Identification of DNA-binding proteins that interact with the 5'-flanking region of the human D-amino acid oxidase gene by pull-down assay coupled with two-dimensional gel electrophoresis and mass spectrometry

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Abstract

D-Amino acid oxidase (DAO) is a flavoenzyme that metabolizes D-amino acids and is expected to be a promising therapeutic target of schizophrenia and glioblastoma. The study of DNA-binding proteins has yielded much information in the regulation of transcription and other biological processes. However, proteins interacting with DAO gene have not been elucidated. Our assessment of human DAO promoter activity using luciferase reporter system indicated the 5'-flanking region of this gene (-4289 bp from transcription initiation site) has a regulatory sequence for gene expression, which is regulated by multi-protein complexes interacting with this region. By using pull-down assay coupled with two-dimensional gel electrophoresis and mass spectrometry, we identified six proteins binding to the 5'-flanking region of the human DAO gene (zinc finger C2HC domain-containing protein 1A; histidine-tRNA ligase, cytoplasmic; molybdenum cofactor biosynthesis protein; 60S ribosomal protein L37; calponin-1; calmodulin binding protein and heterogeneous nuclear ribonucleoprotein A2/B1). These preliminary results will contribute to the advance in the understanding of the potential factors associated with the regulatory mechanism of DAO expression.

Key words: D-amino acid oxidase, 5'-flanking region, DNA-binding protein, DNA-interacting protein, pull-down assay, mass spectrometry.

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49 **1. Introduction**

50 D-Amino acid oxidase (DAO) is a flavoenzyme responsible for the oxidation of D-amino
51 acids to the corresponding imino acids and hydrogen peroxide (H_2O_2). The role of DAO in
52 the degradation of D-serine, a co-agonist of the N-methyl-D-aspartate receptor (NMDAR), is
53 proposed to contribute to the hypofunction of the NMDAR associated with schizophrenia [1-
54 4]. In addition, the H_2O_2 -generating property of DAO showed anti-tumor effect via inducing
55 oxidative stress and apoptosis. In astrocytes, DAO caused the cell death through the effects of
56 its products, not only H_2O_2 but also beta-hydroxypyruvate (HPA) from the D-serine
57 metabolism. The oxidative stress induced by DAO and energy depletion induced by
58 antiglycolytic agents e.g. 3-bromopyruvate (3BP), were combined to cause the anti-glioma
59 effect in the oxidative stress-energy depletion (OSED) therapy [5-7]. Taken together, this
60 flavoenzyme is proposed as an attractive therapeutic protein for glioblastoma and target
61 enzyme for schizophrenia. Thus, comprehensive understanding of the regulatory mechanism
62 of DAO expression and its activity would be important.

63 DNA-interacting proteins play important roles in many biological processes including
64 gene regulation. These proteins bind to DNA sequences or interact with other DNA-binding
65 proteins to regulate the gene expression. Identification of DNA-interacting proteins can lead
66 to comprehension of their role in gene regulation and contribute to the discovery of the
67 regulatory mechanism. Although a large number of investigations on DAO have been carried
68 out for many years, the detailed information of protein bound to DNA still awaits much more
69 research.

70 Here, we prepared proteins from nuclear fraction of LLC-PK₁ cells and isolated by a pull-
71 down assay using a probe corresponding to the 5'-flanking region of the human DAO
72 (hDAO) gene. These proteins were separated on polyacrylamide gel and analyzed by mass

spectrometry (MS). This report describes the novel findings on DNA-binding/interacting proteins of the hDAO gene, which may become one of the potential targets of further studies on regulatory factors of hDAO gene expression.

2. Materials and methods

2.1 Cell line

LLC-PK₁ cells, derived from pig kidney proximal tubules, were cultured in Medium 199 (Gibco, Life Technologies, New York, USA) supplemented with 5% FBS at 37°C under an atmosphere of 5% CO₂.

2.2 Generation of the luciferase reporter constructs

The clone 423G4 of RPCI-11.C human bacterial artificial chromosome (BAC) library, containing the hDAO gene, was obtained from Research Genetics (Invitrogen, Life Technologies, California, USA). The plasmid DNA was isolated using the alkaline-SDS method. In order to research for the 5'-flanking region of the hDAO gene, 4433 bp (-4289/+143) of the hDAO gene including the 5'-flanking region-exon1 was inserted into pGL4.15-luc2P/Hygro vector (Promega Corporation, Madison, USA). After the hDAO (-4289/+143) luc2P construct was established, it was purified with the small-scale preparation of the plasmid. The Endotoxin-free Plasmid Maxiprep kit (Sigma Aldrich, St. Louis, USA) was then used to prepared plasmid DNA in large quantities. The purity and concentration of plasmid DNA were determined using a spectrophotometer (DU®640 Spectrophotometer, Beckman, Ramsey, USA). The plasmid was then precipitated with isopropanol, adjusted to a final concentration of 1 mg/ml and ready to use for transient transfection.

2.3 Transient transfection and luciferase assays

All transfections were carried out in Opti-MEM medium (Gibco, Life Technologies, New York, USA) using the NEPA21 Electroporator Gene Transfection System (Nepa Gene Co., Ltd., Chiba, Japan). A total of 1×10^6 cells were co-transfected with 10 µg of plasmid DNA

of the hDAO (-4289/+143) luc2P construct and *Renilla* luciferase vector (phRL-TK, Promega Corporation, Madison, USA) at a ratio of 10:1. The pGL4.15-luc2P/Hygro vector was used in these experiments as a negative control. The pGL4.15-CMVp-luc2P vector containing the CMV promoter for high translational expression was used as a positive control. These vectors were also co-transfected with *Renilla* luciferase vector and assayed along with the hDAO (-4289/+143) luc2P. The cells after being transfected were cultured in 96-well plates. At 6 h post-transfection, the cells were washed with phosphate-buffered saline (PBS) and lysed with 50 μ l Passive Lysis Buffer (Promega Corporation, Madison, USA). The activities of firefly and *Renilla* luciferases were measured by using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, USA) according to the manufacturer's protocol. The luminescence intensities of firefly and *Renilla* luciferases were recorded using a microplate reader (Infinite M200, Tecan Austria GmbH, Grödig, Austria). Results were presented as relative firefly luciferase activity after normalization to internal control *Renilla* luciferase activity. All results were obtained in at least three independent experiments.

2.4 Preparation of proteins from the nuclear fraction

Nuclear fractionation was performed as described previously [8, 9] with some modifications. Briefly, LLC-PK₁ cells were seeded onto 15-cm dishes and incubated at 37°C, 5% CO₂ until cells reached 80% confluence. Cells were washed twice with cold PBS and harvested by scraping. Cell pellets were collected by centrifugation at 1,000 rpm, 4°C for 5 min, then resuspended in 1 ml of cold hypotonic buffer (42 mM KCl, 10 mM HEPES, 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride) with protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche Diagnostics GmbH, Mannheim, Germany) and homogenized while being incubated on ice for 30 min. The supernatant (cytosolic fraction) and pellets were separated by centrifugation at 600 x g, 4°C for 10 min. The pellets were resuspended in 1 ml of cold extraction buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in 2X PBS, 0.1 mM phenylmethylsulfonyl fluoride) with protease inhibitor cocktail. The pellets were broken by sonication. The protein extract was obtained as the supernatant (nuclear

fraction) by centrifugation at 99,000 x g, 4°C for 30 min.

Validation of the purity of the nuclear fraction was carried out by Western blots. The protein concentration was estimated using BCA kit (Thermo Scientific, Rockford, USA). Ten micrograms of protein from both nuclear fraction and cytosolic fraction were run side-by-side on a polyacrylamide gel (SuperSep™ Ace, Wako Pure Chemical Industries, Ltd., Japan) and transferred onto a polyvinylidene fluoride membrane (Immobilon®-P membrane, Millipore Corporation, Massachusetts, USA). Proteins were then probed with monoclonal antibodies for Histone H3 (1:2000, Cell Signaling Technology, Inc., Massachusetts, USA), GAPDH (1:50000, Sigma Aldrich, St. Louis, USA). The bound primary antibodies were detected by incubating with horseradish peroxidase-conjugated secondary antibodies (1:10000; GE Healthcare UK Ltd., Buckinghamshire, UK). Protein bands were visualized by ECL method (GE Healthcare UK Ltd., Buckinghamshire, UK).

2.5 Pull-down assay

The 5'-flanking region of the hDAO gene was amplified by PCR using 5'-biotin-labeled forward primer. The primer sequences were as follows: forward primer, 5'-GAGCTCTTAAGTGTCTGGCTG-3', reverse primer, 5'-TATAAGCTTCGGAAGTACCAAAGTCTGG-3'. The 5'-biotinylated DNA of the 5'-flanking region of the hDAO gene was immobilized to streptavidin beads following the manufacturer's protocol (Dynabeads® kilobase BINDER™ kit, Invitrogen Dynal AS, Oslo, Norway). Proteins in the nuclear fraction were incubated with 5'-biotinylated DNA beads on a rotating shaker at 4°C overnight. Following this incubation, the supernatant was removed. The beads were washed 3 times with cold PBS. After the last wash, the pull-down mixture was resuspended in distilled water at 70°C for 3 min to break the bond between streptavidin and biotin. The eluted proteins from the beads were subjected to polyacrylamide gel electrophoresis (PAGE) or two-dimensional gel electrophoresis followed by Coomassie blue or silver nitrate staining and MS analyses. The eluted proteins from the beads without the biotinylated DNA probe were used as control.

2.6 PAGE and Coomassie stain

The eluted proteins from pull-down assay were separated on a polyacrylamide gel (SuperSep™ Ace, Wako Pure Chemical Industries, Ltd., Japan) at 20 mA for 80 min. The gels were stained with Coomassie G-250 (GelCode Blue stain reagent, Thermo Scientific, Rockford, USA) following the manufacturer's protocol. Briefly, the gel was fixed in fixing solution (50% methanol, 5% acetic acid) for 20 min. The gel was washed with distilled water to remove the fixing solution. The gel was stained in Coomassie G-250 staining solution overnight. The stained gel was rinsed with distilled water several times.

2.7 Two-dimensional gel electrophoresis

The immobilized pH gradient (IPG) strips (Immobiline DryStrip pH 3-10, 7cm, GE Healthcare UK Ltd., Buckinghamshire, UK) were used to analyze the DNA-binding proteins to the 5'-flanking region of the hDAO gene. The IPG strips were rehydrated overnight at room temperature in rehydration buffer (7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.002% bromophenol blue, 2% IPG buffer 3-10). Proteins obtained from the pull-down assay were treated with the 2-D Clean-Up Kit (GE Healthcare Life Sciences) before being used for two-dimensional gel electrophoresis. A total of 5 µg of proteins was loaded onto the IPG strips and focused using an Ettan IPGphor II (GE Healthcare UK Ltd., Buckinghamshire, UK) with a ceramic manifold as follows: step and hold at 300 V for 1 h, gradient to 1000 V for 30 min, gradient to 5000 V for 30 min, step and hold at 4000 V for 30 min. Prior to PAGE, the IPG strips were equilibrated in equilibration buffer containing 6 M urea, 2% SDS, 23% glycerol, 0.002% bromophenol blue, 1% DTT, and 75 mM Tris-HCl pH 8.8 for 15 min, followed by equilibration in buffer containing 6 M urea, 2% SDS, 23% glycerol, 0.002% bromophenol blue, 5% iodoacetamide, and 75 mM Tris-HCl at pH 8.8 for 15 min. The second dimension separation was run on 10% polyacrylamide gels at 10 mA per gel for 15 min, 20 mA per gel for 80 min. After electrophoresis, gels were fixed and stained with silver nitrate.

2.8 Silver nitrate staining

The gel was fixed in fixing solution (50% methanol, 5% acetic acid) for 20 min followed by washing with washing solution (50% methanol) for 10 min and with distilled water for 10 min. The gel was soaked in a sensitization solution (0.02% sodium thiosulfate) for 1 min and washed three times in distilled water (about 1 min each) to increase the sensitivity and contrast of staining. The gel was incubated in 0.1% silver nitrate (Wako Pure Chemical Industries, Ltd., Japan) for 2 h at 4°C. It was then washed in distilled water three times (about 1 min each) and developed in developing solution (2% sodium carbonate, 0.04% formaldehyde) until the protein spots become visible. The gel was then washed three times with distilled water (about 1 min each) before it was incubated with stopping solution (5% acetic acid) for 10 min. The gel was washed again in distilled water three times (about 5 min each) and stored at 4°C in a plastic sealing bag until trypsin digestion.

2.9 Sample preparation for MS analysis

After silver staining, visible spots on the experimental and control gels were compared. The unique spots on the experimental gel were excised and prepared for MS analysis. The gel piece was destained with 100 µl of destaining solution (15 mM potassium ferricyanide, 50 mM sodium thiosulfate) for 10 min and washed with 500 µl of distilled water for 15 min. The above destaining-steps were repeated twice. Then the gel piece was dehydrated in 100 µl of dehydration solution (50% acetonitrile in 25 mM ammonium bicarbonate) for 10 min. The gel piece was further incubated for 5 min after adding 100 µl of acetonitrile. The gel was then dried in a centrifugal concentrator (Micro Vac, Tomy Seiko Co. Ltd., Tokyo, Japan). In-gel trypsin digestion was started by the addition of 15 µl of a 10 ng/µl trypsin solution (Sequencing Grade Modified Trypsin, Promega Corporation, Madison, USA) in 40 mM ammonium bicarbonate and incubated on ice for 45 min. The trypsin on the outside of the gel piece was removed. The gel was covered with 10 µl of 25 mM ammonium bicarbonate and incubated overnight at 37°C. To elute the peptides, 50 µl of elution buffer (50% acetonitrile, 5% trifluoroacetic acid (TFA) was added to the gel and incubated for 10 min. The supernatant

was collected and concentrated to a final volume of 10 μ l in a centrifugal concentrator. Desalting was subsequently performed using Zip-tip (ZiptipC18, Merck Millipore Ltd., County Cork, Ireland). Peptides were eluted from the Zip-tip with 2 μ l of matrix solution (5 mg/ml α -cyano-4-hydroxycinnamic acid, 50% acetonitrile, 0.1% TFA) and spotted on the MALDI plate ABI 01-192-6-AB (Applied Biosystems, California, USA).

2.10 MS analysis and database search

The samples were analyzed using the MALDI-TOF/TOF mass spectrometer (4700 Proteomic Analyzer, Applied Biosystems, California, USA). Mass spectra were recorded in the positive-ion mode, averaging 2500 laser shots per spectrum. To identify the proteins, mass spectra (excluding trypsin autolytic peptides and other known background ions) were searched against the MASCOT search engine (Matrix Science Limited), run on an in-house server using other primates or other mammalia Swiss-Prot database. The search was performed using trypsin digestion allowing 0 or 1 missed cleavage, specifying carbamidomethyl-Cys as a fixed modification, and setting a peptide mass tolerance of ± 1.2 Da. Candidates with either Mascot score greater than 51 (other primate database) or 54 (other mammalia database) were considered significant ($p < 0.05$).

3. Results and discussion

3.1 5'-flanking region of the hDAO gene contains the regulatory sequence for its expression

To map regulatory functions of the 5'-flanking region of the hDAO gene, we subcloned 4433 bp (-4289/+143) of the hDAO gene including the 5'-flanking region-exon1 into pGL4.15-luc2P/Hygro vector. This reporter construct was co-transfected with *Renilla* luciferase vector (as a control for transfection efficiency) into LLC-PK₁ cells. This pig kidney proximal tubules cell line (LLC-PK₁) was specifically chosen because this has been used as an appropriate in vitro model for DAO research. The empty vector (pGL4.15-luc2P/Hygro) and CMV promoter vector (pGL4.15-CMVp-luc2P) were used as a negative and positive

control, respectively. The results showed the luciferase activity promoted by fragment (-4289/+143) was approximately 38.1-fold higher than that of empty vector (Fig. 1). Based on this observation, the 5'-flanking region (-4289 bp) is considered to contain the regulatory sequence for hDAO gene expression.

3.2 Identification of DNA-binding/interacting proteins of the hDAO gene by MS analysis: 6 candidates may bind to or interact with the hDAO gene

Since the existence of regulatory sequence in the 5'-flanking region was expected, we focused our research on this region. We employed an approach to identify DNA-binding proteins using pull-down assay coupled with two-dimensional gel electrophoresis and MS (Fig. 2).

The results from PAGE followed by Coomassie blue staining revealed that many unknown proteins bound to the 5'-flanking region of the hDAO gene (Fig. 3A). To determine the binding proteins, we performed two-dimensional gel electrophoresis followed by silver nitrate staining and MS analyses. The unique spots present in the experimental gel, but not in the control were numbered and marked by circles (Fig. 3B). These protein spots were submitted to MS analyses. Proteins with a significant Mascot score ($p < 0.05$) were shown in Table 1. In summary, we could identify ten proteins.

Of the ten identified proteins, we considered the six proteins described below as potential interacting factors with the hDAO gene based on Gene Ontology annotations (Uniprot database, <http://www.uniprot.org>). Mass spectra of these six proteins were shown in Fig. 4. Zinc finger C2HC domain-containing protein 1A (spot 1) was annotated as a metal ion binding protein; it may be a component of a metal ion-regulated sequence-specific DNA binding protein (GO: 0046872). Histidine-tRNA ligase, cytoplasmic (spot 4) and molybdenum cofactor biosynthesis protein (spot 8) were annotated as nucleotide binding proteins (GO: 0000166). 60S ribosomal protein L37 (spot 5) was annotated as an RNA binding protein (GO: 0003723). Calponin-1 (spot 7) was annotated as an actin binding protein (GO: 0003779) and calmodulin binding protein (GO: 0005516), which may be related to

protein binding and/or transcription factor activity. Heterogeneous nuclear ribonucleoprotein A2/B1 (spot 10) was annotated as an RNA binding protein (GO: 0003723) and nucleotide binding protein (GO: 0000166). Thus, we proposed these proteins as candidates for the DNA-binding/interacting proteins of the 5'-flanking region of the hDAO gene.

These proteins were involved in protein biosynthesis or translation (histidine-tRNA ligase, cytoplasmic and 60S ribosomal protein L37), signal transduction (GTP binding-molybdenum cofactor biosynthesis protein), or multiple cellular processes including transcription and translation (heterogeneous nuclear ribonucleoprotein A2/B1). Two other proteins may be involved in the regulation of gene expression via regulated sequence-specific DNA (zinc finger C2HC domain-containing protein 1A) or through protein-protein interactions to modulate transcription (calponin-1). Although the actual functions of these proteins in the regulation of DAO gene expression remains unclear, we provided a novel insight into the search for the DNA interacting proteins of DAO. These preliminary results will contribute to advances in our understanding of the potential factors associated with the regulatory mechanisms of DAO expression.

4. Conclusion

We used a method based on pull-down assay coupled with two-dimensional gel electrophoresis and MS for the identification of DNA-binding/interacting proteins of the 5'-flanking region of the hDAO gene. Here, we have identified six potential DNA-binding/interacting proteins, which may modulate hDAO gene expression via the regulatory sequence existing in 5'-flanking region. Although it has yet to be confirmed whether these DNA-binding/interacting proteins are linked to brain pathology with DAO, our results contribute to a deeper understanding of the importance of cis-acting sequences in DAO transcription and trans-acting factors involved in DAO gene expression.

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FIGURE LEGENDS

Fig. 1 The 5'-flanking region of the hDAO gene contains the regulatory sequence for gene expression. (A) The structure of the hDAO gene comprises 11 exons and 10 introns [10]. The top panel illustrates hDAO mRNA. The middle panel displays the structure of hDAO gene. Exons are numbered and indicates by boxes. Closed and open boxes illustrate coding and untranslated regions, respectively. ATG indicates the translation starting codon. TGA indicates the translation stop codon. The bottom panel shows the hDAO 5'-flanking-exon1 region, inserted into pGL4.15-luc2P/Hygro to generate the hDAO (-4289/+4929) luc2P construct employed for luciferase assay and further proteomics research. The arrow represents the transcription initiation site. (B) The hDAO (-4289/+143) luc2P construct demonstrated that the 5'-flanking region of the hDAO gene contains the regulatory sequence for its expression. pGL4.15-luc2P/Hygro and pGL4.15-CMVp-luc2P were used as a negative (Empty) and positive control (CMV), respectively. Luciferase activities were measured 6 h post-transfection and calculated by the average of normalized luciferase activities (the ratio of firefly to *Renilla* luciferase values). Values are represented as means $\pm$ SE from at least three independent experiments.

Fig. 2 Working flow for the identification of DNA-binding/interacting proteins of the 5'-flanking region of the hDAO gene. (A) PCR product of the 5'-biotinylated DNA of the 5'-flanking region of the hDAO gene was immobilized on streptavidin magnetic beads and used as a probe in pull-down assay. (B) Nuclear fraction and cytosolic fraction of LLC-PK₁ cells were run side-by-side on the same blot and then probed separately against each of two primary antibodies, Histone-H3 (marker protein of nuclear fraction), GAPDH (marker protein of cytosolic fraction), respectively, to validate fraction purity. Proteins in the nuclear fraction were subjected to the bead complex in pull-down assay. (C) Flow chart for the pull-down assay coupled with gel electrophoresis and mass spectrometry.

Fig. 3 DNA-binding/interacting proteins of the 5'-flanking region of the hDAO gene were identified by proteomics analyses. Proteins eluted from the beads in pull-down assay were subjected to gel electrophoresis followed by staining and/or mass spectrometry analysis. (A) The results of polyacrylamide gel electrophoresis after staining with Coomassie blue. (B) The results of two-dimensional gel electrophoresis after staining with silver nitrate. The unique spots present in the experimental gel, but not in the control gel were collected for mass spectrometry analysis. These spots are numbered and marked by circles.

Fig. 4 Mass spectra of six DNA-binding/interacting proteins. This figure only shows the mass peaks that match to peptides from the identified proteins.

TABLE

Table 1 A list of protein spots identified by mass spectrometry analysis.

Spot Number ^a	Protein name	Gene name	Mascot score ^b	pI/MW (Da)		p value
				Theoretical	Estimated	
1	Zinc finger C2HC domain-containing protein 1A	ZC2HC1	52(51)	9.9/35683	4.0/42000	0.044
2	Fatty acid-binding protein, heart	FABP3	62(54)	7.8/14862	5.5/60000	0.008
3	Cyclin-G1	CCNG1	60(54)	9.2/34598	6.0/56000	0.012
4	Histidine-tRNA ligase, cytoplasmic	HARS	56(51)	5.5/57904	6.5/44000	0.018
5	60S ribosomal protein L37	RPL37	60(54)	11.7/11299	7.0/44000	0.012
6	Transferrin receptor protein 1	TFRC	62(54)	5.9/86467	7.5/40000	0.008
7	Calponin1	CNN1	60(54)	8.9/33377	8.0/48000	0.013
8	Molybdenum cofactor biosynthesis protein	MOCS1	56(54)	9.2/70356	9.0/65000	0.033
9	Glutamate receptor ionotropic, kainate 1	GRIK1	53(51)	6.6/104688	9.2/46000	0.036
10	Heterogeneous nuclear ribonucleoprotein A2/B1	HNRNPA2B1	62(54)	8.7/36041	10.0/36000	0.008

^a Protein spots were excised from the gel shown in Fig. 3.

^b Two different databases from Swiss-Prot were selected: other primates or other mammalia. Mascot scores greater than 51 (other primates database) or 54 (other mammalia database) were significant ($p < 0.05$).

Figure(s)

A

B

Figure 1

Figure 2

A

B

Figure 3

Figure 4