N-terminal Extension of Canine Glutamine Synthetase Created by Splicing Alters Its Enzymatic Property*

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It was found that an extra exon exists in the first intron of glutamine synthetase gene, generated by means of alternative splicing (Shin, D., Park, S., and Park, C. (2003) Biochem. J. 374, 175–184). Inclusion of this exon decreased the translation of glutamine synthetase (GS) in human, dog, and mouse. When translated in vitro with the canine GS transcript containing the exon, we obtained two different species of GS enzymes. Besides the known 45-kDa protein, the extended form of GS was identified with additional amino acids on its N-terminal end. An upstream ATG in the extra exon served as a translation initiator for the long form of GS. When the long transcript was translated in vivo in animal cells, only the long GS was expressed. On the other hand, the long GS is less predominant relative to the short one in certain tissues including brain and liver. Subcellular fractionation of canine brain revealed that the long GS is present in all cellular compartments as is the short one, which is consistent with fluorescence microscopy data obtained with green fluorescent protein fused to GS. The short (SGS) and long (LGS) forms of canine GS were purified in Escherichia coli and shown to have similar $K_m$ values for L-glutamate and hydroxylamine. However, the $K_m$ values for ATP were slightly altered, 1.3 and 1.9 mM for the short and long GSs, respectively. The $K_s$ for L-methionine-S-sulfoximine (MSOX), a highly potent ATP-dependent inactivator of GS, were considerably different such that the values are 0.067 and 0.124 mM for the short and long GSs, respectively. When the intrinsic fluorescence of tryptophans were monitored upon bindings of chloride and metal ions without any effect on the oligomeric state, the pattern of quenching in LGS was significantly different from that of SGS. Taken together, the N-terminal extension in the long isoform of GS induces a conformational change of core enzyme, leading to a change in affinity to its substrates as well as in the effector-induced conformational alterations.

Glutamine synthetase (GS\(^{1}\); EC 6.3.1.2; L-glutamate ammonium ligase) is an enzyme that catalyzes the ATP-dependent conversion of glutamate and ammonia into glutamine, and thus plays a critical role in eliminating the excitotoxins glutamate in animal brains (1). The expression of eukaryotic GS is regulated at transcriptional and post-transcriptional levels and becomes unstable with oxidation mediated by metal or free radical peptides, generated from a fragmentation of $\beta$-amyloid, and by growth hormone or glutamine (2).

The mammalian GS protein has been reported to form an eight-subunit oligomer (3) with unknown three-dimensional structure. The bindings of chloride and magnesium ions to allosteric and activator sites of GS, respectively, cause changes in GS conformation, without affecting its oligomerization (4). As an essential trace metal in vivo, the manganese ion is mostly (~80%) bound to GS in astrocytes (5), although its concentration is variable in other mammalian tissues. Magnesium is also bound to mammalian GS in vivo (6). L-methionine-S-sulfoximine (MSOX), a structural analogue of L-glutamate, is converted to MSOX-phosphate, mimicking the tetrahedral intermediate formed between an enzyme-bound $\gamma$-glutamyl phosphate and ammonia at the active site of GS (7). This reaction product becomes an irreversible and noncovalent inhibitor of the enzyme. MSOX was originally isolated from nitrogen chloride-treated zein and characterized as a toxin causing induction of convulsions, hysteria, and epileptic fits in a number of animals (8). The $K_s$ values of MSOX are 105, 1, 161, and 100 $\mu$M for GS proteins of sheep, Escherichia coli, pea, and spinach, respectively (8). Despite its crucial role in many neurological diseases (9–13), information on mammalian GS is rather scarce, and inconsistencies are found in various reports on its mass and subunit arrangements (8).

In vertebrate, compartment-specific GS isozymes are produced from a single gene and targeted to either mitochondria or cytosol in a tissue-specific manner (14). The N terminus of GS is variable in size and sequence and serves as a subcellular targeting signal (15). Besides the known 44-kDa protein, the GS-like protein of 54 kDa was reported from human brain. This enzyme is mostly found in crude mitochondrial fractions and possesses higher hydroxylamine-L-glutamate transferase activity than the 44-kDa GS (16, 17).

Modulation of the translational efficiency of mammalian GSs by 5′ transcript leader region has been suggested as a mechanism for regulating its expression (18). This phenomenon was studied in detail with the canine GS, a 45-kDa enzyme with 373 amino acids, which is translated from the gene of ~10 kb in length, being organized into seven exons and six introns. An extra exon is found in the first intron of glutamine synthetase gene, which is subjected to an alternative splicing. The long transcript with extra 5′-UTR is translated less efficiently than the short one. This translational regulation is partially due to an abortive initiation at the upstream ATG located in the extra exon (18). It is known that the 5′ transcript leader regions in most vertebrate mRNAs are less than 100 nucleotides long.

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(19). However, two thirds of the oncogenes and other genes associated with growth and differentiation have several hundred nucleotides of transcript leader region. An AGT within this leader sequence is suggested to be involved in the regulation of downstream translation either by generating a peptide (20) or by promoting an alternative in-frame translation (21).

During the analysis of translation of GS gene in canine brain tissue, the long transcript with the extra 5′UTR is exclusively translated into the 49-kDa of GS protein. This protein was characterized to have different enzymatic properties, presumably due to a conformational alteration.

**EXPERIMENTAL PROCEDURES**

Reagents and Primers—All reagents used in the present study were purchased from Sigma, unless otherwise stated. PCR primers for subcloning were synthesized by Genotech co. (Taejon, Korea). PCR products were separated by agarose gel electrophoresis, purified using QIAquick Gel Extraction kits (Qiagen). All plasmid constructs were sequenced using the Big Dye termination kits for the ABI- Prism 3100 DNA sequencer (PerkinElmer Life Sciences).

Animal Tissues—Tissues from the frontal lobe, heart, skeletal muscle, kidney, and liver of dog (Sapsari, a Korean breed, 22) were obtained from an adult animal, with an approval by the University Animal Care and Use Committee at KAIST (Taejon, Korea). Rat brains were obtained from an adult animal of the FVB strain, which was purchased from Daehan Breeding Center Co. Ltd (Seoul, Korea) and housed under controlled temperature and lighting (22 °C with 12-hour light-dark cycle) with free access to food and water, according to the National Institutes of Health guideline for the care and use of laboratory animals. The brain tissue of human was a mixture of samples from the parietal lobe, cortex, and white matter, kindly provided by Chong-Jai Kim (Seoul National University, College of Medicine, Seoul, Korea), with an informed consent of 16-year-old male who died of primitive neuroectodermal tumor in the chest wall and with an approval by the Ethical Committee of Seoul National University Hospital (Seoul, Korea).

**In Vitro Transcription and Translation**—The short and long transcripts of canine GS, covering the exon 1 to exon 7, were reverse transcribed with oligo(dT)12-18, amplified with primers cGSBanHI-f (5′-GGGGATCCAGCGAGCCGAGGAAGCGG-3′; AF544242, nt 1–20) and cGSXhoI-r1 (5′-GGGTCGACCTTAGTCTGTCGAC-3′; AF544242, nt 128–142) and cGSXhoI-r2 (5′-GGGCTCGAGGTTTGGTACTGGAGG-3′; AF544242, nt 1366–1349), and subcloned into the BamHI and Xhol sites of pcDNA3.1 (+) (Invitrogen), yielding pSGS and pLGS, respectively. The linearized templates of 5 μg by digestion with XhoI restriction enzyme were used in *in vitro* transcription using the mMESSAGE mMACHINE™ kit (Ambion) to obtain capped transcripts. The integrity of transcripts was assessed by visualizing on denaturing 1.5% agarose gel electrophoresis. The capped transcripts were carried out in rabbit reticulocyte lysates (Promega). Equimolar amounts of capped in *in vitro* transcripts were used as templates for protein synthesis in a 50 μl reaction mixture containing 35 μl of reticulocyte lysate, 20 μCi of [35S]methionine (Amersham Biosciences), amino acid mixture (without methionine), and 40 units of RNasin (Promega). After 90 min incubation at 30 °C, the synthesized protein products were analyzed by electrophoresis on 10% SDS-polyacrylamide gel and visualized by autoradiography.

Western Blotting Analysis—Protein samples were heat denatured in the presence of 2-mercaptoethanol and SDS and separated electrophoretically on a 10% SDS-polyacrylamide gel under denaturing conditions. The proteins were then transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell), and reacted sequentially with a mouse anti-glutamine synthetase monoclonal antibody (CHEMICON international) and an horseradish peroxidase-conjugated mouse anti-mouse IgG secondary antibody (Amersham Biosciences). The GS protein bands were visualized with ECL™. Western blotting detection reagents (Amersham Biosciences) on an x-ray film. The mixture contained of 100 μm imidazole-HCl (pH 7.2), 50 μM sodium l-glutamate (pH 7.2), 25 μM 2-mercaptoethanol, 10 mM ATP (pH 7.2), 20 mM MgCl2, and 125 μM of freshly titrated hydroxylamine (pH 7.2). The reaction mixture of 500 μl was pre-incubated for 2 min at 37 °C to allow formation of an amorphous magnesium hydroxide precipitate of the enzyme. The reaction was quenched at 15 min with 750 μl of 0.3 M FeCl3/0.67 M HCl/0.2 M trichloroacetic acid, and the absorbance was read at 535 nm against a blank lacking the enzyme. The controls containing purified GS without ATP or l-glutamate showed no absorbance. All absorbance measured were within the range of the FeCl3/0.67 M HCl/0.2 M trichloroacetic acid standard curve. The unit of GS activity was defined as the amount of enzyme catalyzing the synthesis of 1 μmol of product in 1 min at 37 °C under the conditions given above. The reaction velocity was expressed as units per mg of protein.

**Fluorescence Spectroscopy—**1 μg of GS protein in 10 mM HEPES-
**RESULTS**

**Novel Translation Initiation from the Alternative Transcript of Canine Glutamine Synthetase Gene**—It was found previously that alternatively spliced GS transcript acquiring extra 5'-UTR decreased the normal translation (18). In an effort to obtain further evidences on this observation, we carried out an *in vitro* translation of transcripts made from the GS plasmids in rabbit reticulocyte lysate. Although the short transcript generated a 45-kDa protein (SGS), the long one with extra 5'-UTR gave rise to a 49-kDa protein (LGS) with a small amount of SGS (Fig. 1A). This indicates that translation occurs from an upstream ATG (uATG) at −120 (Fig. 2) located in the extra exon, resulting in an in-frame 40 amino acids extension of the N-terminal SGS (Fig. 2).

Because the rabbit reticulocyte, a highly specialized cell, is known to have ill-balanced translation system (28), expressions of the GSs were analyzed transiently in 293T cells (Fig. 1B). The 293T cells transfected with the plasmid containing cDNA for SGS (pSGS) produced a band of expected size (45 kDa) on Western blot, whereas the LGS plasmid (pLGS) generated only the 49-kDa protein band, which is different from the result of *in vitro* translation (Fig. 1A). When both pSGS and pLGS were transfected, equal amounts of proteins were expressed. The same patterns of GS expressions were obtained for cDNAs of SGS and LGS in SK-N-SH cells (data not shown), suggesting that the primary role of long GS transcript is to produce the LGS protein in vivo.

**The Long Form of GS Is Found in Canine Brain and Liver Tissues**—To examine the presence of LGS in the canine tissue, soluble extracts from the frontal lobe, heart, skeletal muscle, kidney, and liver tissues were separated on SDS-PAGE and blotted with monoclonal antibody raised against the mouse GS. The canine GSs are highly expressed in brain, moderately in kidney, and liver tissues were separated on SDS-PAGE and blotted with monoclonal antibody raised against the mouse GS. The canine GSs are highly expressed in brain, moderately in kidney, and liver, and barely in other tissues (Fig. 3B). To examine the presence of LGS in the canine brain and liver tissues, soluble extracts from the frontal lobe, heart, skeletal muscle, kidney, and liver tissues were separated on SDS-PAGE and blotted with monoclonal antibody raised against the mouse GS. The canine GSs are highly expressed in brain, moderately in kidney, and liver, and barely in other tissues (Fig. 3B). To examine the presence of LGS in the canine brain and liver tissues, soluble extracts from the frontal lobe, heart, skeletal muscle, kidney, and liver tissues were separated on SDS-PAGE and blotted with monoclonal antibody raised against the mouse GS. The canine GSs are highly expressed in brain, moderately in kidney, and liver, and barely in other tissues (Fig. 3B). To examine the presence of LGS in the canine brain and liver tissues, soluble extracts from the frontal lobe, heart, skeletal muscle, kidney, and liver tissues were separated on SDS-PAGE and blotted with monoclonal antibody raised against the mouse GS. The canine GSs are highly expressed in brain, moderately in kidney, and liver, and barely in other tissues (Fig. 3B). To examine the presence of LGS in the canine brain and liver tissues, soluble extracts from the frontal lobe, heart, skeletal muscle, kidney, and liver tissues were separated on SDS-PAGE and blotted with monoclonal antibody raised against the mouse GS. The canine GSs are highly expressed in brain, moderately in kidney, and liver, and barely in other tissues (Fig. 3B). To examine the presence of LGS in the canine brain and liver tissues, soluble extracts from the frontal lobe, heart, skeletal muscle, kidney, and liver tissues were separated on SDS-PAGE and blotted with monoclonal antibody raised against the mouse GS. The canine GSs are highly expressed in brain, moderately in kidney, and liver, and barely in other tissues (Fig. 3B).
in 293T cells. The 293T cells transfected with the plasmid containing cDNA for the short transcript of human GS produced the band of expected size (45 kDa), so did the long one with less amount of protein (data not shown). The short transcript of mouse GS expresses the 45-kDa protein, whereas the long one produces none (not shown), presumably due to translational repression as described previously (18).

Intracellular localization of GS protein was analyzed by subcellular fractionation of the canine brain, which were blotted with GS antibody. Absolute amounts of both enzymes were varied in different fractions, i.e. cytosol (S3), endoplasmic reticulum/plasma membrane (P3), nuclei (P1), and other particulate fraction (P2). However, their ratios are about the same, suggesting that the extra 40 amino acids are not associated with specific targeting. This pattern of SGS/LGS distribution is consistent with the result of subcellular localization of the GS proteins fused to GFP, produced from pEGFP-SGS and pEGFP-LGS. When two constructs were transfected into Madin-Darby canine kidney cells, the GS-GFP fusion proteins were expressed evenly in all subcellular regions (Fig. 4B).

### Comparisons of $K_m$ and $V_{max}$ in SGS and LGS

| Substrate | SGS | LGS |
|-----------|-----|-----|
| $K_m$ | 0.067 ± 0.0030 | 0.124 ± 0.0051 |
| $V_{max}$ | 0.32 ± 0.0157 | 0.31 ± 0.0090 |

Units for $V_{max}$ are $\mu$mol/min per nM of protein, while units for $K_m$ are millimolar. Calculated errors correspond to the 99% confidence intervals.

The Glutamine Synthetase Isoforms Differ in Their Enzymatic Properties—The two different canine GS genes were cloned into an inducible pET21b vector, from which the enzymes were purified to homogeneity. The colorimetric synthetase reaction assay with l-glutamate, hydroxylamine, and ATP as substrates revealed that $K_m$ values of SGS and LGS are 1.1 ± 0.12 and 1.3 ± 0.14 mM for l-glutamate, 1.6 ± 0.41 and 1.7 ± 0.31 mM for hydroxylamine, and 1.3 ± 0.24 and 1.9 ± 0.23 mM for ATP, respectively (Table I). The values for l-glutamate and hydroxylamine are similar in both enzymes, but the affinity for ATP is higher in SGS than in LGS. It is known that MSOX, with the presence of ATP, is phosphorylated by GS, leading to an irreversible and non-covalent inhibition of the enzyme (27). When MSOX was titrated in the presence of 50 mM l-glutamate, the $K_i$ values were 0.067 and 0.124 mM for SGS and LGS, respectively (Table I), indicating that the affinity for MSOX is lower in LGS than in SGS. The affinity changes for ATP and MSOX suggest that the binding sites for both...
substrates are altered due to the presence of N-terminal 40 amino acids of LGS.

Fluorescence Detection of Conformational Differences in GSs upon Bindings of Various Effectors—Maurizi et al. (4) demonstrated that bindings of various effectors, such as chloride, manganese, and magnesium, to allosteric or activator site of the bovine brain GS cause a conformational change as monitored by fluorescence quenching, without affecting the oligomerization state. There are seven tryptophans and fifteen tyrosines in the short GS protein, whereas the long one contains an extra tryptophan in the N-terminal peptide (Fig. 2). To assess whether the extended peptide of LGS affects the conformation of GS, we monitored fluorescence quenching by the presence of KCl, MnSO₄, or MgSO₄, each treated alone or combined together. The fluorescence changes are shown here as the ratio of fluorescence (F) for the sample to that of SGS.
enzyme itself (Fig. 5, $F_p$). When excited at 295 nm for tryptophan, addition of MnSO$_4$ to SGS resulted in fluorescence quenching of about 15%, whereas MgSO$_4$ increased fluorescence of 30% (Fig. 5). In contrast, LGS shows 34% quenching with MnSO$_4$, and only 8% increase with MgSO$_4$. Nevertheless, the patterns of fluorescence changes are similar. However, the tryptophan fluorescence of SGS increased −133% by an addition of 10 mM chloride, whereas no change was observed in LGS. Further addition of MnSO$_4$ and MgSO$_4$ to the chloride-treated SGS resulted in 85% quenching relative to the untreated. For LGS, quenching was 17% with MnSO$_4$, whereas MgSO$_4$ increased fluorescence of about 5−6%. The notable differences in fluorescence between SGS and LGS were observed upon chloride binding, with enormous increase in SGS. Based on the fact that chloride ion binds to the allosteric sites of GS, such fluorescence changes may reflect conformational differences between the two enzymes. On the other hand, the basal levels of tryptophan fluorescence are different between SGS and LGS such that the emission of LGS at 338 nm is 1.5-fold higher than that of SGS. The difference could be due to either the presence of extra tryptophan residue in LGS or the change in microenvironment of other tryptophans, or both. To exclude the possibility that the LGS is caused by its oligomerization, we examined oligomeric states of the enzymes by gel-filtration chromatography. As shown in Fig. 6, the degrees of multimerization in both proteins are similar, in which the majority exists as octamer of 70−73%. This suggests that the fluorescence changes in the GS proteins are not associated with their quaternary structures.

**DISCUSSION**

We characterized two isoforms of GS proteins, 45 and 49 kDa, translated from one functional gene. The long form was generated by in-frame translation from the upstream ATG located in the extra exon of long GS transcript. The extra peptide did not reveal any sequence similarity in the data base search. Because the sequence analysis predicted O-linked glycosylation site for N-acetylglucosamine at +40 position (threonine) of LGS, we treated LGS with β-acetyl hexosaminidase or with sodium hydroxide for β-elimination (29). However, the result was negative (data not shown), although we cannot completely rule out the possibility of other modifications including phosphorylation.

In a previous study (18), it was shown that the ratio of the short to long transcripts of canine GS was similar in various brain tissues. However, relative ratio of the proteins was different, with far more SGS (Fig. 3A), implying that there is a difference in stability or translation between the transcripts of LGS and SGS. The known regulators of GS expression at transcriptional level include glutamine, growth hormone, and oxidations mediated by metal or free radical peptides generated by a fragmentation of β-amylod (2).

Most eukaryotic mRNAs contain a short recognition sequence that facilitates initial binding of mRNA to the small subunit of the ribosome (19). The sequence information of the long UTR (Fig. 2) as well as the analyses of in vitro (Fig. 1A) and in vivo (Fig. 1B) translations indicated that the synthesis of LGS depends on the uATG codon located at −120. Efficiency of translation from the ATG codon in eukaryotes relies on an optimal translation initiation context (particularly $A_u^3$ and/or $G_t^4$). Unlike the regular ATG at +1, the translation start site of LGS (−120 ATG) has a G at +4, but not A at the −3 position, presumably serving as less efficient translation initiators. Nevertheless, LGS seems to be expressed from the long transcript at higher concentration in vitro than SGS. Interestingly, the long GS transcript produces only LGS in vivo (Fig. 1), although the SGS contains an ATG codon with conserved Kozak sequence (both $A_u^3$ and $G_t^4$). This might be due to a read-through translation of LGS from uATG without stopping at the SGS initiation codon (+1 position in Fig. 2).

In the case of human GS, two uATGs exist at −326 and −317 nucleotides upstream of the authentic ATG (18). However, a stop signal is found at −248 position, which is 78 and 69 nucleotides downstream of the uATGs. This stop signal may allow the re-initiation of SGS translation in the long transcript of human GS. In the mouse GS exon, one uATG is located at −83 nucleotides upstream of ATG, which is in out-of-frame from the coding sequence. The translation from uATG may encounter a stop codon appeared after the initiation ATG, and thus we speculated that the abortive translation from uATG inhibits the authentic SGS translation by skipping the normal initiation.

The heterogeneity of GS, i.e. existence of several forms inside one taxon, is well known and widespread (14). Most of subtypes of GS are compartmentalized in different subcellular organelles (15). The human genome contains at least five GS homologs (30). There are at least two enzymes possessing GS activities in the human brain, one oligomeric enzyme consisting of 44 kDa subunits (GS) and the other with 54 kDa subunits (GS-like protein) that is enriched in mitochondrial fraction (16). One may speculate that the addition of N-terminal amino acids affects functional activities or intracellular location of the GS protein. When we examine subcellular localizations of SGS and LGS in mammalian cell or brain tissue, we were unable to find any differences in compartmental localization between LGS and SGS in canine brain (Fig. 4).

We compared enzymatic properties of the two enzymes, in which the affinities for l-glutamate and hydroxylamine are similar, although those for ATP and MSOX were different (Table I). This may indicate that there is a difference in enzyme conformation, at least in the substrate binding sites for ATP and MSOX. To further investigate this, we examined chloride and divalent metal ion bindings to allosteric and activator sites, respectively, which induce the conformational changes of GS (4). The most prominent differences in fluorescence change were found in the chloride binding, in which a considerable increase was observed for the short protein compared with the long one, suggesting a conformational difference between the two proteins.

Because the fluorescence change could also be due to a nonspecific binding of chloride on the substrate site if there is a fluorophore located in the substrate site of GS, we examined the fluorescence changes upon bindings of ADP, l-glutamate, and MSOX. The marginal difference in fluorescence was detected below the level of 5%, indicating that there is no tryptophan residue in the substrate site that significantly alters its fluorescence upon substrate binding (data not shown). On the other hand, to assess whether the fluorescence change is due to the oligomerization of enzyme, we detected fluorescence in the sample with only monomers, obtained by treating 1% CHAPS (witterionic detergent). Here, the fluorescence change was less than 10% of the values obtained for purified protein with mostly oligomeric forms, indicating that the tryptophan fluorescence is not probing the oligomerization (data not shown).

We attempted to detect any structural difference due to an extension of N-terminal amino acids in LGS using circular dichroism spectroscopy. However, an analyzable spectra were not obtained because of the high propensity of GS oligomerization that disturbs circular dichroism measurement. At any rate, we already presented other enzymatic and spectroscopic data indicating the conformational alteration by an extension of N-terminal amino acids in LGS, although physiological
relevance of this structural variation still remains to be elucidated.

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