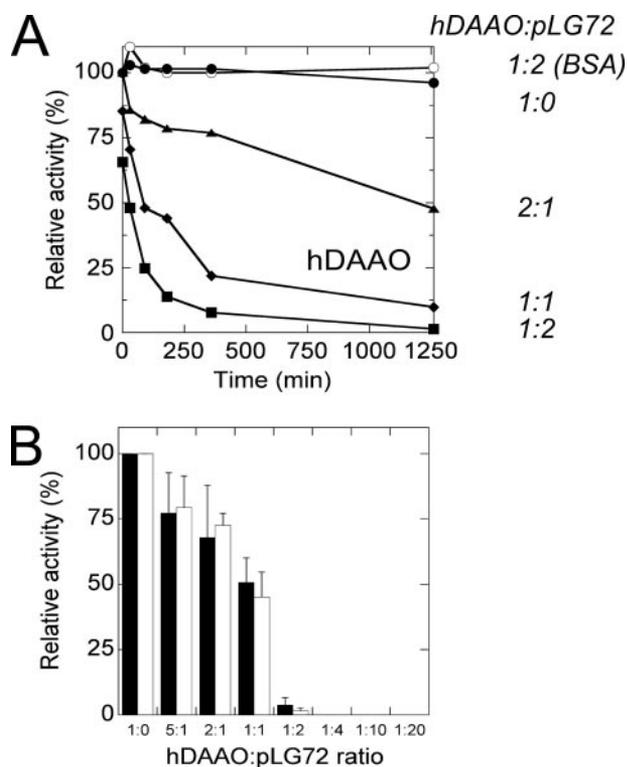
increasing hDAAO concentrations (see the supplemental materials).

The apparent affinity for the hDAAO-pLG72 complex formation was assessed by means of surface plasmon resonance using the BIAcore technology. By fluxing pLG72 solutions at different concentrations over a sensor chip surface with immobilized hDAAO, a specific signal was detected (supplemental Fig. 1B); an apparent  $K_D$  of  $8.3 \times 10^{-6}$  M for the hDAAO-pLG72 complex formation was calculated. The injection of several other protein solutions (CK2 $\alpha$ , CK2 $\beta$ , Bud32) and also native and denatured BSA (boiled or incubated at 50 °C for 30 min) as negative controls did not induce any significant signal (data not shown). The same experiments reported in supplemental Fig.

1B were also performed using the apohDAAO, resulting in similar experimental traces (not shown). The BIAcore experiments were also conducted by fluxing soluble hDAAO on an immobilized pLG72-chip; this approach confirmed the  $K_D$  value for the hDAAO-pLG72 complex and demonstrated the specificity of pLG72 binding to hDAAO as RgDAAO displayed a very low binding capability (supplemental Fig. 1C) even when injected at a 10-fold higher concentration.

**Effect of pLG72 Binding on hDAAO Stability**—The effect of pLG72 on hDAAO stability was analyzed by mixing a fixed amount of hDAAO (50  $\mu$ M) with increasing concentrations of pLG72 (at a 0.06% NLS concentration, at which pLG72 is fully soluble and hDAAO is stable, supplemental Fig. 2B). In the

## Modulation of Human D-Amino Acid Oxidase



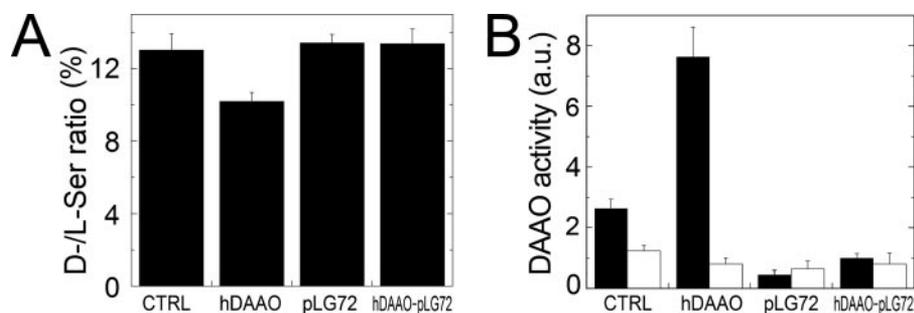
**FIGURE 2. Effect of pLG72 binding on hDAAO activity.** *A*, effect of pLG72 on the time course of enzymatic activity of hDAAO (50 nmol/ml); hDAAO:pLG72 ratios 1:0 (●), 2:1 (▲), 1:1 (◆), and 1:2 (■) in buffer A containing 0.06% NLS. For the sake of comparison, the effect of a 2-fold molar excess of boiled BSA on hDAAO activity is also reported (○). When not shown, the error bars are smaller than the symbols used. It is noteworthy that the inactivation depended on the percentage of NLS in the incubation mixture; at 0.01% NLS the DAAO enzymatic activity was not altered by adding pLG72 because at this concentration of detergent pLG72 is not soluble. *B*, effect of pLG72 on hDAAO activity after 30 min of preincubation. The enzymatic activity was measured by the oxygen consumption method on 100 mM D-serine using fixed amount of hDAAO (0.1 nmol/ml) to which increasing amounts of pLG72 were added 30 min before the assay. The activity was measured with (black bars) and without (white bars) 200  $\mu$ M free FAD in the assay mixture. The same experiment was carried out by measuring the residual DAAO activity without preincubation with pLG72 (see panel C of supplemental Fig. 2).

absence of pLG72, hDAAO maintained more than 90% of its initial activity after 21 h of incubation at 4 °C, whereas in the presence of increasing amounts of pLG72 the enzyme activity rapidly decreased (Fig. 2A). The effect of pLG72 on hDAAO stability was similar, but the enzyme was more rapidly inactivated when incubated at 25 or 37 °C. The presence of different amounts of free FAD in the incubation mixture (10 or 500  $\mu$ M) did not modify the time course of enzyme activity. As an additional control and under the same experimental conditions, neither the addition of pLG72 to RgDAAO nor the addition of BSA (soluble and denatured) to hDAAO modified the time course of enzyme (in)activation (Fig. 2A). SDS-PAGE analyses showed that the effect of pLG72 on the time course of loss of hDAAO activity was not due to proteolysis or to precipitation of the enzyme (no protein was detected in the pellet after centrifugation at up to 24 h of incubation). Further evidence of a destabilizing effect on hDAAO native conformation induced by pLG72 binding was derived from limited proteolysis experiments. In fact, the half-time for cleavage of hDAAO by 10% trypsin largely decreased from 7 to 3.5 min after adding an equimolar amount of pLG72.

**Effect of pLG72 Binding on hDAAO Activity**—As a preliminary investigation, the initial rate of hDAAO reaction was measured using the oxygen consumption assay on 100 mM D-serine (a saturating substrate concentration) in the presence of increasing concentrations of pLG72. As shown in supplemental Fig. 2C, the enzymatic activity only increased slightly in the presence of a large amount of pLG72 and an excess of free FAD in the assay mixture. By way of comparison, the activity decreased at increasing pLG72 concentrations in the absence of exogenous FAD in the assay mixture, *i.e.* under conditions at which a fraction of hDAAO in the assay solution is present in the apoprotein form (19). Interestingly, when the same measurement was performed after preincubating hDAAO and pLG72 for 30 min at 25 °C, the enzymatic activity always showed a decrease and was completely abolished in the presence of an excess of pLG72 (at a hDAAO:pLG72 ratio  $\geq$  1:2, Fig. 2B). The apparent kinetic parameters on D-serine, the putative physiological substrate of hDAAO determined at 25 °C and 21% oxygen saturation (19), were not changed after the addition of pLG72 (exogenous FAD did not affect these values either; see supplemental Table 1).

The presence of pLG72 decreased the amount of hDAAO-bound FAD that can be reduced by the substrate (*i.e.* the active enzyme form). In fact, after incubation for at least 30 min (the time required to achieve anaerobic conditions) with a stoichiometric amount of pLG72, only 55% of FAD was converted into the corresponding reduced form when a large excess of D-serine was added under anaerobic conditions with respect to the flavin reduced in the absence of pLG72 (supplemental Fig. 3A). This value is further decreased at higher pLG72 concentrations. Such an effect was not due to the depletion of FAD in solution because of its binding to pLG72 (see below), as the same result was obtained when an excess of free FAD was present in the assay mixture; *i.e.* it represents an inhibition of the enzymatic activity. This result correlates with the residual enzyme activity measured after 30 min of incubation (see above and Fig. 2B).

**FAD and CPZ Binding to hDAAO and pLG72**—hDAAO contains one molecule of noncovalently bound FAD per protein monomer that can be easily isolated from the apoprotein by dialysis in the presence of 1 M KBr (19). By measuring the changes in protein fluorescence during titration of the apoprotein with FAD, a  $K_d$  of  $10 \pm 4 \times 10^{-6}$  M in 50 mM sodium pyrophosphate buffer, pH 8.3, was estimated (19). This value is significantly higher than the one measured for any other known DAAO (in the  $10^{-7}$ – $10^{-8}$  M range) (4, 5), raising the (unanswered) question of how much (active) holoenzyme of hDAAO is present *in vivo*. In the presence of buffer A containing 0.06% NLS, the  $K_d$  for FAD binding to apohDAAO increased to  $40 \pm 3 \times 10^{-6}$  M. Under the same conditions, the coenzyme binding was not significantly affected by the presence of a stoichiometric amount or by a 4-fold molar excess of pLG72 ( $K_d$  value is  $41.2 \pm 2.3$  and  $41.3 \pm 6.2$   $\mu$ M, respectively). Interestingly, the control experiments performed under the same conditions and in the absence of hDAAO showed that the protein fluorescence (at 340 nm) of pLG72 decreases at increasing FAD concentrations (see supplemental Fig. 4A); a  $K_d$  value of  $40 \pm 8$   $\mu$ M has been estimated. The pLG72·FAD interaction was confirmed by gel permeation chromatography on a Sephadex G25 column;



**FIGURE 3. Dependence of D-serine concentration and DAO activity in U87 cells transfected with hDAAO and/or pLG72.** The data are reported as the means  $\pm$  S.E. *A*, summary histogram of the D-/L-serine ratio (percentage) in U87 control cells and in the same cells transfected with hDAAO, pLG72, or co-transfected with both plasmids ( $n = 5$ ); see supplemental Fig. 5 for details. The change was demonstrated to be significant for hDAAO ( $p = 0.004$ ) and not significant for pLG72 and co-transfected cells. *CTRL*, control. *B*, summary histogram of the DAO activity (determined by means of the Amplex UltraRed assay and expressed as fluorescence change at 490 nm per 10,000 cells) in U87 control cells and in the same cells transfected with hDAAO, pLG72, or co-transfected with both plasmids ( $n = 6$ ). *Black and white bars* report the activity values determined in the absence and in the presence of 165  $\mu\text{M}$  sodium benzoate as DAO inhibitor, respectively. The change in activity with respect to the control was demonstrated to be statistically significant for hDAAO ( $p = 0.012$ ) and not significant for pLG72 and co-transfected cells.

the cofactor elutes in the same fraction of pLG72 (the absorbance spectrum of the eluate is shown in supplemental Fig. 4B).

The kinetic assay used to establish the amount of hDAAO-bound FAD (see above) was also employed to assess the effect of pLG72 on the rate of flavin binding to the apoprotein form of hDAAO. The presence of a 1–5-fold molar excess of pLG72 did not significantly affect the rate constant of flavin binding to the apoprotein of hDAAO ( $k_{\text{obs}} \sim 0.008 \pm 0.002 \text{ s}^{-1}$ ) but did decrease the amount of reconstituted holoenzyme, reaching values similar to those observed under static conditions (as reported in Fig. 2B).

Using the same fluorescence analysis employed for detecting the FAD binding, we demonstrated that pLG72 possesses a binding site for molecules with large hydrophobic moieties. In fact, a  $K_d$  value of  $65 \pm 13$  and  $26 \pm 3 \mu\text{M}$  was estimated for the binding of FMN and riboflavin, respectively, whereas no binding was observed using AMP (which lacks the hydrophobic isoalloxazine ring). Even the drug CPZ interacts with apo-hDAAO and pLG72 (supplemental Fig. 4C); in both cases a  $K_d$  of about  $5 \mu\text{M}$  was estimated. CPZ is an aliphatic phenothiazine and a widely used antipsychotic drug in the treatment of schizophrenia (its chemical structure resembles that of the isoalloxazine ring of FAD) that has been shown to be an FAD competitive inhibitor of pkDAAO (25, 26). The interaction between hDAAO or pLG72 and CPZ is not tight enough to be pharmacologically relevant. The spectral analyses of the pLG72 interaction to hDAAO have been published as supplemental data on the JBC web site.

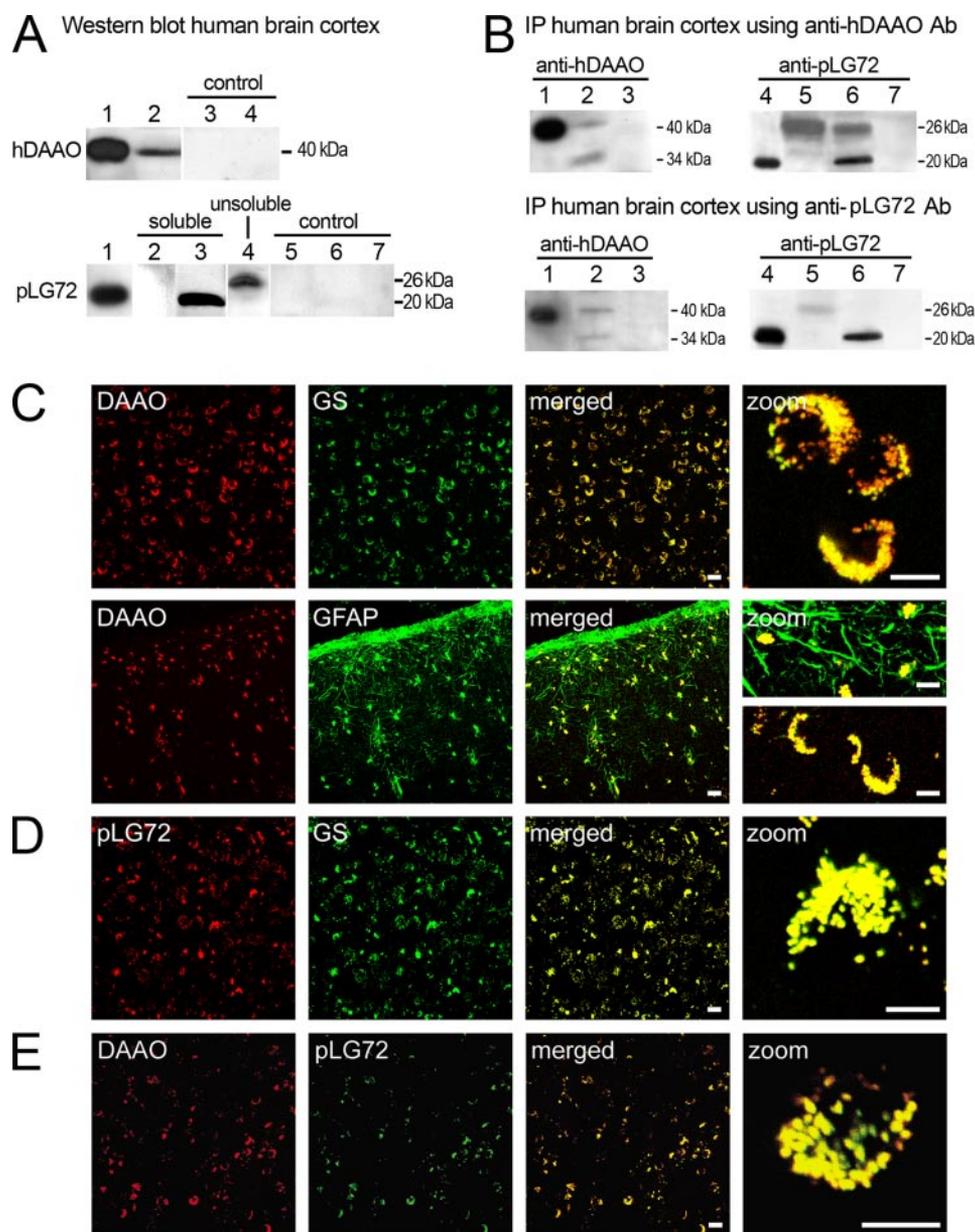
**Effect of pLG72 or hDAAO Transient Transfection on D-Serine Concentration**—To establish the relationship between pLG72/hDAAO and the cellular concentration of D-serine, human glioblastoma U87 cells were transfected with pEGFP-C3 expression vectors encoding for EGFP, EGFP-hDAAO, and EGFP-pLG72 proteins (average transfection yield was  $\sim 30$ – $40\%$ ; see supplemental Fig. 5A). D- and L-serine concentrations in the transfected cells were determined by HPLC analyses (supplemental Fig. 5B). Because the concentration of the serine isomers showed a significant sample-dependent variability, the value was normalized to the L-serine concentration

by using the D-/L-serine ratio. The D-/L-serine ratio determined on control cells (nontransfected or transfected with pEGFP-C3 plasmid = mock transfection) decreased in the cells transfected with the plasmid pEGFP-C3-hDAAO encoding for hDAAO from  $13.0 \pm 0.9$  to  $10.2 \pm 0.5\%$  ( $n = 5$ ,  $p < 0.05$ ) and did not show a statistically significant change in those transfected with the plasmid pEGFP-C3-pLG72 encoding for pLG72 and in the cells cotransfected with the two plasmids (showing in both cases a value of  $\sim 13.4\%$ ,  $n = 5$ ,  $p > 0.05$ , Fig. 3A). Furthermore, the amount of hDAAO activity in U87 control and transfected cells was assessed using

the Amplex UltraRed assay. As shown in Fig. 3B, the fluorescence signal corresponding to hDAAO activity increases in U87 cells transfected with the pEGFP-C3-hDAAO plasmid, whereas it is practically negligible in cells transfected with pEGFP-C3-pLG72 plasmid and similar to the control in cells cotransfected with the two plasmids. In samples that show a substantial enzymatic activity (control and hDAAO-transfected cells), the addition of the hDAAO inhibitor benzoate significantly decreases the observed activity values. These results indicate that the level of D-serine decreases after transient transfection of hDAAO in U87 cells according to the function of this flavooxidase in D-serine catabolism; this effect is not observed after the transfection of pLG72 in the same cells, further indicating that pLG72 is not an activator of hDAAO.

**Localization of hDAAO and pLG72**—The results of all the aforementioned experiments suggest that pLG72 interacts with hDAAO and acts as an endogenous modulator of this enzyme. We first checked for the presence of the two proteins in human cortex. Western blot analysis on crude extracts from human brain cortex samples using anti-DAAO antibodies showed the presence of a band at a molecular mass of  $\sim 40$  kDa (lane 2 in Fig. 4A, top panel; detection limit of  $0.005 \mu\text{g}$ ) corresponding to that of native hDAAO ( $M_r = 39,411$ ). When the same analysis was performed using anti-pLG72 antibodies, no bands were evident in the crude extract (detection limit of  $\sim 0.02 \mu\text{g}$ ), whereas a band at  $\sim 26$  kDa, corresponding to pLG72 (18), was observed in the corresponding insoluble fraction (see lane 4 in Fig. 4A, bottom panel). When the same analyses were performed in the presence of preabsorbed antibodies, no signals were detected (see controls in Fig. 4A), thus confirming the specificity of the recognition.

Immunoprecipitation of hDAAO from human cortex extracts was performed using an amount of sample 50-fold higher than in the Western blot experiments (10 versus 0.2 mg of protein), rabbit anti-hDAAO antibodies, and the TrueBlot system; the precipitated proteins were analyzed by SDS-PAGE and Western blot. By using anti-hDAAO antibodies, two bands were identified; one at  $\sim 40$  kDa (the intact hDAAO) and another at an  $\sim 6$ -kDa lower molecular mass (that we propose



**FIGURE 4. hDAAO and pLG72 are both present in astrocytes of the human cortex.** *A*, immunoblot analysis of protein extracts from human cortex. *Top panel*, 0.01  $\mu\text{g}$  of purified hDAAO (*lanes 1 and 3*) and 200  $\mu\text{g}$  of total proteins of extracts from human cortex (*lanes 2 and 4*) were analyzed using anti-DAAO antibodies. For *lanes 3 and 4*, the antibodies were preadsorbed with a 1:350 molar ratio of hDAAO. Immunolabeling with anti-DAAO antibodies demonstrates the presence of a band at 40 kDa in the crude extracts (*lane 2*). *Bottom panel*, 0.05  $\mu\text{g}$  of purified pLG72 (*lanes 1 and 5*) and 200  $\mu\text{g}$  of total proteins of soluble extracts (*lanes 2, 3, and 6*) and pellets (*lanes 4 and 7*) from human cortex were analyzed using anti-pLG72 antibodies (*lanes 1–4*) and anti-pLG72 antibodies pre-adsorbed with a 1:1200 molar ratio of pLG72 (*lanes 5–7*). Purified recombinant pLG72 was added in sample 3. Immunorecognition with anti-pLG72 antibodies showed bands at  $\sim 20$  kDa in the presence of purified recombinant pLG72 (*lanes 1 and 3*) and at  $\sim 26$  kDa in the insoluble fraction (*lanes 4*), thus indicating that the amount of soluble pLG72 in crude extracts is below the detection limit ( $<0.02 \mu\text{g}/\text{lane}$ ). When antibodies were preabsorbed before immunolabeling, no signal was detected (*lanes 5–7*). *B*, co-immunoprecipitation of hDAAO and pLG72 using anti-hDAAO antibodies (*upper*) and anti-pLG72 (*lower*), 500  $\mu\text{l}$  of human cortex extract (20 mg protein/ml; *lanes 2, 3, and 5*) and the TrueBlot system. The membrane corresponding to the precipitated sample was at first developed using anti-hDAAO antibodies (*lanes 1–3*) and, after stripping, using anti-pLG72 antibodies (*lanes 4–7*). *Lane 1*, 0.01  $\mu\text{g}$  of purified hDAAO; *lane 4*, 0.05  $\mu\text{g}$  of purified pLG72. *Lane 6 upper*, the same sample as in *lane 5* (crude extract) with 0.05  $\mu\text{g}$  of purified recombinant pLG72 showing the bands corresponding to recombinant (20 kDa) and native (26 kDa) pLG72. *Lane 6 lower*, immunoprecipitation performed using anti-pLG72 antibodies on 0.04  $\mu\text{g}$  of purified pLG72 treated as the crude extract and showing the band at 20 kDa corresponding to recombinant pLG72. *Controls*, *lanes 3 and 7* contain the same amount of anti-hDAAO (*upper*) or anti-pLG72 (*lower*) antibodies (*Ab*) used for immunoprecipitation (*IP*). *C and D*, confocal analysis of the cellular distribution of hDAAO and pLG72 in the human cortex. *Red channel*, Alexa Fluor 594; *green channel*, Alexa Fluor 488. hDAAO and pLG72 (*red channel*) are both found throughout the cerebral wall. GS, GFAP, and S100 $\beta$  (not shown) are also present (*green channel*) in the cerebral wall. Note that GS is present in the cell bodies, whereas GFAP (and S100 $\beta$ ) stain is also localized in the processes of astrocytes. The results reported in *panels C and D* suggest that both hDAAO and pLG72 reside in the same cells. The analysis of the distribution of the two proteins reported in *panel E* support this hypothesis; *red* (hDAAO) and *green* (pLG72) channels overlay revealed a net co-localization of signal patterns, strongly indicating the presence of hDAAO and pLG72 inside the same astrocytes. Overlays of red and green channels revealed strong *yellow spots* that support co-localization and the presence of hDAAO and pLG72 in astrocytes. *Scale bars* = 25  $\mu\text{m}$  for all panels except for zooms (= 10  $\mu\text{m}$ ).

as a proteolytic fragment of hDAAO and which is currently being characterized) (Fig. 4B, top, left panel, lane 2). The same membrane was then developed by using anti-pLG72 antibodies; a band at ~26 kDa was detected, corresponding to that identified in the insoluble fraction from crude extracts of human brain cortex and confirming a lower electrophoretic mobility of native, nonrecombinant pLG72 (lane 4, Fig. 4A, bottom panel). The same results were obtained when the experiment was carried out using anti-pLG72 antibodies to coimmunoprecipitate the protein complex (see Fig. 4B, bottom panel). No signals were detected if the primary antibodies or the crude extracts were omitted (IgG-only, see lanes 3 and 7 in Fig. 4B), thus confirming the specificity of the recognition. This finding indicates that hDAAO and pLG72 interact in human cortex.

We next analyzed the cellular distribution of the two proteins in slices from human cortex. Immunostaining displayed a homogeneous distribution of DAAO throughout the layers of the human cerebral wall, showing a pattern that seemed to highlight parts of certain cell bodies (Fig. 4C, red channel). It has been proposed that DAAO localizes in astrocytes in the central nervous system (26–28). By using double immunostaining experiments in which hDAAO labeling is paired with GS or with GFAP, two well known markers of the astroglial lineage, we demonstrated the presence of DAAO in astrocytes. The pattern of GS staining was quite similar to that of DAAO throughout the cerebral wall (Fig. 4C, top panel, green channel). On the other hand, GFAP staining revealed clearly labeled cell bodies with processes (Fig. 4C, bottom panel, green channel). Although the GFAP staining is found throughout the cerebral wall, it is more pronounced and denser at the surface of the cortex at the level of the subpial layer. Comparison of the two glial markers with hDAAO confirmed the strictly astrocytic localization of this flavoenzyme. On the double immuno-stains, DAAO was absent in the subpial layer (Fig. 4C, merged panels). No signal for hDAAO, GFAP, or GS was observed in control experiments (data not shown). Immunofluorescent staining for pLG72 revealed the presence of the protein throughout the cerebral wall, with a pattern resembling that of hDAAO (compare the red channels on Fig. 4, C and D). A comparison with GS, GFAP, or S100 $\beta$  (a further glial marker) clearly demonstrates that pLG72 is distributed in the astrocytes (not shown). At higher magnifications hDAAO and pLG72 were both concentrated mostly in the cell bodies, with no signal detected in the processes of astrocytes (Fig. 4, C and D, zoom panels). Experiments in brain slices suggested that both proteins should be present in the same astrocytes. We confirmed this hypothesis by showing a clear colocalization of the two proteins in the same cells (Fig. 4E, merged panels).

To further analyze the subcellular distribution of pLG72 and hDAAO, we next used cultured human astrocytes. For hDAAO an abundant and punctuate signal was found in the cultured astrocytes (Fig. 5); the protein was widely distributed throughout the cytoplasm. The DAAO flavoprotein was previously identified as a resident protein of peroxisomes (27, 29, 30) that can be unmasked by PMP70, a component of peroxisomal membranes. In astrocytes, PMP70 was abundant, yielding a signal pattern that resembled that of hDAAO (Fig. 5A, red channel). In fact, the overlay of the red channel onto the green one

revealed a net (although partial) colocalization of hDAAO and PMP70, particularly in the perinuclear region of the cell (Fig. 5A, top panel, merged, and zoom panels). This latter observation confirmed that in human astrocytes DAAO is largely present in the peroxisomes but also revealed that a significant amount of this flavoenzyme is localized outside these organelles. Importantly, the putative nonperoxisomal DAAO form might possibly be related to the electrophoretically more mobile form of the protein observed in the immunoprecipitation experiment on the crude human cortex extract (see Fig. 4B); further analyses will be undertaken to characterize this protein form or hDAAO proteolytic product. On the other hand, an only marginal overlap in signal was observed between hDAAO and 58K protein (a marker of the Golgi complex) and MitoTracker green FM (a mitochondrion-selective stain) (Fig. 5, B and C).

Concerning the subcellular distribution of pLG72, immunofluorescent staining demonstrated a perinuclear and tubular pattern of distribution that resembles the Golgi apparatus network (Fig. 5A, bottom panel). Indeed, pLG72 has previously been proposed to be present in this compartment (3). Thus, we analyzed the distribution of pLG72 versus the pattern of a 58K Golgi protein and versus GM130, a protein associated with the membrane of the cis-Golgi network (31). Although GM130 also displays its typical tubular pattern, no colocalization of pLG72 with GM130 was detected in the cultured astrocytes (not shown), thus excluding the presence of pLG72 inside the cis-Golgi network. On the other hand, a partial perinuclear overlapping in the signal pattern was observed for the 58K protein (Fig. 5B, bottom panel, merged panel), suggesting that pLG72 could transiently pass through the Golgi apparatus. Furthermore, a strong punctuate immunostaining for pLG72 is present outside this subcellular compartment, indicating a cytosolic localization of pLG72 (Fig. 5B, bottom panel, merged panel). No significant overlap in signal was observed between pLG72 and PMP70 (Fig. 5A, bottom panel) or between pLG72 and MitoTracker green FM (Fig. 5C, bottom panel). Furthermore, no colocalization was detected between hDAAO or pLG72 and lysosome-associated membrane protein 2 as a marker of the lysosomes (data not shown).

Finally, we compared the distribution of hDAAO with that of pLG72 (Fig. 5D). Here, we only rarely observed colocalization patterns in resting cells between pLG72 and hDAAO and, therefore, could not accurately quantify this distribution. In particular, we observed that only 5% of the cells display colocalization patterns, with a mean average of 8.1% colocalization; the cell displayed in Fig. 5D shows a partial overlap of 10% between hDAAO and pLG72. Thus, it is more likely that pLG72 interaction with hDAAO is driven by specific spatiotemporal stimuli, the features of which remain to be discovered.

## DISCUSSION

Schizophrenia is a severe psychiatric disorder that affects nearly 1% of the world population and accounts for about 2.5% of healthcare costs. With the discovery of the correlation between G72 and DAAO genes and schizophrenia (both as an individual gene and synergistically), a biochemical system was proposed that could be responsible for the major symptoms of this psychiatric disorder (3). After the report by Chumakov

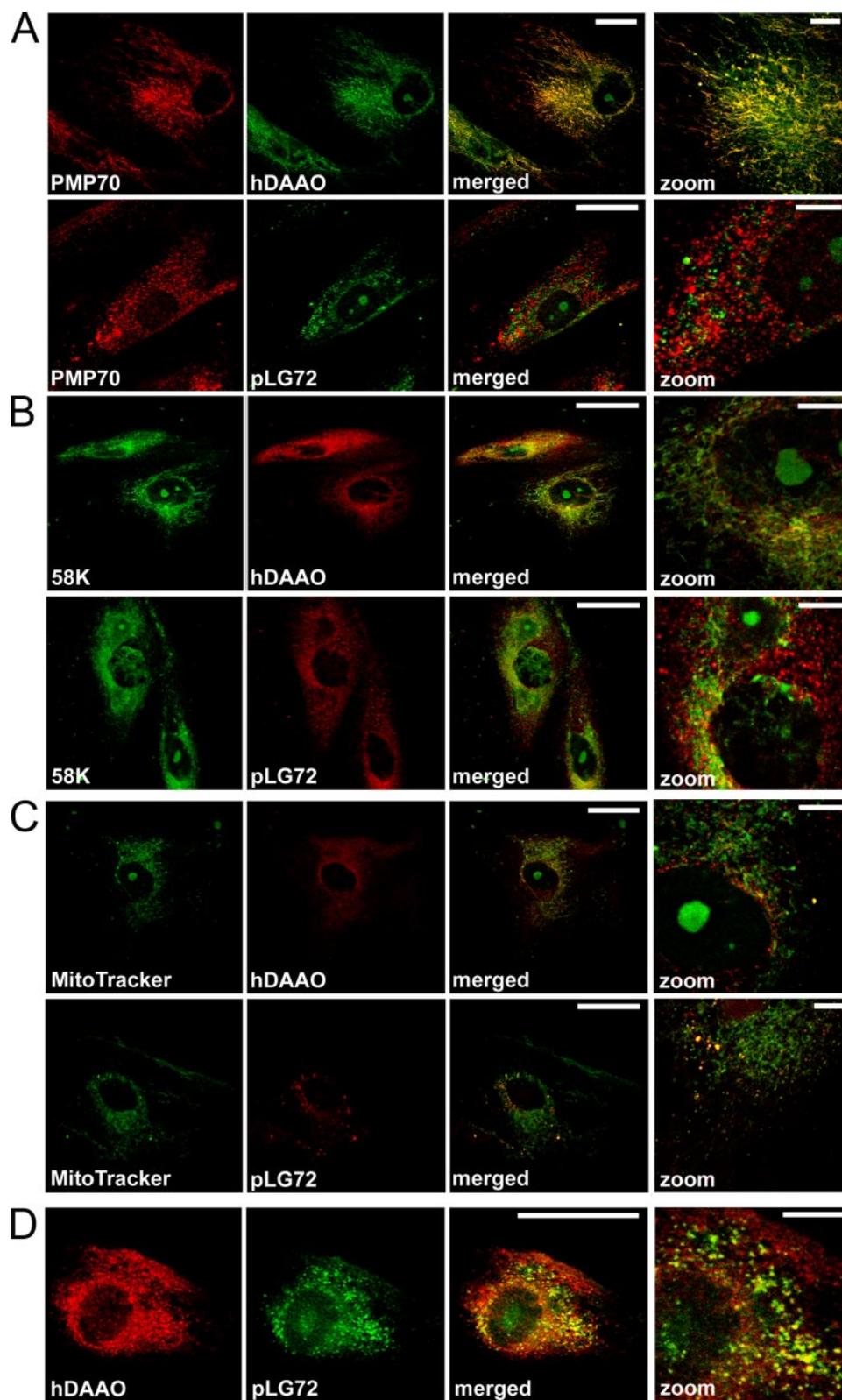
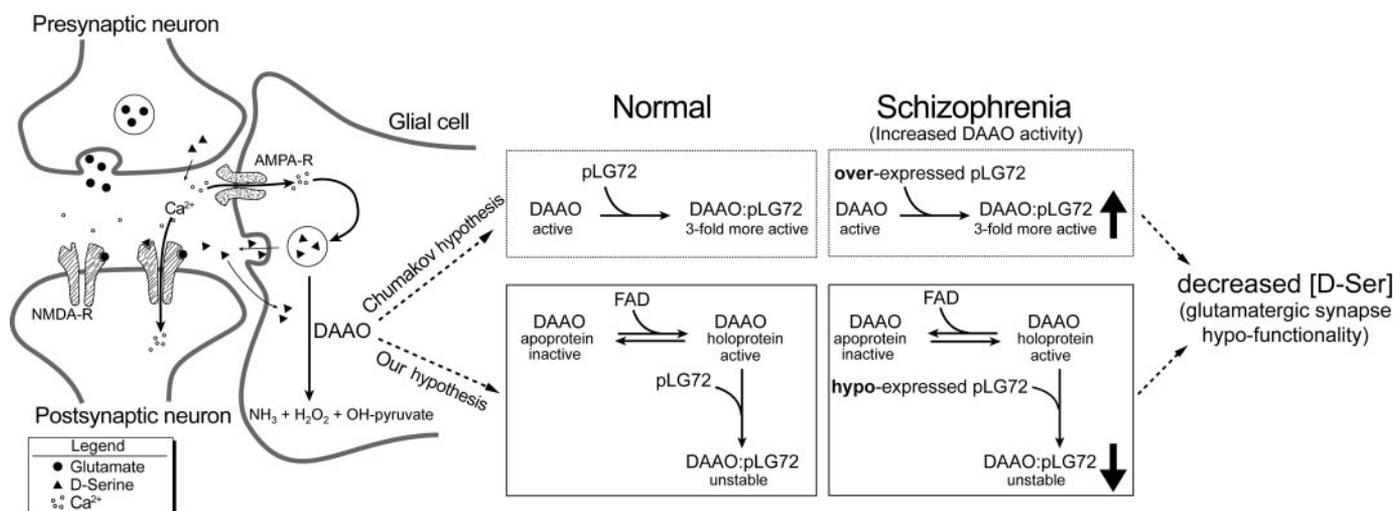


FIGURE 5. **Confocal analysis of the subcellular distribution of hDAAO and pLG72 in cultured human astrocytes.** *Red channel*, Alexa Fluor 594 or 647; *green channel*, Alexa Fluor 488. *A*, the immunofluorescence staining of hDAAO and pLG72 (*green channel*) are compared with the distribution of the peroxisomal marker PMP70 (*red channel*). The *merged* and *zoom* panels show no overlap in signal between pLG72 and PMP70 staining and highlight a co-localization of hDAAO and PMP70, also revealing that a significant amount of hDAAO is present outside peroxisomes. *B*, subcellular distribution of hDAAO and pLG72 (*red channel*) with respect to 58K Golgi protein (*green channel*). The overlay of the two channels in the *merged* and *zoom* panels demonstrates a partial perinuclear overlapping in pLG72 and 58K protein signal patterns even though a strong punctuate cytosolic immunostaining is also observed. Only marginal overlap in signals is observed between hDAAO and 58K protein. *C*, the immunofluorescence signals of hDAAO and pLG72 (*red channel*) are compared with MitoTracker green FM mitochondrion-selective stain (*green channel*). For both hDAAO and pLG72, no significant co-localization with MitoTracker is observed. *D*, comparison of the subcellular distribution of hDAAO (*red channel*) and pLG72 (*green channel*). In the *merged* and *zoom* panels a partial co-localization (~10%) is evident. Scale bars = 40  $\mu\text{m}$  for all panels except for zooms (=10  $\mu\text{m}$ ).



**FIGURE 6. Hypothesis of the role of hDAAO and pLG72 on the D-serine bioavailability at glutamatergic synapses under normal and pathological conditions.** Glutamate is released by the presynaptic neuron into the synaptic space after the depolarization of nerve terminals, yielding the activation of non-NMDA receptors on the membrane of the postsynaptic neuron and, subsequently, an increase in cytoplasmic concentration of  $\text{Ca}^{2+}$ . D-Serine is then released by the glial cells and, together with glutamate, activates NMDA receptors on the membrane of the postsynaptic neuron, leading to the opening of ion channels. The  $\text{Ca}^{2+}$  entry induces several possible intracellular responses, e.g. it activates nitric-oxide synthase that produces NO, which can diffuse to the neighboring cells where it is proposed to inhibit serine racemase and (probably) activate DAAO, thus decreasing the local concentration of D-serine (8). *Upper loop*, hypothesis of involvement of hDAAO and pLG72 in schizophrenia according to Chumakov *et al.* (3) in which pLG72 is an activator of hDAAO; under pathological conditions an overexpression of pLG72 leads to an increase in hDAAO activity that decreases the local concentration of D-serine. *Lower loop*, our hypothesis, in which pLG72 modulates the amount of active hDAAO acting on the stability of the holoenzyme. An abnormal, low expression of pLG72 under pathological conditions could result in hyperactivation of hDAAO and decrease D-serine concentration. Importantly, in both hypotheses the decrease in D-serine concentration results in a lower amount of activated NMDA receptors (NMDA-R) and, thus, in a hypofunction of the glutamatergic neurotransmission. Filled dots, glutamate; triangles, D-serine; open dots,  $\text{Ca}^{2+}$ ; AMPA-R,  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazolopropionic acid receptor.

*et al.* (3), at least 11 genetic studies reported on the association between markers near *G72* and *DAAO* genes and various phenotypes. In particular, a significant association of *G72* with both schizophrenia and bipolar affective disorders and of *DAAO* with schizophrenia in at least two different ethnic groups was reported; for a recent review, see Ref. 32. Among the identified single nucleotide polymorphisms, no changes in the coding region of hDAAO were identified that can relate loss/gain of functionality and/or altered levels of expression with schizophrenia susceptibility. Concerning *G72*, only single nucleotide polymorphism rs2391191, corresponding to M-15 in the report from Chumakov *et al.* (3), contains a mutation (Arg  $\rightarrow$  Lys) in the coding region; indeed, a recent study did not find an association between this polymorphism and schizophrenia (33). Identification of the levels of hDAAO or pLG72 expression and of hDAAO activity in schizophrenia patients will help validate the inferred physiopathological model.

Our investigations confirm the interaction between human proteins pLG72 and DAAO but failed to reproduce the effects of pLG72 binding to pkDAAO reported by Chumakov *et al.* (3); specifically, pLG72 did not activate pkDAAO. By using a variety of experimental methods, we demonstrated that pLG72 interacts specifically with both the holo- and apoprotein forms of mammalian DAAO, yielding an  $\sim 200$ -kDa complex constituted by 2 hDAAO homodimers ( $2 \times 80$  kDa) and 2 pLG72 molecules ( $2 \times 20$  kDa);  $K_d$  for the complex formation is  $\sim 8 \times 10^{-6}$  M.

pLG72 binding did not affect the kinetic parameters of the reaction catalyzed by hDAAO on D-serine, the affinity for coenzyme, or the rate constant of FAD binding to the apoprotein. The main effect observed was a faster time course of hDAAO inactivation when an excess of pLG72 was present, which we

attributed to the decrease in the holoenzyme (active) form. In fact, the amount of cofactor bound to the enzyme (the only FAD molecules that can be reduced by the substrate) decreased after the pLG72·hDAAO complex was formed (compare Fig. 2B and supplemental Fig. 3A). Visible absorbance and near-UV CD spectroscopy also showed that the binding altered the tertiary structure of hDAAO.

In human brain slices from cortex, we identified hDAAO and pLG72 in the same astrocytes. Furthermore, the coimmunoprecipitation of the two proteins from human cortex extracts and the partial subcellular colocalization of hDAAO and pLG72 in human glial cells further supports the possibility of interaction between these two proteins *in vivo*, suggesting that pLG72 might indirectly modulate NMDA receptor function (at least in cortex), controlling the levels of D-serine by acting on hDAAO.

Chumakov *et al.* (3) proposed pLG72 as an activator of hDAAO (Fig. 6, top), and therefore, they hypothesized that the onset of schizophrenia may result from the overexpression of pLG72, which induces hyperactivation of DAAO and, ultimately, decreases D-serine concentrations at the synapse (and the amount of NMDA receptors that can be activated). In our opinion, the role of pLG72 is more complex. Initially, pLG72 binding does not affect hDAAO functionality but, rather, modifies its tertiary structure and results in a time-dependent inactivation of the flavoenzyme. We propose that this binding switches off hDAAO activity and under normal conditions prevents glial D-serine from being fully degraded. This "slow" modification of hDAAO activity by pLG72 binding correlates with the low, inferred *in vivo* efficiency of this flavoenzyme (due to the weak cofactor binding and low turnover number) (19) and with the long half-life ( $\sim 16$  h) of D-serine in brain (8). We propose that alterations in the expression of pLG72 (e.g. a hypoe-

pression in schizophrenia patients) could significantly decrease the D-serine concentration in brain due to abnormally high activity of hDAAO (Fig. 6, *bottom*). Importantly, our hypothesis also explains the beneficial effects of D-serine treatment in schizophrenia patients (17). Moreover, our findings and those of Chumakov *et al.* (3) contradict the conclusions recently reported by Kvajo *et al.* (34), which suggested that pLG72 has a role in modulating mitochondrial functions.

The availability of the pLG72-hDAAO protein complex in solution and under physiological conditions represents an ideal system for finding small molecules that inhibit and/or modulate this protein-protein interaction. Demonstration of a specific correlation between the D-serine-pLG72-hDAAO biochemical pathway, glutamatergic neurotransmission, and relevant psychiatric disorders such as schizophrenia will promote therapeutic approaches targeting the molecular pathogenesis rather than the symptoms.

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