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Expression of alpha subunit of alpha glucosidase II in adult mouse brain regions and selective organs

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Abstract

Alpha glucosidase II (GII), a resident of endoplasmic reticulum (ER) and an important enzyme in folding of nascent glycoproteins, is heterodimeric consisting of alpha (GII α) and beta (GII β) subunits. The catalytic GII α subunit with the help of mannose 6-phosphate receptor homology (MRH) domain of GII β sequentially hydrolyzes two α -1-3-linked glucose residues in the 2nd step of N-linked oligosaccharide-mediated protein folding. The soluble GII α subunit is retained in the ER through its interaction with the HDEL-containing GII β subunit. N-glycosylation and correct protein folding is crucial for protein stability, trafficking, and cell surface expression of several proteins in the brain. Alterations in N-glycosylation lead to abnormalities in neuronal migration and mental retardation, various neurodegenerative diseases, and invasion of malignant gliomas. Inhibitors of GII are used to inhibit cell proliferation and migration in a variety of different pathologies such as viral infection, cancer and diabetes. In spite of the widespread usage of GII α inhibitory drugs and the role of GII α in brain function little is known about its expression in brain and other tissues. Here, we report generation of a highly specific chicken antibody to GII α subunit and its characterization by Western blotting and immunoprecipitation using cerebral cortical extracts. Using this antibody we show that the GII α protein is highly expressed in testis, kidney, and lung, with the least amount in heart. GII α polypeptide levels in whole brain were comparable to spleen. However, higher expression of GII α protein was detected in cerebral cortex reflecting its continuous requirement in correct folding of cell surface proteins.

Keywords

Chicken antibody; Western blot analysis; Immunoprecipitation; Cerebral Cortex; GII alpha; N-glycosylation; Protein Folding; Mouse

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Introduction

In the endoplasmic reticulum (ER), folding of newly synthesized glycoproteins is initiated by a sequential removal of three glucose molecules from N-linked glycans conjugated to asparagine residues of the nascent polypeptide (Turco et al. 1977) (Liu et al. 1979) (Grinna and Robbins 1979) (Hubbard and Robbins 1979). Glucosidase I trims the first glucose residue while the inner two glucose residues are removed by glucosidase II (Ugalde et al. 1978; Ugalde et al. 1980) (Scher and Waechter 1979) (Grinna and Robbins 1979; Grinna and Robbins 1980). If the glycoprotein is not correctly folded, then the protein is reglucosylated and becomes a substrate of GI and GII (Parodi 2000; Trombetta et al. 1996). Glucosidase I consists of a single polypeptide with a membrane integration sequence (Bause et al. 1989; Hettkamp et al. 1984; Shailubhai et al. 1987; Shailubhai et al. 1991). In contrast, glucosidase II is heterodimer complex consisting of two polypeptides, alpha (GII α) and beta (GII β) subunits (D'Alessio et al. 1999; Pelletier et al. 2000; Trombetta et al. 1996). GII α , a soluble protein is retained in the ER via its interaction with ER membrane integrated GII β subunit (Arendt and Ostergaard 1997; Arendt and Ostergaard 2000; Trembl et al. 2000; Trombetta et al. 1996). Immunoelectron microscopy data support the presence of GII α in endoplasmic reticulum (Brada et al. 1990; Lucocq et al. 1986). GII α protein purified from a variety of mammalian and non-mammalian tissues and organisms including yeast has been characterized and sub-cloned. Based upon these data, GII α is a highly conserved protein from yeast to mammals with a molecular mass of 116 kDa in mouse and it can be endoH-sensitive and/or -insensitive (Arendt and Ostergaard 1997; Brada et al. 1990). The GII α subunit has the catalytic activity but requires the assistance of mannose 6-phosphate receptor homology (MRH) domain of the GII β subunit for this action (Hu et al. 2009; Stigliano et al. 2011).

Glucosidases I and II are important enzymes required for folding of glycoproteins that are to be exported out of the cells or are integrated into cell membranes (Roth et al. 2003; Roth et al. 2010; Trombetta and Parodi 2003). Interestingly studies involving the use of GI/GII inhibitors reported a substantial reduction in cell proliferation/migration (Pili et al. 1995) and incorporation of incorrectly folded proteins into the cell membranes (Chapel et al. 2007) prompting their use as drugs to curtail cell proliferation and migration in diseases such as cancer, viral infection, and diabetes (Asano 2003; Chapel et al. 2007; Hwu et al. 2003; McLaughlin and Vandenbroeck 2011; Pili et al. 1995; van de Laar 2008).

Given the importance of GII enzyme in cell physiology, little is known about its expression in various tissues including brain even though its presence in the brain was first reported in 1979 (Scher and Waechter 1979; Tulsiani et al. 1990). Hence in this study we examined the expression of catalytic subunit, GII α at the polypeptide level in different regions of mouse brain and selective organs. GII α antibodies raised in rabbit obtained from three independent sources had low titer. Therefore we raised GII α antibody in chicken against two promising antigenic epitopes. Purification and characterization of GII α chicken antibody using various methods showed it to be a specific antibody with high titer. Using this antibody, we demonstrate for the first time that expression of GII α in whole brain was comparable to spleen. However when examined in cerebral cortex, hippocampus, and cerebellum, the relative expression of GII α protein was higher than spleen and was comparable in all three

brain regions. Testis, ovary, liver and kidney expressed the highest level of GII α protein while heart expressed the least amount.

Material and Methods

1. Generation of Antibody against GII α in Hen

- a. **Prediction and Selection of Antigenic Peptides:** The amino acid sequence of GII α was analyzed by Aves Labs (Tigard, OR) using their proprietary Immunogenicity Algorithm® that looks for a combination of hydrophilicity, chain flexibility, and specific residues. As a cross-check, the sequence was run against various standard algorithms, including Hopp-Woods and Kyte-Doolittle (hydrophilicity), Emini (surface probability), and Karplus-Shulz (chain flexibility), and Jameson-Wolf (a combination of attributes). The sequence was then scanned for likely sites of post-translational modifications using the Prosite database (Sigrist et al. 2013). Based upon these analyses, several different regions of GII α protein appeared to be immunogenic and these peptides were ranked based upon their hydrophilicity, chain flexibility, surface probability, and lack of phosphorylation sites. Two potentially highly immunogenic domains (peptide I sequence = RIDELEPRRPRYRVPD, and peptide III sequence = DKPEETQEKA EKDE) were selected to raise antibodies against GII α subunit. The amino acid sequence of peptide I is identical in mouse and human while the amino acid sequence of peptide III is unique for mouse. Both peptides were synthesized *in vitro* with an additional cysteine (“C”) and aminocaproic acid (“Z”) residues at the N-terminus ends for conjugating synthetic peptides to a carrier protein, keyhole limpet hemocyanin (KLH).
- b. **Peptide Injection:** The KLH conjugated GII alpha synthetic peptides were injected as a mixture into two hens (identified as #6362 and #6363) four times as a single injection with two week interval time. Two months later, three booster injections were given. Eggs were collected twice for a week, once two weeks after the 4th peptide injection and then after the last booster injection.
- c. **Antibody Purification, Titer and Specificity**
 - i. **Antibody Purification:** Egg yolks were recovered and pooled from two batches of eggs collected after immunization independently from two hens and immunoglobulins were precipitated out by Aves labs. After determining the presence of GII α antibody in immunoprecipitated immunoglobulins by ELISA assay, total immunoglobulins were processed for affinity purification of the GII α antibody using synthetic peptide III (amino acid sequence = DKPEETQEKA EKDE) by Aves labs. Pre-immune immunoglobulins were also

recovered independently from eggs collected prior to immunization of two hens used in the present study.

- ii. ELISA Assays: were performed using peptides I and III independently. Peptides I and III were first conjugated to N-maleimide activated ovalbumin (ThermoFisher Sci.) according to the manufacturer's protocol and then absorbed onto an ELISA plate (NUNC) (100 μ l per well of 1.0 μ g/ml in 1 X phosphate buffered saline) (PBS) in an overnight incubation at room temperature. As a negative control, some wells were incubated with ovalbumin only. Next day, 100 μ l of 1.0% BlokHen® (Aves Labs, Tigard, OR) in 1 X PBS was added to each well and incubated further for 1 hour at room temperature to block non-specific sites. Following extensive washes with deionized distilled water for 10 min, the plates were then incubated for 1 hour at room temperature with 100 μ l of various dilutions of GII α chicken antibody (purified from hyperimmune eggs) or preimmune antibodies (purified from egg yolks collected prior to immunization of hens). Following extensive washing with water as above, wells were incubated with a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-chicken IgY (Aves Labs) for a 1h at room temperature. After water-washing steps, HRP enzymatic activity in each well was quantified using o-phenylenediamine (OPD; ThermoFisher Sci) according to the instructions of the manufacturer. Kinetic analysis of HRP enzymatic activity was performed using a ELX808IU kinetic microplate reader, analyzed by KC Junior software (Bio-Tek Instruments, Winooski, VT), and plotted on a log scale.

- iii. Dot Blot Assay: Peptides I and III were dissolved independently in 1 x PBS at a final concentration of 100ng/ μ l. Mouse kidney microsomes prepared by differential centrifugation (see below: isolation of microsomal fraction) were mixed with sodium dodecyl sulfate (to 1%), alternative NP 40 (to 1%), and Triton X100 (to 1%) in buffer A and incubated overnight at 4 °C with continuous gentle mixing. Next day, kidney lysate was centrifuged at 4 °C for 10 min at 16,100 x g and clear supernatant was recovered.

Increasing concentration of two peptides (50 ng, 100 ng, 250 ng, and 500 ng) and kidney microsomes (1 μ g, 5 μ g, 10 μ g, and 20 μ g) were independently blotted onto nitrocellulose membrane using BioRad dot blot apparatus. After blocking for two hours at room temperature in 5% goat serum prepared in 1X PBS, membranes were incubated overnight at

4 °C with either pre-immune immunoglobulins (1:1,000 dilution) or partially purified GII α antibody (1:1,000 dilution) or affinity purified GII α antibody (1:5,000 dilution) or affinity purified GII α antibody pre-incubated with peptide III (1:1 ratio in μ g) (1:5,000 dilution). Next day, membranes were washed three times with 1X PBS containing 0.05% Tween 20 (10 min each wash) and incubated at room temperature for 2h with HRP-conjugated goat anti-chicken (1:2,500 dilution). Membranes were washed again and immunoreactive spots were visualized by incubation with ECL plus reagent (GE Healthcare) and scanning on a PhosphorImager.

- iv. Optimization of Western Blot Assay Conditions: Western blot analysis conditions were optimized using microsomes from cerebral cortex (CC) of adult mice, 8% SDS-polyacrylamide gels and Laemmli's buffers, and ECL plus solution (Laemmli 1970). Initially conditions were optimized for nitrocellulose transfer membrane and goat serum in 1X PBS as a blocking reagent. To remain within the linear range of detection, conditions for primary antibody, secondary antibody, and CC protein amount were optimized sequentially. PhosphorImager.

Briefly, protein samples were separated on 8% SDS-polyacrylamide gels using Laemmli's buffer system, transferred to nitrocellulose membrane, and blocked in 5% goat serum in 1X PBS for 2 hours at room temperature. Membranes were incubated overnight at 4 °C with appropriate dilution of GII α chicken antibody (Laemmli, 1970). Next day, membranes were washed three times with 1X PBS containing 0.05% Tween 20 (10 min each wash) and incubated with appropriate dilution of HRP-conjugated goat anti-chicken for 2h at room temperature. Following washing, immunoreactive bands were detected by incubation of membranes with ECL plus reagent (GE Healthcare) and scanning on a PhosphorImager.

- v. Peptide Blocking Experiment: The GII α antibody was mixed with peptide III dissolved in 1X PBS (w/w) and incubated at room temperature for 12h or at 4 °C for 24h. At the end of the incubation, the antibody was diluted to 1:80,000 and used for detection of GII α polypeptide in mouse cerebral cortical microsomes by optimized Western blotting conditions (see above).

- vi. **Thrombin Digestion Experiment:** Microsomes from cerebral cortex (CC) of adult mouse (20 µg protein) were digested overnight at 22 °C with a serine protease, thrombin according to the instructions of the supplier (GE Healthcare). At the end of the digestion, Laemmli's sample buffer was added to terminate the enzyme activity. Digested and undigested samples were incubated for 3 min in boiling water and then processed for detection of GIIα polypeptide using optimized Western blotting conditions (see above).
2. **GIIα Protein Immunoprecipitation:** Because the source of GIIα antibody was chicken for both immunoprecipitation (IP) and detection of immunoprecipitated GIIα protein by Western blotting, we utilized co-immunoprecipitation kit (Pierce, Thermo Scientific) that does not allow elution of primary antibody in the IP experiment. Microsomal proteins from CC (1 mg protein) were incubated with 0.75% Triton X100 for 30 min on ice with occasional gentle mixing and then centrifuged to obtain clear supernatant. Pellet (P1) was retained to detect insoluble GIIα protein. Clear supernatant (S1) was used to immunoprecipitate GIIα protein. Supernatant (S1) was first incubated with control agarose resin for 30 min at 4 °C with constant mixing to remove any non-specific binding of lysate proteins to the agarose resin. Control agarose beads were separated from the supernatant (S2) by centrifugation at 1,000 x g for 1 min. The S2 fraction was then incubated overnight at 4 °C with constant mixing with 13 µg affinity purified GIIα chicken antibody immobilized onto agarose beads according to instructions of the supplier. Next day, unbound protein fraction (UP) was recovered by centrifugation (1,000 X g for 1 min). The antigen-primary antibody-agarose bead complexes were washed stringently (200 µl per wash - 3 X with lysis buffer, 2 X with 1X PBS, and once with 1X conditioning buffer) and non-covalently bound GIIα proteins were eluted twice using elution buffer (30 µl per elution). Eluted protein samples (20 µl) and other samples retained during different steps of IP experiment (20 µl per sample irrespective of the sample volume except entire pellet P1 was loaded onto the gel) were processed to detect GIIα protein by optimized Western blot conditions (8% SDS-polyacrylamide gels, 1:80,000 dilution of affinity purified GIIα chicken antibody and 1:7,500 HRP conjugated secondary antibody). Immunoreactive bands were detected by incubation of membranes with ECL plus reagent (GE Healthcare) and scanning on a PhosphorImager.
3. **Isolation of Microsomal Fraction from Various Tissues:** Adult male and female mice (C57 BL/6) were used in accordance with institutional guidelines and procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University. Animals were sacrificed and different tissues (liver, kidney, small intestine, lung, heart, spleen, ovary, testis, and brain) and three brain regions (cerebral cortex, hippocampus and cerebellum) were dissected out. Tissues were rinsed in 1 X PBS, minced on ice and homogenized in glass homogenizers with tight fitted pestles (Wheaton) in buffer A (10 mM Tris.HCl buffer, pH7.4 containing 1 mM potassium acetate, 1.5 mM magnesium acetate, 0.25 M sucrose,

1X anti-protease cocktail (RPI, IL), and anti-phosphatases (PhosSTop - Roche, Indianapolis, IN). Homogenates were centrifuged at 1,000 rpm for 10 min at 4 °C in eppendorf microcentrifuge. Pellet containing nuclei and unbroken cells were discarded. Supernatants were layered over a 1.5 ml 30% sucrose cushion and centrifuged at 100,000 $\times g$ for 150 min at 4 °C in 55Ti rotor (Beckman Coulter). Pellets containing mitochondria, endoplasmic reticulum, Golgi complex, cell membranes, endosomes etc. were labeled as microsomal fraction and suspended in buffer A containing 1 X anti-proteases and anti-phosphatases. Aliquots of samples were stored at -80 °C until further processing. Protein content in microsomal fractions was determined by Bradford assay (Bradford 1976).

4. **GII α Protein Expression in Mouse Brain Regions and Selected Mouse Organs:** was examined by Western blotting. A known amount of microsomal proteins from various tissues or different brain regions of adult mouse along with pre-stained protein molecular weight markers (NEB) were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Nitrocellulose membranes were cut into two parts at the visible 60 kDa protein molecular weight marker band. The membrane above 60 kDa band was probed with GII α antibody while the lower half of the membrane was probed with β -actin antibody. Membranes were processed appropriately to detect immunoreactive GII α band using optimized Western blotting conditions for GII α (see section iv) or β -actin band using monoclonal antibody to β -actin (Sigma) (Anji and Kumari 2006).

Western blot analysis experiment was repeated four times using tissue extracts prepared on four different occasions. Intensity of immunoreactive GII α band quantitated using ImageQuant software was expressed in arbitrary units. Data points are expressed as a mean $\pm$ standard error. Tissue expression of GII α was not normalized with β -actin as expression of β -actin varies across tissues. However, expression of GII α in different brain regions was normalized with expression of β -actin for equal loading of samples as expression of β -actin did not vary with brain region. Statistical analyses were performed using ANOVA and Scheffe's test (* $p < 0.05$).

Results

Antigenic Domains of GII α Polypeptide

Primary sequence of amino acids of murine GII α (Accession Number U92793 and version U92793.1 GI:2104688) was analyzed using different software (see materials and methods) to detect potential immunogenic regions that can be used to raise GII α antibody in hens. Based upon these analyses, four distinct domains appeared to be potentially highly immunogenic. Two domains overlapped with human sequence while two domains were unique for mouse. Out of these four domains, two domains spanning amino acid (aa) residues from 102-117 (peptide I – identical aa sequence in mouse and human) and 231-244 (peptide III – unique aa sequence in mouse) of GII α polypeptide were selected for raising GII α antibody as these domains matched the stringent criterion used for their identification (Figure 1). Two selected peptides were synthesized *in vitro* with an additional cysteine ("C")

and aminocaproic acid ("Z") residues at the N-terminus to improve the efficiency of conjugation of synthetic peptides to carrier protein, KLH.

GII α Antibody Specificity and Titer

Total immunoglobulins purified from eggs collected from two different birds were tested independently for the presence of GII α antibody by ELISA assay. Antibodies (total immunoglobulins) from two hens showed cross reactivity to peptides I and III. The horse radish peroxidase (HRP) activity increased in an antibody concentration dependent manner when total immunoglobulins purified from two hens (#6362 and #6363) were used with either peptide I (Figure 2A) or peptide III (Figure 2B). In contrast, the pre-immune antibodies from both hens (6362 Pre-immune IgY and 6363 Pre-immune IgY) produced negligible HRP activity with peptide I (Figure 2A) and peptide III (Figure 2B). The equilibrium dissociation constant values (K_d's) ranged between 2-5 μ g/ml for both antibody preparations (6362 Immune IgY and 6363 Immune IgY).

To confirm ELISA assay data and to further test the specificity of the antibody against the native protein, we employed dot blot assay. Increasing concentrations of peptide I, peptide III, and soluble kidney microsomal fraction were blotted on to nitrocellulose membrane, blocked, and incubated overnight at 4 °C with either pre-immune immunoglobulins or total immunoglobulins. Next day, membranes were washed and probed with HRP conjugated goat anti-chicken. Antigen-antibody complexes were detected using ECL plus reagent. As a negative control, buffer was blotted onto the nitrocellulose membrane and processed them simultaneously for incubation with total immunoglobulins. As expected, no immune complex was detected when buffer was blotted onto the membrane (data not shown here). Pre-immune immunoglobulins from hen #6362 reacted with soluble fraction of kidney microsomes (Figure 3: Panel 1) and this cross-reaction was subsequently confirmed by Western blotting (data not shown here). In contrast, no antigen-antibody complex formation was detected with pre-immune immunoglobulins from hen #6363 (Figure 3: Panel 3). Total immunoglobulins from hen #6362 displayed better binding to peptide I than peptide III and kidney microsomal fraction (Figure 3: Panel 2). Total immunoglobulins from hen #6363 produced a high intensity immune complex with both peptides (I & III) and kidney microsomes (Figure 3: Panel 4). Antibodies from hen #6362 were discarded as the pre-immunized antibodies cross-reacted with kidney microsomes. Antibodies from hen #6363 were purified further by affinity method (hereafter referred as GII α antibody). In a dot blot assay, the GII α antibody produced an antigen-antibody complex in an antigen concentration dependent manner (Figure 3: Panel 5). Although the GII α antibody was affinity purified using peptide III, it cross-reacted with peptide I and this may be due high concentration of GII α antibody used in this experiment. To test this hypothesis, the GII α antibody was neutralized by incubation with peptide III prior to its use for dot blot assay. Pre-incubation of GII α antibody with peptide III obliterated the formation of antigen-antibody complex (Figure 3: Panel 6). Irrespective of the temperature employed for incubation of peptide with GII α antibody, similar results were obtained (Figure 3: Panel 6).

The GII α antibody titer was determined by Western blotting. Prior to conducting this experiment, Western blot conditions were optimized in terms of nitrocellulose versus PVDF

membrane, blocking solutions, and incubation temperature/duration with primary and secondary antibodies. Based upon these data, nitrocellulose membrane was the best support membrane and 5% goat serum in 1X PBS obliterated the high background noise (data not shown). The Western blot conditions were initially optimized to remain within the linear range of detection. Increasing amounts of microsomal protein samples from cerebral cortex of adult mouse were separated on 8% SDS-PAGE, blotted, and probed with GII α antibody. Based upon this data, 20 μ g of cortical microsomal protein was found to be optimal (data not shown). As a next step, optimal dilution factor for GII α antibody was determined. Overnight incubation with low dilution of GII α antibody (1:10,000 to 1:40,000) at 4 °C detected several bands (Figure 4A: Lanes 1-4). But as the dilution of GII α antibody increased (up to 1:100,000), two to three immunoreactive bands were detected with an apparent molecular mass of 116, 50.5, and 34 kilodaltons (kDa) respectively (Figure 4A: Lanes 6-10). The 116 kDa immunoreactive band is the full length GII α polypeptide. The optimal dilution of GII α antibody using cerebral cortex microsomal protein fraction was 1:80,000 for Western blot analysis. As a next step, the secondary antibody concentration was optimized and 1:7,500 dilution with incubation at room temperature for 2 h was found to be optimal to remain within the linear range of detection of GII α polypeptide (data not shown).

Next we examined the specificity of primary antibody-antigen interaction by employing three complementary approaches by determining whether: 1) secondary antibody alone can detect any immunoreactive band(s), 2) neutralization of primary antibody prior to its use for Western blotting can detect GII α polypeptide, and 3) the primary antibody can recognize GII α peptide fragment of correct molecular mass following its digestion with an endopeptidase that cuts GII α only once.

Microsomal protein samples from cerebral cortex of adult mouse were separated on 8% SDS-PAGE, blotted on to nitrocellulose membrane and incubated with secondary antibody only. Washed membranes were incubated with ECL plus reagent and scanned on PhosphorImager. No immunoreactive bands were detected indicating that three bands detected by Western blotting are due to specific interaction of GII α antibody with the antigen in the microsomes (Figure 4B).

To further confirm the specificity of GII α antibody, the affinity purified GII α antibody was incubated with peptide III in equal proportion (w/w) at room temperature for 12 h to neutralize its GII α protein binding sites. Increasing concentration of microsomal proteins separated on 8% SDS-polyacrylamide gel and blotted on to nitrocellulose membranes were probed with either neutralized affinity purified GII α antibody or affinity purified GII α antibody at 1:80,000 dilution. Incubation with secondary antibody and detection of immunoreactive bands was same as above for Western blotting. A 116 kDa immunoreactive band of GII α protein was detected using affinity purified GII α antibody. The intensity of GII α immunoreactive band increased with increase in the amount of sample loaded on the gel (Figure 4C: Lanes 1-3). In contrast, no GII α immunoreactive band was detected when affinity purified GII α antibody was neutralized prior to its use for Western blotting (Figure 4C: Lanes 4-6) suggesting that the GII α antibody is specific for the detection of GII α protein in tissue lysates.

Analysis of GII α amino acid sequence using PeptideCutter software (Artimo et al. 2012) showed that thrombin, a serine protease, digests GII α once at position 834 (Figure 1) thereby generating two peptide fragments of 834 and 132 amino acids long with theoretical masses of 94.626 kDa and 14.728 kDa respectively (PeptideMass software) (Artimo et al. 2012). The apparent molecular mass of these peptide fragments is expected to be higher due to their post-translational modifications (Flura et al. 1997; Strous et al. 1987). Following digestion of microsomal proteins with thrombin and analysis by Western blotting highlighted an immunoreactive band with an apparent molecular mass of a 101 kDa with GII α antibody (Figure 4D: Lane 2). As expected, the full length GII α polypeptide of 116 kDa size was detected in undigested microsomal protein sample (Figure 4D: Lanes 1 & 3).

Immunoprecipitation of GII α Polypeptide

Often antibodies raised against synthetic peptides are not able to immunoprecipitate the respective protein in spite of being highly specific. Since the GII α chicken antibody in the present study was raised against two synthetic peptides, it was therefore reasonable to determine whether GII α antibody can immunoprecipitate its cognate protein from cortical microsomes. Because rabbit GII α antibody was not able to detect low levels of immunoprecipitated GII α protein, the GII α antibody was covalently coupled to an amine-reactive agarose resin using Pierce co-immunoprecipitation kit. In this protocol, antibody bound proteins are eluted without co-elution of antibody at the end of the immunoprecipitation experiment. Detergent solubilized microsomal proteins from mouse cerebral cortex were pre-cleared by incubation with control agarose resin. The pre-cleared microsomal protein mixture was incubated with GII α antibody coupled to agarose resin overnight at 4 °C with gentle mixing. Following stringent washing, proteins bound to GII α antibody-agarose resin were eluted without co-elution of GII α antibody. Eluted proteins were separated on 8% SDS-polyacrylamide gel along with other fractions retained during immunoprecipitation, blotted and probed with GII α chicken antibody as above. Four immunoreactive bands were detected with an apparent molecular mass of approximately 116, 75, 42, and 27.4 kDa respectively (Figure 5a: Bands A-D). The faint band of ~116 kDa size is the full length GII α protein (Figure 5a: Band A). The major band at ~75 kDa appeared after overnight incubation of pre-cleared supernatant (S2) with GII α antibody coupled agarose (Figure 5a: Band B in lane 4) and hence could be a degradation product of full length GII α protein. The 42 & 27.4 kDa bands may also be a degradation product of two immunoreactive bands (50.5 kDa and 34 kDa bands) detected by Western blotting in Figure 4A.

To ensure that none of the immunoreactive bands (Figure 5a: Bands B-D) are the co-eluted heavy/light chains of GII α antibody, the membrane was stripped of antibodies by incubation with Tris-HCl buffer pH6.7 containing 100 mM β -mercaptoethanol and 2% SDS for 2h at 50 °C. The antibody stripped membrane was washed, exposed to ECL plus reagent and scanned on PhosphorImager to ensure complete stripping of antibodies. No immunoreactive bands were detected after antibody stripping (Figure 5b). The membrane was now probed with the secondary antibody, HRP conjugated goat anti-chicken. Incubation with the secondary antibody was carried out at room temperature for 2h and immunoreactive bands were detected using ECL plus reagent as above. No immunoreactive bands were detected when

washed membrane was incubated with secondary antibody alone (Figure 5c). This data suggests that the 75 kDa band and other low molecular weight fragments are not the GII α antibody chains but may possibly be degradation products of the full length GII α protein.

Expression of GII α Protein in Mouse Tissues

To determine expression of GII α protein in various tissues of adult mouse, microsomal fractions from selected tissues were prepared. Tissue samples (20 μ g proteins) were electrophoresed on 8% SDS polyacrylamide gels and blotted. Following blotting, the nitrocellulose membranes were cut into two halves at 60 kDa size molecular weight marker. Membrane above 60 kDa size was probed with GII α antibody using optimized Western blot conditions as above and the membrane below 60 kDa size were used for detection of β -actin (see method section). Intensity of β -actin was not used to normalize equal loading of samples since the expression level of β -actin was highly variable across tissues. A 116 kDa size immunoreactive band of GII α polypeptide was detected in all tissue samples (Figure 6A). However, the intensity of GII α polypeptide was different in various tissues suggesting variability in its expression (Figures 6A, 6B). Least amount of GII α protein was present in heart and highest levels were detected in testis. Based upon intensity of immunoreactive band, the relative expression of GII α subunit was as follows: testis>kidney>lung>ovary>liver> whole brain=spleen>small intestine>heart (Figure 6B).

Expression of GII α Protein in Mouse Brain Regions

To determine expression in various regions of adult mouse brain, microsomal fractions were prepared from cerebral cortex, hippocampus, and cerebellum. Tissue was pooled from several animals to isolate microsomes from hippocampus and cerebellum. Twenty μ g proteins from each brain region were electrophoresed on 8% SDS polyacrylamide gel and blotted. The membrane was cut in to two parts at 60 kDa size molecular weight marker and probed with GII α antibody or β -actin antibody using optimized Western blot conditions (see above). A 116 kDa size immunoreactive band of GII α polypeptide was detected in all brain regions as expected (Figure 6C). Data was normalized for equal loading of samples using intensity of β -actin as intensity of β -actin immunoreactive band did not vary with brain region. Although the intensity of GII α polypeptide in cerebral cortex appears to be the highest, analysis of data showed that the levels of immunoreactive GII α protein were not different from each other in three brain regions examined in the present study (Figure 6D).

Discussion

Glucosidase II consisting of alpha (GII α) and beta (GII β) subunit is an important enzyme for folding and re-folding of glycosylated nascent proteins in the endoplasmic reticulum (Parodi 2000; Roth et al. 2010; Trombetta and Parodi 2005). In this manuscript we demonstrate for the first time high level of GII α expression in three different brain regions by Western blotting using a specific, high-titer GII α antibody generated in chicken. However, when the level of GII α expression was examined in whole adult mouse brain its expression was comparable to spleen. Of all the non-nervous tissues examined, GII α expression was highest in testis and least in heart.

The soluble GII α subunit is the catalytic subunit although it requires mannose 6-phosphate receptor homology (MRH) domain of GII β subunit for effective hydrolysis of 1,3-linked glucosyl residues and is retained in lumen of ER by interaction with HDEL containing GII β subunit (Quinn et al. 2009; Stigliano et al. 2009). The GII α subunit has been purified from a variety of animal and plant tissues. Invariably two methods have been employed previously to characterize the GII α protein during and after purification: a) GII α enzyme activity assay; and b) Western blotting using GII α rabbit antibodies. Although these antibodies were specific for GII α subunit, their titers were low. Some mammalian proteins often present themselves as poor antigens in rabbits and it is possible that GII α protein falls in this category. We therefore used chickens in the present study as phylogenetic differences make them an excellent alternative host to raise antibodies (Carlander et al. 1999; Larsson et al. 1993; Larsson and Sjoquist 1990).

In the past, short synthetic peptides have been successfully used to raise specific, high titer antibodies (Kaumaya et al. 1992). We analyzed the GII α amino acid sequence as the full length GII α protein was not available to us and identified four antigenic determinants. Out of these, two epitopes (peptide I: 102-117 aa and peptide III: 231-244 aa) were selected and their synthetic analogues were used to raise antibodies in two hens identified as #6362 and #6363. Total immunoglobulins purified from egg yolks of both immunized birds showed specific binding to peptides I and III with a high titer but not the immunoglobulins from non-immunized hens in an ELISA assay. A dot blot assay, however, identified specific interaction between immunoglobulins from non-immunized hen #6362 and kidney microsomal proteins indicating that this hen was exposed to mammalian protein(s) prior to immunization with GII α peptides. Thus antibodies from this hen were discarded and total immunoglobulins from hen #6363 were further processed to purify by affinity method using peptide III. The affinity purified antibody was highly specific as it prevented immunodetection of peptides I/III and GII α protein in kidney microsomes upon neutralization with peptide III prior to its use in dot blot assay. The affinity purified antibody had a high titer as it detected GII α protein at 1:100,000 dilution in a Western blot assay.

The GII α antibody raised in the present study recognized three proteins with an apparent molecular mass of 116, 52, and 33 kDa respectively in a Western blot assay. The 116 kDa protein is the full length GII α protein with post-translational modifications (Arendt and Ostergaard 1997) and its identity was confirmed by digestion of cortical microsomal proteins with thrombin. Thrombin digests GII α once at position 834 generating two peptide fragments with theoretical masses of 94.626 kDa and 14.728 kDa respectively without posttranslational modifications. Following thrombin digestion, a 101 kDa size protein band was detected with GII α antibody. A difference in molecular mass by ~ 7 kDa (from 94.626 kDa predicted mass to 101 kDa apparent mass) is likely due to posttranslational modifications of 834 aa long peptide (Flura et al. 1997; Strous et al. 1987). The specificity of the GII α antibody was further confirmed by neutralization of binding sites on the antibody. Affinity purified antibody was incubated with peptide III in equal proportion (w/w) prior to its use in Western blotting. As expected, neutralization of GII α antibody did not detect the 116 kDa GII α immunoreactive band in microsomes. The two additional bands detected by Western blotting with an apparent molecular mass of 52 kDa and 33 kDa respectively are possibly degradation products of GII α protein as observed previously in

liver extracts by Lucocq and colleagues (Lucocq et al. 1986). The 52 kDa and 33 kDa bands detected by Western blotting using GII α antibody are not members of family 31 glycosylhydrolases as the selected immunogenic epitopes are unique for GII α polypeptide (D'Alessio et al. 2010).

Antibodies raised against peptides are generally unable to immunoprecipitate their cognate proteins. In contrast to this observation, the chicken antibody raised in the present study was able to immunoprecipitate GII α protein from cortical microsomes. Four immunoreactive bands with apparent molecular masses of 116, 75, 42, and 27.4 kDa were detected in an IP experiment. The intensity of full length GII α protein was much less than the other bands. The 75 kDa protein band with highest intensity is a degradation product of GII α protein as it appeared after overnight incubation with GII α antibody coupled agarose beads. It is also unlikely that the smaller immunoreactive bands observed in an IP experiment are part of heavy and light chains of immunoglobulin Y (IgY) as their molecular masses are 65.1 and 18.7 kDa respectively (Leslie and Clem 1969). Nevertheless to ensure that 75, 42 & 27.4 kDa immunoreactive bands were not actually the IgY fragments co-eluted with the GII α protein, the nitrocellulose membrane was stripped and re-probed with the secondary antibody alone. No immunoreactive bands were detected with the secondary antibody alone suggesting that low molecular weight immunoreactive bands were indeed degradation products of the full length GII α protein.

Enzymes glucosidase I (GI) and II (GII), and mannosidases play an important role in folding of nascent glycoproteins in the rough endoplasmic reticulum (Herscovics 1999). Often these enzyme levels are altered in cancerous tissue such as human lung tumor (Suradej et al. 2013). Such reports led to the use of inhibitors of these enzymes as drugs to control cell proliferation, infectivity and migration seen in retroviral infection, cancer, and diabetes (Hadwiger-Fangmeier et al. 1989; Kumar et al. 2010; McLaughlin and Vandenbroeck 2011; Pili et al. 1995; Qu et al. 2011; Sasak et al. 1985). Despite the wide use of GI/GII inhibitors, normal level of expression in various tissues has not been examined to date. Hence in the present study we examined the expression of GII α subunit in various adult mouse tissues by Western blotting. Differential expression of GII α analyzed by ImageQuant software showed the following order of relative expression of GII α subunit: testis>kidney>lung>ovary>liver>whole brain = spleen>small intestine>heart. It is not clear whether the least amount of GII α protein observed in heart is due to a difficulty in homogenizing muscle fibers or due to its low level of expression. The high level of GII α expression in certain tissues suggests that these tissues are engaged in rapid glycoproteins biosynthesis. For instance, in the testis Sertoli cells are actively engaged in secretion of proteins, ions, and water into the lumen of seminiferous tubules and this fluid is necessary for sperm transport to excurrent ducts (Griswold 1988; Rato et al. 2010; Shabanowitz and Kierszenbaum 1986). Continuous production of spermatozoa in testis requires active biosynthesis of glycoproteins and hence the high abundance of GII α subunit (present study) and beta subunit (Naaby-Hansen et al. 2010). Other non-neuronal tissues expressing high levels of GII α such as kidney, lungs, and liver may have a high turn-over rate of membrane glycoproteins and/or their rate of protein secretory activity is high as compared to other tissues employed in this study.

N-glycans from glycoproteins in the brain are quite complex and differ in general from other organs in the body but there is no difference in N-glycan of glycoproteins among rodents and human (Albach et al. 2001). N-glycosylation is an important post-translational process and its importance in protein folding is recognized at many levels of biological processes. For instance, incomplete glycosylation of α -dystroglycan, results in abnormal migration of neurons during development and this leads to mental retardation (Waite et al. 2009). Alpha-dystroglycan is a cell membrane integrated protein that links β -dystroglycan and extra cellular matrix components (Waite et al. 2009). Function of integrins, another family of proteins involved in neuronal cell migration during cerebral cortex development and cell adhesion, is affected by N-glycosylation (Schmid and Anton 2003) (Janik et al. 2010). Integrins and several other proteins are also involved in invasion of glioblastomas (Moskal et al. 2009) (Vogetseder et al. 2013). Assembly and cell surface expression of norepinephrine and serotonin transporters, formation of NR1/NR2A channel domain, maturation of AMPA receptors and their protection from proteolytic degradation correlates well with N-glycosylation of the respective protein (Blakely et al. 1994) (Sanchez-Perez et al. 2005) (Jiang et al. 2006). Abnormal folding of proteins in specific brain regions such as Alzheimer's disease is known to cause loss of cell-cell communication in the brain (Yankner et al. 1990) (Tiraboschi et al. 2004). In some instances such as certain neurodegenerative diseases and alcohol addiction, changes in protein folding and/or glucosidases in specific brain regions have been identified (Hashimoto et al. 2003). For example, there is a significant increase in GII β , the non-catalytic subunit of alpha glucosidase II, in cultured cortical neurons and cerebral cortex of chronic alcohol exposed mice (Anji and Kumari 2006) (Anji and Kumari 2011). It has been known since 1988 that the enzyme, GII is absolutely required for the development and differentiation of oligodendrocytes (Bhat 1988) (Bhat and Zhang 1994). These studies thus highlight the importance of N-glycosylation and protein folding enzymes in the brain. Lack of GII expression studies in different brain regions prompted the present study. Since GII α subunit has the catalytic activity, in the present study we first examined its expression in the whole brain. The level of GII α expression in whole brain was comparable to spleen. However when examined independently the level of GII α expression in cerebral cortex was comparable to hippocampus and cerebellum. This GII α polypeptide expression data corroborates the importance of GII in glycoprotein folding in these brain regions.

In summary, a specific and high titer antibody to a mammalian protein, GII α was generated in hens. A difference in relative expression of GII α protein was observed in various adult mouse tissues using this antibody in a Western blot study. Highest level of GII α protein was found in testis and lowest level in heart, a highly muscular tissue. Three distinct regions of adult mouse brain express similar levels of GII α protein. This data provides a baseline value to compare a deviation from normal tissue such as that seen in cancer, viral infection, and neurodegenerative diseases.

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MAAIAVAAR RRRSWLSLVL AYLGVCLGIT LAVDRSNFKT CDESSFCKRQ 50

RSIRPGLSPY RALLDTLQLG PDALTVHLIH EVTKVLLVLE LQGLQKNMTR 100

IRIDELEPRR **PRYRVPD**VLV ADPPTARLSV SGRDDNSVEL TVAEGPYKII 150

LTAQPFRLDL LEDRSLLLSV NARGLMAFEH QRAPRVPFSD KVSLALGSVW 200

DKIKNLFQRQ ESKDPAEGNG AQPEATPGDG **DKPEETQEKA** **EKDE**PGAWEE 250

TFKTHSDSKP YGPTSVGLDF SLPGMHVYV IPEHADSLRL KVTEGGEPYR 300

LYNLDVFQYE LNNPMALYGS VPVLLAHSFH RDLGIFWLNA AETWVDISSN 350

TAGKTLFGKM LDYLQGSGET PQDIRWMSE SGIIDVFLML GPSVFDVFRQ 400

YASLTGTQAL PPLFSLGYHQ SRWNYRDEAD VLEVDQGFDD HNMPCDVIWL 450

DIEHADGKRY FTWDPTRFPQ PLNMLEHLAS KRRKLVAIVD PHIKVDSGYR 500

VHEELRNHGL YVKTRDGSY EGWCWPGSAS YPDFTNPRMR AWWSNMFSD 550

NYEGSAPNLY VWNDMNEPSV FNGPEVTMLK DAVHYGGWEH RDIHNIYGLY 600

VHMATADGLI QRSGGIERPF VLSRAFFSGS QRFGAVWTGD NTAEDHDLKI 650

SIPMCLSLAL VGLSFCGADV GGFFKNPEPE LLVRWYQMG A YQPFRAHAH 700

LDTGRREPWL LASQYQDAIR DALFQRYSL PFWYTLFYQA HKEGFPVMP 750

LWVQYPEDMS TFSIEDQFML GDALLIHPVS DAGAHGVQVY LPGQEEVWYD 800

IQSYQKHHGP QTLYL PVTLS SIPVFQGGT IVP**R**WMRVRR SSDCMKDDPI 850

TLFVALSPQG TAQGELFLDD GHTFNYQTRH EFLLRFSFS GSTLVSSAD 900

PKGHELEPIW IERVVIMGAG KPAAVVLQTK GSPE SRLSFQ HDPETSVLIL 950

RKPGVSVASD WSIHLR 966

Figure 1. Sequence of mouse alpha subunit of alpha glucosidase II (GII α)

The amino acid (aa) sequence of murine GII α (Accession Number U92793 and version U92793.1 GI:2104688) is shown here. Based upon antigenicity criteria, two regions of GII α , 102-117 aa and 231-244 aa (underlined bold letters) were selected for raising antibodies against GII α protein in hens. Based upon analysis by PeptideCutter software (ExPASy Proteomics Server), thrombin cuts once at position 834 (bold and underlined arginine residue) resulting in two peptide fragments of unequal size (1-834 aa and 835-966 aa). The predicted mass of the larger peptide fragment following thrombin digestion is

94.684 kDa without glycosylation and 101.257 kDa with glycosylation (Protein Calculator v3.3).

2A

Figure 2. GIIa chicken antibody titer by ELISA assay

Titer of antibodies raised in two hens was independently tested by ELISA assay using standard procedures (see methods for more details). Total immunoglobulins from egg yolks of pre-immunized (negative controls) (6362 Pre-immune IgY and 6363 Pre-immune IgY) and hyperimmunized (6362 Immune IgY and 6363 Immune IgY) hens were used independently to detect peptide I or III conjugated to ovalbumin. Wells coated with ovalbumin only served as additional negative control. Antigen-primary antibody interaction was detected by incubation with secondary antibody conjugated to horse radish peroxidase (HRP) and quantified by measuring enzymatic activity of bound HRP using o-phenylenediamine in a microplate reader. Data was analyzed by KC Junior software. 2A: Data obtained using peptide I as a source of antigen and Pre-Immune and Immune IgYs; 2B: Data obtained using peptide III as a source of antigen and Pre-Immune and Immune IgYs.

Figure 3. Specificity of GII α chicken antibody by dot blot assay

Increasing concentrations of peptide I, peptide III, and kidney microsomal fraction were blotted on to nitrocellulose paper, blocked in 5% goat serum in 1X PBS and incubated overnight at 4 °C with either pre-immune immunoglobulins (IgY) from hen #6362 (Panel 1) or total immunoglobulins from hen #6362 (Panel 2), or pre-immune immunoglobulins from hen #6363 (Panel 3) or total immunoglobulins from hen #6363 (Panel 4) or affinity purified immunoglobulins from hen #6363 (Panel 5) or neutralized affinity purified 6363 immunoglobulins (Panel 6). Next day, membranes were stringently washed and incubated with HRP conjugated goat anti-chicken and processed further to detect immunoreactivity using ECL plus reagent and scanning on a PhosphorImager.

4A **4B**

Figure 4. Titer and specificity of GII α antibody

(A) Determination of optimal GII α antibody titer for Western blotting. Twenty micrograms of microsomal protein from cerebral cortex of adult mouse separated on 8% SDS-polyacrylamide gels were blotted onto nitrocellulose membrane, blocked in 5% goat serum in 1X PBS and incubated with increasing concentrations of GII α antibody overnight at 4 °C. Next day, membranes were washed, incubated with HRP conjugated goat anti-chicken for 2h at room temperature. Immunoreactive bands were visualized using ECL Plus reagent and scanning on a PhosphorImager. The position of 116 kDa GII α immunoreactive band is indicated by an arrow on the right. Size of broad range protein molecular weight markers (Lane M) is shown on the left. Dilution of affinity purified GII α chicken antibody used in this Western blot is as follows: Lane 1: 1:10,000; Lane 2: 1:20,000; Lane 3: 1:30,000; Lane 4: 1:40,000; Lane 5: 1:50,000; Lane 6: 1:60,000; Lane 7: 1:70,000; Lane 8: 1:80,000; Lane 9: 1:90,000; and Lane 10: 1:100,000). (B) Specificity of primary antibody-antigen interaction. Microsomal proteins (20 μ g) separated on SDS-PAGE were processed as in 4A for the detection of GII α immunoreactive band except that incubation with primary antibody was omitted. Immunoreactive bands were visualized using ECL Plus reagent and scanning on a PhosphorImager. Lane 1: microsomal fraction from cerebral cortex of adult mouse; Lane M: Broad-range protein molecular weight markers with size indicated on the left. (C) Specificity of GII α antibody by neutralization of GII α antibody. GII α antibody was pre-incubated overnight at 4 °C with peptide III in equal proportion (w/w) and then utilized for the detection of GII α protein by Western blotting as in 4A. Increasing concentration of microsomal protein from cerebral cortex of adult mouse were separated on SDS-PAGE and processed as in 4A to detect GII α protein by probing with 1:80,000 dilution of either GII α antibody (Lanes 1-3) or neutralized GII α antibody (Lanes 4-6). GII α immunoreactive band is indicated by solid arrow on the right. Lane M: Broad-range protein molecular weight

markers with size indicated on the left. **(D)** Specificity of GII α antibody by thrombin digestion. Microsomal proteins (20 μ g) from cerebral cortex of adult mouse were digested with thrombin overnight at 22 °C. Thrombin action was stopped by the addition of Laemmli's sample buffer. Both undigested (Lanes 1 and 3) and thrombin digested (Lane 2) samples were processed to detect GII α immunoreactive band by Western blotting as in 4A using 1:80,000 dilution of GII α antibody. Solid line on the right indicates the full length 116 kDa immunoreactive GII α protein band while solid arrow on the right points to a 101 kDa immunoreactive GII α protein band obtained following thrombin digestion. Lane M: Broad-range protein molecular weight markers with size indicated on the left.

Figure 5.

GII α immunoprecipitation. A known amount of microsomal proteins (1 mg) were solubilized by incubation with Triton X100 and centrifuged to obtain clear supernatant. Clear supernatant was first incubated with control agarose resin to prevent non-specific binding of proteins and then incubated overnight at 4 °C with GII α chicken antibody covalently coupled to agarose beads. Following stringent washing, GII α protein bound non-covalently to primary antibody-agarose beads was eluted twice. Eluted proteins and other fractions retained during immunoprecipitation and protein molecular weight markers were

separated on SDS-PAGE and probed with GII α chicken antibody (1:80,000 dilution). Following washing and incubation with secondary antibody, immunoreactive bands were visualized using ECL Plus reagent and scanning on a PhosphorImager. **Panel a:** Four immunoreactive bands were detected. The full length 116 kDa GII α immunoreactive band is indicated by a solid arrow A on the right. Three additional immunoreactive bands detected by GII α chicken antibody are indicated by solid arrows B, C, and D respectively. Lane 1: Pellet P1 containing detergent insoluble GII α protein (entire fraction); Lane 2: Soluble fraction (S1); Lane 3: Pre-cleared soluble fraction (S2); Lane 4: Unbound protein fraction (UP); Lane 5: 1st wash; Lane 6: 6th wash; Lane 7: 1st eluted sample; Lane 8: 2nd eluted sample; and Lane M: Broad-range protein molecular weight markers (NEB) with size indicated on the left. **Panel b:** Nitrocellulose membrane in 'Panel a' was washed appropriately to remove bound antibodies, incubated with ECL Plus reagent and scanned on PhosphorImager to detect any residual staining of immunoreactive bands retained prior to re-probing of the membrane. **Panel c:** Washed membrane in Panel b was incubated with goat anti-chicken HRP-conjugated secondary antibody for 2h at room temperature. Following washing, immunoreactive bands were visualized using ECL Plus reagent and scanning on a PhosphorImager.

Figure 6.

GIIα expression in mouse tissues. A known amount of microsomal protein (20 μg) from selective adult mouse tissues were separated on SDS-polyacrylamide gels, blotted, and the nitrocellulose membranes were cut at the 60 kDa protein molecular weight marker. The upper half of the membrane was blocked in 5% goat serum in 1X PBS and probed with GIIα chicken antibody (1:80,000 dilution). The lower half of the membrane was blocked in 5% milk in 10 mM Tris-HCl buffered saline containing 0.01% Tween 20 and incubated with β-actin antibody (1:7,000 dilution). Membranes were washed and incubated with appropriate

secondary antibodies and immunoreactive bands were visualized using ECL Plus solution. **A:** GII α expression in selective adult mouse tissues. The position of the 116 kDa GII α immunoreactive band is indicated by an arrow on the right. Lane Marker: Broad-range protein molecular weight markers with size indicated on the left. **B:** Quantitation of GII α immunoreactive band was performed using ImageQuant TL software. Data are expressed as arbitrary units (mean $\pm$ SEM of four independent experiments). Statistical analysis was performed by ANOVA and Scheffe's tests (* $p < 0.05$). **C:** GII α expression in three different regions of adult mouse brain. Lane 1: cerebral cortex, Lane 2: hippocampus, and Lane 3: cerebellum. The position of the 116 kDa GII α immunoreactive band is indicated by an arrow on the right. Lane M: Broad-range protein molecular weight markers with size indicated on the left. **D:** Quantitation of GII α immunoreactive band was performed using ImageQuant TL software. The signal intensity of GII α immunoreactive band was divided by the signal intensity of the β -actin immunoreactive band from the same lane. Data are expressed as arbitrary units (mean $\pm$ SEM of three independent experiments). Statistical analysis was performed by ANOVA and Scheffe's post-hoc tests (* $p < 0.05$).