Molecular and Cellular Neuroscience 48 (2011) 20–28

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Evidence for the interaction of D-amino acid oxidase with pLG72 in a glial cell line

Silvia Sacchi [a,b], Pamela Cappelletti [a,b], Stefano Giovannardi [a], Loredano Pollegioni [a,b,*]

[a] Dipartimento di Biotecnologie e Scienze Molecolari, Università degli studi dell'Insubria, via J. H. Dunant 3, 21100 Varese, Italy
[b] "The Protein Factory", Centro Interuniversitario di Ricerca in Biotecnologie Proteiche Politecnico di Milano and Università degli studi dell'Insubria, Italy

ARTICLE INFO

Article history:
Received 16 February 2011
Revised 29 April 2011
Accepted 1 June 2011
Available online 12 June 2011

Keywords:
D-serine
Neurotransmission
Schizophrenia
Flavoprotein
NMDA receptor
Subcellular localization

ABSTRACT

Accumulating genetic evidence indicates that the primate-specific gene locus G72/G30 is related to schizophrenia: it encodes for the protein pLG72, whose function is still the subject of controversy. We recently demonstrated that pLG72 negatively affects the activity of human D-amino acid oxidase (hDAAO, also related to schizophrenia susceptibility), which in neurons and (predominantly) in glia is expected to catabolize the neuromodulator D-serine. The D-serine regulation mechanism relying on hDAAO–pLG72 interaction does not match with the subcellular localizations proposed for hDAAO (peroxisomes) and pLG72 (mitochondria). By using glioblastoma U87 cells transfected with plasmids encoding for hDAAO and/or pLG72 we provide convergent lines of evidence that newly synthesized hDAAO, transitorily present in cytosol before being delivered to the peroxisomes, colocalizes and interacts with pLG72 which we propose to be exposed on the external membrane of mitochondria. We also report that newly synthesized cytosolic hDAAO is catalytically active, and therefore pLG72 binding—and ensuing hDAAO inactivation—plays a protective role against D-serine depletion.

Introduction

Astrocytes release numerous molecules that can modulate synaptic transmission and synchronize neuronal network activity. One such signaling molecule is D-serine. This "atypical" neuromodulator is produced through the isomerization reaction catalyzed by the enzyme serine racemase (SR) from L-serine (Wolosker et al., 1999) and degraded by the α,β-elimination reaction of SR and/or by oxidative deamination catalyzed by the FAD-containing flavoprotein D-amino acid oxidase (DAAO) (Baumgart and Rodríguez-Crespo, 2008; Pollegioni et al., 2007; Pollegioni and Sacchi, 2010). D-Serine binds to the "glycine site" of the N-methyl-D-aspartate subtype of glutamate receptors (NMDAr), which, together with the glutamate site, must be occupied for the receptor to operate. D-Serine plays key roles in excitatory synaptic transmission and in several pathological conditions and is important for a number of physiological processes involved in neuronal plasticity (Martineau et al., 2006; Oliet and Mothet, 2009; Schell, 2004).

Different studies indicated that D-serine levels are decreased in schizophrenic patients (Bendikov et al., 2007; Hashimoto et al., 2003) and D-serine greatly ameliorated the symptoms of schizophrenia when administered together with conventional neuroleptics (Heresco-Levy et al., 2005). Schizophrenia is a complex disease: a collection of signs and symptoms of unknown etiology, primarily defined by observed signs of psychosis. At least 43 candidate genes associated with a risk for schizophrenia have been identified, but individual effect sizes are modest (Insel, 2010). Among the possible schizophrenia susceptibility genes, polymorphisms, and haplotypes in the genes encoding for DAAO, pLG72 and SR have been identified, reviewed in (Pollegioni and Sacchi, 2010). The human G72 gene encodes for pLG72, a protein present only in primates, which interacts with human DAAO (hDAAO) in astrocytes (Chumakov et al., 2002; Sacchi et al., 2008). We recently demonstrated that *in vitro* pLG72 inactivates hDAAO (Molla et al., 2006a,b) and that cellular concentrations of D-serine depend on expression of the active form of this flavooxidase. Therefore, we proposed a model for schizophrenia susceptibility in which a decrease in pLG72 expression yields an anomalously high level of DAAO activity and induces an excessive decrease in the local concentration of D-serine (Sacchi et al., 2008). Here, the subcellular localization of hDAAO and pLG72 represents a major concern: DAAO is a well-known peroxisomal enzyme targeted to this compartment by the C-terminal PTS1 sequence (Arnold et al., 1979; Moreno et al., 1999; Sacchi et al., 2008; Pollegioni and Sacchi, 2010). However, there is controversy concerning the subcellular distribution of pLG72: it has been proposed as a mitochondrial protein (Benzel et al., 2008; Kvajo et al., 2008), but in human cultured astrocytes it showed a perinuclear and tubular distribution (Sacchi et al., 2008). Indeed, only 5% of the latter cells displayed (cytosolic) overlapping signals for pLG72 and hDAAO, with a mean average of 8% colocalization. These observations suggested that pLG72 interaction with hDAAO is probably driven by specific spatiotemporal stimuli, whose features were elusive.

* Corresponding author at: Dip. Biotecnologie e Scienze Molecolari, Università degli studi dell'Insubria, Varese, Italy. Fax: +39 332 421500.
E-mail address: loredano.pollegioni@uninsubria.it (L. Pollegioni).

1044-7431/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.mcn.2011.06.001

In the present study we investigated the subcellular localization and interaction of hDAAO with pLG72 by using U87 glioblastoma cells transiently or stably transfected with plasmids encoding for fluorescent-tagged pLG72 and/or hDAAO. We solved the apparent discrepancy between the different subcellular localization of the two proteins and their putative interaction, enlightening the effect of this interaction on D-serine metabolism.

Results

hDAAO and pLG72 (apparently) localize to different cellular compartments

The hDAAO–pLG72 interaction has been previously studied *in vitro* by using different biochemical approaches (Chumakov et al., 2002; Sacchi et al., 2008). We also demonstrated that fusion of the GFP protein with hDAAO does not alter its biochemical properties (Caldinelli et al., 2010). As native hDAAO, the EGFP–hDAAO is fully active, binds one molecule of FAD per protein monomer (with a K_d of 7 μM), and interacts with pLG72, yielding a complex constituted by two hDAAO homodimers and two pLG72 molecules; indeed, the time course for inactivation of the EGFP–hDAAO–pLG72 complex closely resembles that observed for native hDAAO. To investigate subcellular localization (and interaction) of hDAAO and pLG72 in depth, U87 glioblastoma cells were transfected with plasmids encoding for the corresponding tagged proteins (see Supplemental Materials). U87 Clones stably expressing EYFP–hDAAO (U87 EYFP–hDAAO cells), hDAAO–EYFP (U87 hDAAO–EYFP cells), or pLG72–ECFP (U87 pLG72–ECFP cells) were then isolated in D-MEM medium to which G418 was added. Confocal microscopy shows that, in the U87 pLG72–ECFP clone, the signal corresponding to the expressed pLG72–ECFP protein displays a peculiar "spaghetti like" distribution around the nucleus (Fig. 1A). In contrast, the EYFP–hDAAO signal is near a punctuate subcellular structure (Fig. 1B) and differs from the (cytosolic) one observed for the U87 hDAAO–EYFP cells (in which the C-terminal peroxisomal targeting signal -SHL of the over-expressed protein is masked by the fused EYFP, Fig. 1C). Since DAAO is known to be a peroxisomal enzyme (Arnold et al., 1979; Moreno et al., 1999; Sacchi et al., 2008; Pollegioni and Sacchi, 2010), we used U87 EYFP–hDAAO cells in the present study, in addition to U87 pLG72–ECFP cells, as a cellular system model. In order to exclude an effect of the specific tag on the cellular distribution of the overexpressed proteins, similar experiments were also carried out using U87 cells stably expressing pLG72–FLAG and untagged hDAAO proteins (panels D and E of Fig. 1). In these cells the signal distribution of both proteins closely resembled that observed for pLG72–ECFP and EYFP–hDAAO, respectively.

Expression of the full-length, fluorescent fusion proteins in the selected, stable clones was confirmed by Western blot analysis. A single band corresponding to EYFP–hDAAO (66.4 kDa) is detected in U87 EYFP–hDAAO (Fig. 1F, lane 3, α-DAAO panel). A significant expression of the full-length pLG72–ECFP in the U87 pLG72–ECFP clone (Fig. 1F, lane 5, α-GFP panel) is also apparent: in addition to the 46-kDa band of the pLG72–ECFP fusion protein, a ~28-kDa band corresponding to the ECFP moiety was also detected.

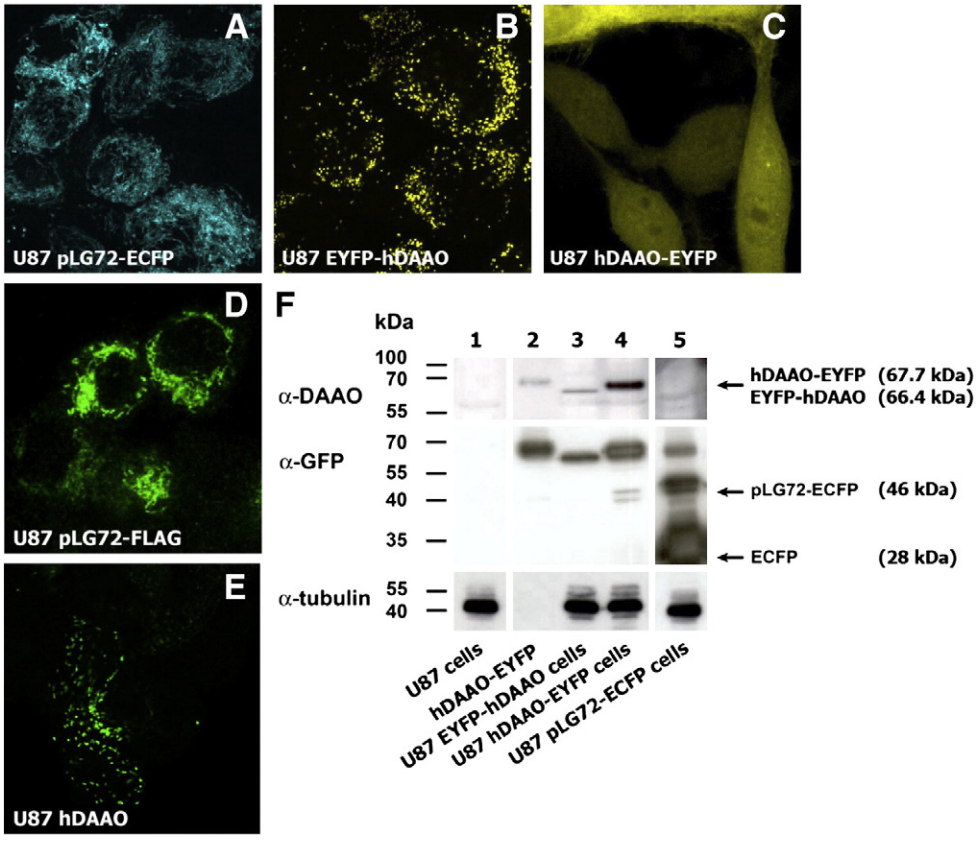

Fig. 1. Expression of chimeric fluorescent hDAAO and pLG72 proteins in stably transfected U87 glioblastoma cells (A–E). Confocal analysis showing distribution of the different fluorescent chimeric proteins (panels A–D) and untagged hDAAO (panel E) in selected U87 clones. pLG72–ECFP and pLG72–FLAG display a "spaghetti like", perinuclear distribution (A and D), while the punctuate localization of EYFP–hDAAO and untagged hDAAO (labeled using sheep anti-hDAAO and donkey anti-sheep Alexa 488 antibodies) indicate internalization into organelles (B and E). The hDAAO–EYFP shows a diffused, cytosolic distribution since the PTS1 signal at the C-terminus of hDAAO is masked by EYFP (C). F) Western blot analysis performed using rabbit anti-hDAAO and mouse anti-GFP antibodies confirms expression of the different fluorescent fusion proteins in transfected U87 cells. The same amount of sample (corresponding to 5×10^4 cells) was loaded in each lane, as further confirmed by using an anti-α-tubulin antibody as internal control (bottom panel). hDAAO–EYFP (0.03 μg, lane 2) recombinant purified protein was used as positive control. The level of endogenous hDAAO and pLG72 in U87 control cells is below the detection limit (≤0.005 μg for hDAAO and ≤0.025 μg for pLG72), see lane 1.