

The LGI1/Epitempin gene encodes two protein isoforms differentially expressed in human brain

Sandra Furlan,¹ Federico Roncaroli,² Francesca Forner,^{3,4} Libero Vitiello,⁵ Elisa Calabria,⁶ Salomé Piquer-Sirerol,⁷ Giorgio Valle,⁴ Jordi Perez-Tur,⁷ Roberto Michelucci,⁸ and Carlo Nobile,¹

¹CNR-Istituto di Neuroscienze, Sezione di Padova, c/o Dipartimento di Scienze Biomediche Sperimentali, Università di Padova, Padua, Italy;

²Department of Neuropathology, Imperial College London, London, UK;

³Department of Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, Martinsried, Germany;

⁴CRIBI Biotechnology Centre, ⁵Dipartimento di Biologia, ⁶Dipartimento di Scienze Biomediche Sperimentali, Università di Padova, Padua, Italy;

⁷Unitat de Genètica Molecular, Institut de Biomedicina de València-CSIC, València, Spain;

⁸Dipartimento di Neuroscienze, Divisione di Neurologia, Ospedale Bellaria, Bologna, Italy.

Address correspondence and reprint requests to Carlo Nobile, Istituto di Neuroscienze del CNR, Sezione di Padova, c/o Dipartimento di Scienze Biomediche Sperimentali, Università di Padova, Viale G. Colombo 3, 35121 Padova, Italy. Email: nobile@bio.unipd.it

Abbreviations used: ADLTE, autosomal dominant lateral temporal epilepsy; ADPEAF; autosomal dominant partial epilepsy with auditory features; BCIP, 5-bromo-4-chloro-3-indolyl phosphate;

DMEM, Dulbecco's Modified Eagle's Medium; DTT, dithiothreitol; EAR, epilepsy associated region; ECL, enhanced chemiluminescence; EPTP, epitempin; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3phosphate dehydrogenase; GFP, green fluorescent protein; HEK, human embryonic kidney; LRR, leucine rich repeat; NBT, nitro blue tetrazolium; PMSF, phenylmethylsulfonyl fluoride; ppm, parts per million; SDS, sodium dodecylsulfate; SDS-PAGE, sodium dodecylsulfate-polyacrilamide gel electrophoresis; TBS, tris-buffered saline; TFA, trifluoroacetic acid;

Abstract

The leucine rich, glioma inactivated 1 (LGI1)/Epitempin gene has been linked to two phenotypes as different as gliomagenesis and autosomal dominant lateral temporal epilepsy. Its function and the biochemical features of the encoded protein are unknown. We characterized the LGI1/Epitempin protein product by Western blot analysis of mouse and human brain tissues. Two proteins of about 60 and 65 kDa were detected by an anti-LGI1 antibody within the expected molecular mass range. The two proteins appeared to reside in different subcellular compartments, as they were fractionated by differential centrifugation. The specificity of both polypeptides was validated by cell transfection assay and mass spectrometry analysis. Immunoblot analysis of protein distribution in various zones of the human brain revealed variable amounts of both proteins. Notably, these proteins were more abundant in the temporal neocortex than in the hippocampus, the difference in abundance of the 65-kDa product being particularly pronounced. These results suggest that the two protein isoforms encoded by LGI1/Epitempin are differentially expressed in the human brain, and that higher expression levels of these proteins in the lateral temporal cortex may underlie the susceptibility of this brain region to the epileptogenic effects of LGI1/Epitempin mutations.

Key words: temporal epilepsy; LGI1 protein; Western blot; brain localization

Running title: LGI1 protein expression in human brain

Introduction

The human leucine rich, glioma inactivated 1 (LGI1) gene appears to be involved in malignant progression of gliomas and carries mutations in families with autosomal dominant lateral temporal epilepsy (ADLTE), a rare familial partial epilepsy syndrome. This gene was found to be frequently downregulated and sometimes rearranged in malignant gliomas (Chernova *et al.* 1998), suggesting a possible tumor suppression function. However, neither point mutations of the coding region nor differential methylation of the LGI1 core promoter region have been demonstrated in these tumors (Somerville *et al.* 2000), arguing against a role of LGI1 as a tumor suppressor gene. Recent studies have suggested that LGI1 may have a role in gliomagenesis as a tumor metastasis suppressor gene (Kunapuli *et al.* 2003; Kunapuli *et al.* 2004).

ADLTE, also known as autosomal dominant partial epilepsy with auditory features (ADPEAF), has been recognized as a distinct epileptic condition characterized by typical auditory auras and/or symptoms suggesting a lateral temporal onset, absence of any brain structural abnormality, and benign evolution. Recently, we and others have identified mutations causing ADLTE in LGI1 (Kalachikov *et al.* 2002; Morante-Redolat *et al.* 2002). Numerous additional LGI1 mutations resulting in either protein truncation or single amino acid substitutions have been reported subsequently (see Ottman *et al.* 2004), including a *de novo* mutation (Bisulli *et al.* 2004). Overall, LGI1 mutations have been found in about 50% of ADLTE families (Michelucci *et al.* 2003; Ottman *et al.* 2004; Berkovic *et al.* 2004).

LGI1, which has been renamed Epitempin, is mostly expressed in the brain (Chernova *et al.* 1998). *In situ* hybridisation experiments have shown that expression of the orthologous murine gene is predominantly neuronal (Kalachikov *et al.* 2002). Both in humans and mice, the main transcription product is predicted to encode a protein of 557 amino acids. Computer analysis of the amino acid sequence has identified an amino-terminal signal peptide sequence and two distinct structural domains, each spanning about half of the protein. The N-terminal half of the protein consists of 3.5

leucine-rich repeat (LRR) sequences flanked on both sides by typical cysteine-rich repeat sequence clusters (Kobe and Kajava 2001); the C-terminal half is made of seven copies of a novel repeat of about 45 residues, named epitempin (EPTP; Staub *et al.* 2002) or epilepsy associated region (EAR; Scheel *et al.* 2002), which is reminiscent of the beta-propeller structural domain (Paoli 2001). The same structural disposition of LRR and EPTP/EAR domains has been identified in three putative paralogues, named LGI2, LGI3, and LGI4 (Gu *et al.* 2002; Staub *et al.* 2002; Scheel *et al.* 2002). LRR and beta-propeller motifs are found in many other proteins and often mediate protein-protein interactions.

Functional studies of LGI1/Epitempin are still at early stage. Particularly, the biochemical features, subcellular localization, and distribution in brain tissues of the protein product have not yet been characterized. A few studies have investigated LGI1/Epitempin protein expression in human and mouse brain by immunohistochemistry (Gu *et al.* 2002; Morante-Redolat *et al.* 2002), but signal specificity of the polyclonal antibodies utilized was poorly characterized.

In this study, we characterized the basic biochemical features of the LGI1/Epitempin protein product and investigated its subcellular localization and distribution in various regions of the human brain by Western blot analysis.

Materials and methods

Materials

Reagents for sodium dodecylsulfate-polyacrilamide gel electrophoresis (SDS-PAGE) were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Molecular mass standards for SDS-PAGE (phosphorylase B, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α -lactalbumin, Mr 97, 66, 45, 30, 20,1, and 14,4 kDa, respectively), enhanced chemiluminescence (ECL) Western Blotting Detection Reagents, and autoradiography film were from Amersham Biosciences (Piscataway, NJ, USA). Phenylmethyl-sulfonyl fluoride (PMSF), benzamidine,

leupeptin, bovin serum albumin, EGTA, sucrose, HEPES, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) liquid substrate system, nitro blue tetrazolium (NBT), and anti-mouse IgG conjugated to alkaline phosphatase secondary antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrocellulose was from Sartorius (Hamburg, Germany). Anti-rabbit and anti-goat IgG conjugated to horseradish peroxidase secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA), Opti-MEM and Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA), and trypsin from Promega (Madison, WI, USA).

Antibodies

Six polyclonal antibodies raised against different LGII/Epitempin peptides were tested: the commercially available antibodies sc-9583, sc-9581, and sc-28238 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); a rabbit antiserum developed by Kunapuli and co-workers (2003) (generous gift of Dr. J. K. Cowell); and two additional antisera developed by us (see Morante-Redolat *et al.* 2002). A mouse monoclonal antibody to glyceraldehyde-3phosphate dehydrogenase (GAPDH) was from Chemicon (Temecula, CA, USA).

Mouse tissue preparation

Adult mice (strain C57BL/6J) were used as a source of brain, skeletal muscle, heart, kidney, spleen, and liver. Tissues were frozen immediately in liquid nitrogen and stored at -80°C until use. Whole homogenates were prepared as described previously (Salvatori *et al.* 1997). Briefly, tissues were homogenized by using an Ultraturrax disperser (20,000 RPM) in the presence of a medium containing 3% (wt/vol) sodium dodecylsulfate (SDS), 0,1 mM EGTA, pH 7.0 and a protease inhibitor mixture (PMSF and benzamidine 1 mmol/l, leupeptin 0,1 mmol/l). Homogenates were then boiled for 5 min and clarified at 15,000xg for 10 min. Supernatants were used as whole protein extracts.

For mouse brain fractionation all steps were carried out at 4°C. Brains from adult mice were homogenized in 0,32 M sucrose, 10 mM HEPES-KOH, pH 7.4 at 4 ml/g of tissue in the presence of the above protease inhibitor mixture to minimize protein degradation by using an Ultraturrax disperser (13,500 RPM). A post-nuclear supernatant was prepared by centrifugation of the homogenate at 3,000xg for 10 mins, and spinned again at 25,000xg for 10 mins. A crude membrane fraction was separated from the cytosolic fraction by centrifuging the last supernatant at 35,000xg or 105,000xg for 1 hr. The resulting pellet (microsomal fraction) was resuspended in RIPA buffer (50mM Tris-HCl pH 8.0, 0.15 M NaCl, 1% (vol/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS).

Human brain tissue preparation

Fresh tissue samples of grey matter from superior frontal gyrus, post-central (parietal) cortex, superior temporal gyrus, primary visual (occipital) cortex, and samples from anterior superior cerebellar vermis, hippocampus (Ammon's horn) and putamen were taken from brains of an Italian 80-year-old male (subject 1) died of heart stroke and a British 70-year-old female (subject 2) died of lung carcinoma at the Institute of Anatomic Pathology, Bellaria Hospital, Bologna, Italy and the UK Multiple Sclerosis Tissue Bank, respectively. Sampling was performed 12 hours post-mortem. Neuropathological examination did not reveal significant pathological abnormalities but ageing changes consisting of a few diffuse senile plaques in the frontal and hippocampal cortex. Particularly, no metastatic deposits were seen in the brain from subject 2. After removing leptomeninges, cortical ribbon was dissected using a magnification lens in order to avoid any contamination or white matter. Tissues were frozen in liquid nitrogen straight after sampling and then stored at – 70°C. Whole protein homogenates were obtained as described for mouse tissues.

Cell culture transfection assay

Human embryonic kidney 293 (HEK293) cells were grown in 4 ml DMEM plus 10% (vol/vol) FBS on 25 cm² flasks and transfected with a pcDNA3LGI1 expression construct using Lipofectamine 2000, following the manufacturer instructions. Twenty-four hours after the beginning of transfection cells were washed twice and then re-fed with 4 ml of serum-free medium Opti-MEM. Cells were kept in serum-free medium for about 20 hours. The medium was then collected and centrifuged to pellet cell debris, after which the supernatant was concentrated to about 100 µl using Centricon YM30 concentrators (Millipore, Billerica, MA, USA). Twenty-five µl of the concentrated medium were loaded on an SDS-PAGE gel and analysed by Western blot. In parallel, we also loaded a similarly concentrated medium obtained from cells transfected with pcDNA3 vector alone or a green fluorescent protein (GFP) expression construct. Cell extracts were prepared for immunoblotting using Triton lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% (vol/vol) Triton, 10% (vol/vol) Glycerol, 1mM EDTA) supplemented with proteases inhibitors (Leupeptin 10 µg/ml, Aprotinin 10 µg/ml, PMSF 1mM) and phosphatase inhibitors (20mM NaF, 20 mM Na₂VO₃, β-glycerophosphate).

Protein analysis

Protein concentration was determined as described by Bradford (1976), using bovine serum albumin as standard. Tissue homogenates, brain tissue fractions, and cell lysates (50 µg/lane), and concentrated media were separated on 5-15% SDS-polyacrylamide gels according to Laemmli (1970) and then electroblotted onto nitrocellulose membrane. The integrity of the Western blot was analysed by Red Ponceau staining. Destained membranes were blocked with 10% (vol/vol) skimmed milk in tris-buffered saline (TBS) for 1 hr and then incubated with primary antibody in TBS containing 2% (vol/vol) skimmed milk for 2 hrs at room temperature or, in some cases, ON at 4°C. Proteins immunostained with anti-LGI1 antibody were detected with a horseradish peroxidase-labelled secondary antibody and enhanced chemiluminescence (ECL) reagent and visualized by autoradiography, while GAPDH was detected with an alkaline phosphatase-labeled secondary

antibody and visualized by using BCIP/NBT system. Densitometric analysis was performed using the Scion Image for Windows software, version Beta 4.0.2 (Scion Corp., Frederick, MD, USA), downloaded from the web site: www.scioncorp.com.

Mass spectrometry analysis

Mouse microsomal and cytosolic fraction proteins and HEK293-LGI1 medium proteins were resolved by standard SDS-PAGE prior to cutting the relevant lanes of the gel into slices. Those slices corresponding to the regions of immunoreactivity with sc-9583 were then subjected to mass spectrometry analysis together with control slices. Following de-staining, gel slices were washed with 50 mM ammonium bicarbonate and shrunk with ethanol. Reduction/alkylation of proteins was performed with 10 mM dithiothreitol (DTT) and 55 mM iodoacetamide. After two wash steps with ammonium bicarbonate/ethanol, the gel was dried with ethanol and incubated with 12.5 ng/ μ l trypsin in 50 mM ammonium bicarbonate at 4°C for 15 min. The supernatant was then replaced with fresh 50 mM ammonium bicarbonate and the reaction allowed to proceed overnight at 37°C. The reaction was stopped with 1% (vol/vol) trifluoroacetic acid (TFA), 0.5% (vol/vol) acetic acid and 3% (vol/vol) acetonitrile and the supernatant recovered. Additional peptide extraction steps were performed with 30% (vol/vol) acetonitrile and 100% acetonitrile. Supernatants were concentrated and then diluted with 0.5% (vol/vol) acetic acid, 30% (vol/vol) acetonitrile, 1% (vol/vol) TFA. Peptides were desalted and concentrated on reverse phase C₁₈ StageTips (Rappsilber et al 2003). Liquid chromatography was performed on a 20 cm fused silica capillary column (75 μ m ID) packed in-house with reverse phase C₁₈ material and the eluate was electrosprayed directly into a 7 Tesla LTQ-FT mass spectrometer (Thermo Electron, San Jose, CA, USA). Data were acquired in data-dependent mode. Fragment ions were searched against the mouse IPI database (<ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/>) using the Mascot server with the following parameters: trypsin specificity, two missed cleavages, cysteine carbamidomethylation as a fixed modification, methionine oxidation, protein N-terminal acetylation and N/Q deamidation as variable

modifications. The mass error of all the identified peptides was below 1 parts per million (ppm) and these peptides were matched with the highest score to the LGI1 sequence (“bold red” in Mascot).

Results

Immunoblot analysis of mouse tissues

In humans and mice, the LGI1/Epitempin protein product is highly conserved (97% identity) and has an expected molecular mass of 64 kDa. We tested six polyclonal antibodies raised against LGI1/Epitempin peptides (see Materials and Methods) by Western blot analysis of whole cell extracts from mouse brain. Five of these antibodies detected weak or no bands around 60 kDa (data not shown). The commercially available antibody sc-9583 detected two bands at about 60 and 65 kDa, the 60-kDa product being far more abundant (Fig. 1 A). None of these bands was detected in other mouse tissues (Fig. 1 A), and detection of both proteins was abolished by blocking the primary antibody with the peptide used for immunization (not shown). An additional protein of about 22 kDa was recognized only in mouse skeletal muscle (Fig. 1 A), which may represent a muscle-specific isoform or a cross-reaction product.

Differential centrifugation of mouse brain homogenates

To investigate the subcellular localization of the two brain-specific proteins, we performed immunoblot analysis of crude microsomal and cytosolic fractions obtained by differential centrifugation of whole mouse brain homogenates. The sc-9583 antibody detected the 60-kDa protein exclusively in the microsomal fraction, whereas the 65-kDa product was found in the cytosolic fraction, where it was considerably enriched (Fig. 1 B). Western blots of microsomal and cytosolic fractions were then probed with the other five anti-LGI1 antibodies. The rabbit antiserum developed by Kunapuli and colleagues (2003) detected a band of about 65 kDa in the cytosolic fraction exactly co-migrating with the cytosolic protein identified by sc-9583, whereas no bands

were noted in the microsomal fraction (Fig. 2). Although it cross-reacted with other proteins of various molecular masses, this antiserum was previously shown to recognize an LGI1-specific polypeptide in extracts from glioblastoma cell lines stably transformed with an LGI1-FLAG construct (Kunapuli *et al.* 2003). Therefore, both Kunapuli's and sc-9583 antibodies very likely recognized the same 65-kDa cytosolic protein that was encoded by LGI1.

Cell culture expression of LGI1/Epitempin

The proteins expressed by HEK293 cells transiently transfected with an LGI1/Epitempin expression construct were analysed by immunoblotting. Both cell lysate and concentrated (about 40x) serum-free medium were analysed using the sc-9583 antibody. In the medium, this antibody detected a band similar in size to the 60-kDa protein recognized in mouse brain (Fig. 3). No such band was detected in the lysate of LGI1-transfected cells or in the medium of HEK293 cells transfected with a GFP expression construct as control. The specificity of the protein seen in the medium of LGI1-transfected cells was confirmed by mass spectrometry analysis: several LGI1-specific peptides were identified in a gel-fractionated medium protein sample corresponding to the band immunoreacting with the sc-9583 antibody, altogether spanning 28% of the LGI1/Epitempin protein length (see Supplementary Table S3). Thus, our results show that the LGI1/Epitempin protein produced in transfected HEK293 cells is secreted, confirming the data published recently by Senechal and co-workers (2005).

Mass spectrometry analysis of mouse brain proteins

To gain further, more direct, evidence for the specificity of both the 60 and 65 kDa products to LGI1/Epitempin, we analysed gel-fractionated mouse brain proteins by mass spectrometry. Protein samples resolved by SDS-PAGE and corresponding to the microsomal and cytosolic bands recognized by the sc-9583 antibody were cut out of gel, digested with trypsin and analyzed (see Materials and Methods). Several LGI1-specific peptides were identified in both samples, spanning

25% and 15% of the microsomal and cytosolic protein forms, respectively (Fig. 4; details in Supplementary Tables S1 and S2). This finding combined with the results described above strongly suggest that the two products recognized by the sc-9583 antibody are protein isoforms encoded by LGI1/Epitempin.

Immunoblot analysis of human brain tissues

To check whether the two proteins detected in the mouse brain by sc-9583 were also expressed in the human brain, we performed Western blot analysis of samples of grey matter taken from post-mortem brains of two individuals with no neurological diseases. Tissue samples from cortical areas of the frontal, parietal, occipital, and temporal lobes, and from cerebellum, hippocampus, and putamen were analysed. Tissue extracts subjected to immunoblot contained comparable amounts of total proteins, as determined by Red Ponceau staining and immunostaining with anti-GAPDH antibody (Fig. 5 B). Both the 60- and 65-kDa proteins were shown by the sc-9583 antibody in most human brain samples, and, as in the mouse, the 60-kDa product was found to be more abundant than that of 65 kDa (Fig. 5 A). Expression of the two proteins was considerably different in the various tissue samples from the two individuals analysed (Fig. 5 A). The 65-kDa protein was absent in the cerebellum and was barely detectable in the occipital cortex and hippocampus; higher amounts were observed in the parietal and frontal cortices, putamen, and, particularly, in the temporal neocortex, where this protein was 3 to 5 times more abundant than in the hippocampus, as shown by densitometry (data not shown). Variations in abundance of the 60-kDa product were also considerable in some samples (Fig. 5 A), the highest expression level occurring in the occipital cortex and the lowest in the hippocampus (2:1 densitometric ratio; data not shown). Although some differences in expression pattern were seen, particularly in the cerebellum, comparable band intensities were observed in most tissue samples from the two individuals analysed, suggesting that the expression variations seen in different brain regions were site-specific.

Discussion

The data presented in this paper show that LGI1/Epitempin encodes two proteins of approximately 60 and 65 kDa, which reside in distinct cell compartments and are differentially expressed in various human brain regions.

The finding of two LGI1/Epitempin protein products may be explained in different ways. Given the predicted molecular weight of 64 kDa of the LGI1/Epitempin cDNA translation product, the protein of about 65 kDa detected by the sc-9583 antibody may correspond to the full-length protein, possibly a precursor or an unprocessed form, and the 60-kDa product may result from cleavage of the putative N-terminal signal peptide. Alternatively, the two protein isoforms may result from differential splicing of the LGI1/Epitempin transcript. Because of the small difference between the masses of the two polypeptides, the corresponding mRNA splice isoforms would presumably differ in size by about 100-150 nucleotides, a difference not detectable by Northern blot. However, no ESTs have been identified that are compatible with splice isoforms other than the one predicted to encode a short, 292-amino acid, polypeptide spanning the N-terminal LRR domain (Morante-Redolat *et al.* 2002).

Recently, Senechal and collaborators (2005) have shown that the sc-9583 antibody specifically recognizes the LGI1/Epitempin protein that is secreted by 293T cells transfected with LGI1/Epitempin cDNA. Here, we confirm this result and show that the soluble LGI1/Epitempin protein secreted by transfected HEK293 cells seems to correspond to the 60-kDa product detected in brain tissues (Fig. 3). Yet, the latter protein was found only in the membrane-enriched fraction and not in the soluble fraction obtained from mouse brain tissues. A possible explanation for this is that the 60-kDa LGI/Epitempin product, once secreted outside brain cells, binds to other proteins to form a protein complex bound to membrane structures. Alternatively, this protein may undergo

processes within neuronal cells very different from those occurring in non-neuronal transfected cells, possibly not leading to secretion.

Cell transfection experiments performed by Senechal and collaborators (2005) also demonstrated that the sc-9583 antibody, in addition to recognizing the LGI1/Epitempin product, cross-reacts with the LGI3 protein, which is similar in size to LGI1/Epitempin. Yet, *in situ* hybridisation data by the same authors have shown that LGI3 is expressed at low levels throughout the adult mouse brain, the highest amount of expression being in the facial nerve nucleus (Senechal *et al.* 2005). Consistent with these mRNA expression data, our mass spectrometry analysis of gel-fractionated mouse brain proteins identified several LGI1/Epitempin peptides in both the soluble and membrane protein samples immunoreacting with sc-9583, whereas no peptides from LGI3 or other paralogous proteins were recognized. This finding together with the results of LGI1-transfection experiments, and the detection of the 65-kDa product by two different anti-LGI1 antibodies (sc-9583 and the antiserum by Kunapuli and collaborators (2003)) strongly suggest that both the 60-kDa and 65-kDa protein species are encoded by LGI1/Epitempin.

Northern blot analysis has revealed that LGI1/Epitempin is widely expressed in the human brain, though with varying intensity in different brain regions (Chernova *et al.* 1998). In addition, *in situ* hybridisation experiments have shown that expression of the murine LGI1 gene is higher in some areas, particularly in the neocortex and limbic regions (Kalachikov *et al.* 2002; Senechal *et al.* 2005). In keeping with mRNA expression studies, our immunoblotting data clearly showed variable expression of both LGI1/Epitempin proteins in the human brain regions investigated, suggesting that expression of the LGI1/Epitempin gene may be differentially regulated in different brain regions and that such tissue-specific expression may affect neuronal function. Notably, the abundance of the 65-kDa LGI1/Epitempin product was much higher in the lateral temporal cortex than in the hippocampus, where this protein species was barely detectable; the 60-kDa product also appeared to be more expressed in the temporal neocortex than in the hippocampus, though the

expression difference was less remarkable. Such differential expression of the LGI1/Epitempin proteins in the temporal lobe of normal individuals provides a clue to the understanding of the molecular mechanisms leading to ADLTE: expression of higher amounts of these proteins in the lateral temporal cortex may underlie the susceptibility of this brain region to the epileptogenic effects of LGI1/Epitempin mutations. This possible mechanism should be specific for the lateral temporal neurons, since increased amounts of the LGI1/Epitempin proteins were observed also in other cortical regions such as frontal and parietal cortices, from which focal seizures are unlikely to originate in epileptic patients with LGI1/Epitempin mutations. Presumably, neurons of these latter cortical areas are likely to be involved in circuits different from those involving the temporal cortex and/or may produce additional factors exerting protective effects that prevent seizure onset.

The possibility exists that occurrence of variable amounts of the LGI1/Epitempin proteins in different brain regions results from protein transport to specific regions rather than tissue-specific expression. Functional studies are needed to clarify this point. If their functions resemble that of the structurally homologous *slit* protein, a diffusible LRR protein involved in axon guidance and neuronal migration (Battye et al. 2001; Wu et al. 1999), then the LGI1/Epitempin products, once secreted, are likely to exert their action locally.

In conclusion, we have identified two protein products very likely encoded by LGI1/Epitempin, which are differentially expressed in the temporal cortex, consistent with the lateral temporal localization of seizures in patients with LGI1/Epitempin mutations. Further work is needed to clarify their biochemical differences, and to determine their fine distribution across the human brain.

While this paper was under revision, Schulte and co-workers (2006) showed that an LGI1 protein product of about 60 kDa is complexed with Kv1.1 potassium channels in neurons of rat hippocampus. This protein, the single product recognized by an anti-LGI1 antibody developed by these authors, was found in the membrane-enriched fraction obtained from rat brain homogenate and was shown to be associated to the neuronal membrane possibly through protein-protein

interactions. Because of its molecular mass and subcellular localization, the protein described by Schulte and co-workers very likely corresponds to the 60-kDa LGI1 isoform recognized by the sc-9583 antibody.

Acknowledgments

We would like to thank J.K. Cowell for providing the anti-LGI1 antiserum raised in his laboratory. Brain tissue samples were supplied by the UK Multiple Sclerosis Tissue Bank, funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland, registered charity 207495, and by the Institute of Anatomic Pathology, Bellaria Hospital, Bologna, Italy. We thank S. Salvatori and G. A. Danieli for helpful comments and reading the manuscript. This work was supported by Telethon-Italy (grant n. GGP02339 to C.N. and R.M.), the Genetic Commission of the Italian League Against Epilepsy (R.M. and C. N.), the Spanish Ministerio de Educacion y Ciencia (SAF2002-00060 to J. P.-T.), and by the Cooperative Programme CSIC-CNR (2003IT0018 to J.P.-T. and C.N.). J.P.-T. is part of a Network of Excellence of the Generalitat Valenciana.

References

- Battye R., Stevens A., Perry R.L. and Jacobs J.R. (2001) Repellent signalling by Slit requires the leucine-rich repeats. *J. Neurosci.* 21, 4290-4298.
- Berkovic S.F., Izzillo P., McMahon J.M. et al (2004) LGI1 mutations in temporal lobe epilepsies. *Neurology* 62, 1115-1119.

- Bisulli F., Tinuper P., Scudellaro E., Naldi I., Bagattin A., Avoni P., Michelucci R. and Nobile C. (2004) A de novo LGI1 mutation in sporadic partial epilepsy with auditory features. *Ann. Neurol.* 56, 455-456.
- Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-54.
- Chernova O.B., Somerville R.P. and Cowell J.K. (1998) A novel gene, LGI1, from 10q24 is rearranged and downregulated in malignant brain tumors. *Oncogene* 17, 2873-2881.
- Gu W., Wevers A., Schroeder H., Grzeschik K.H., Derst C., Brodtkorb E., de Vos R., and Steinlein O.K. (2002) The LGI1 gene involved in lateral temporal lobe epilepsy belongs to a new subfamily of leucine-rich repeat proteins. *FEBS Lett.* 519, 71-76.
- Kalachikov S, Evgrafov O, Ross B. et al (2002) Mutations in LGI1 cause autosomal dominant partial epilepsy with auditory features. *Nat. Genet.* 30, 335-41.
- Kobe B. and Kajava A.V. (2001) The leucine-rich repeat as a protein recognition motif. *Curr. Opin. Struct. Biol.* 11, 725-732.
- Kunapuli P., Chitta K,S, and Cowell J.K. (2003) Suppression of the cell proliferation and invasion phenotypes in glioma cells by the LGI1 gene. *Oncogene* 22, 3985-3991.
- Kunapuli P., Kasyapa C.S., Hawthorn L. and Cowell J.K. (2004) LGI1, a putative tumor metastasis suppressor gene, controls in vitro invasiveness and expression of matrix metalloproteinases in glioma cells through the ERK1/2 pathway. *J. Biol. Chem.* 279, 23151-23157.
- Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

- Michelucci R., Poza J.J., Sofia V. et al (2003) Autosomal dominant lateral temporal epilepsy: clinical spectrum, new epitempin mutations, and genetic heterogeneity in seven European families. *Epilepsia* 44, 1289-1297.
- Morante-Redolat J.M., Gorostidi-Pagola A., Piquer-Sirerol S. et al (2002) Mutations in the LGI1/Epitempin gene on 10q24 cause autosomal dominant lateral temporal epilepsy. *Hum. Mol. Genet.* 11, 1119-1128.
- Ottman R., Winawer M.R., Kalachikov S., Barker-Cummings C., Gilliam T.C., Pedley T.A. and Hauser W.A. (2004) LGI1 mutations in autosomal dominant partial epilepsy with auditory features. *Neurology* 62, 1120-1126.
- Paoli M. (2001) Protein folds propelled by diversity. *Prog. Biophys. Molec. Biol.* 76,103-130.
- Rappsilber J., Ishihama Y. and Mann M. (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* 75, 663-670.
- Salvatori S., Biral D., Furlan S. and Marin O. (1997) Evidence for localization of the myotonic dystrophy protein kinase to the terminal cisternae of the sarcoplasmic reticulum. *J. Muscle Res. Cell. Motil.* 18, 429-440.
- Scheel H., Tomiuk S. and Hofmann K. (2002) A common protein interaction domain links to recently identified epilepsy genes. *Hum. Mol. Genet.* 11, 1757-1762.
- Schulte U., Thumfart J.O., Klocker N. et al. (2006) The epilepsy-linked Lgi1 protein assembles into presynaptic Kv1 channels and inhibits inactivation by Kvbeta1. *Neuron* 49, 697-706.
- Senechal K.R., Thaller C. and Noebels J.L. (2005) ADPEAF mutations reduce levels of secreted LGI1, a putative tumor suppressor protein linked to epilepsy. *Hum. Mol. Genet.* 14, 1613-1620.

- Somerville R.P., Chernova O.B., Liu S., Shoshan Y. and Cowell J.K. (2000)

Identification of the promoter, genomic structure, and mouse ortholog of LGI1. *Mamm. Genome* 11, 622-627.

- Staub E., Perez-Tur J., Siebert R., Nobile C., Moschonas N.K., Deloukas P. and

Hinzmann B. (2002) The novel EPTP repeat defines a superfamily of proteins with implications in epileptic disorders. *Trends Biochem. Sci.* 27, 441-444.

- Wu W., Wong K., Chen J.-H., Jiang Z.-H., Dupuis S., Wu J.Y. and Rao Y. (1999)

Directional guidance of neuronal migration in the olfactory system by the protein Slit. *Nature* 400, 331-336.

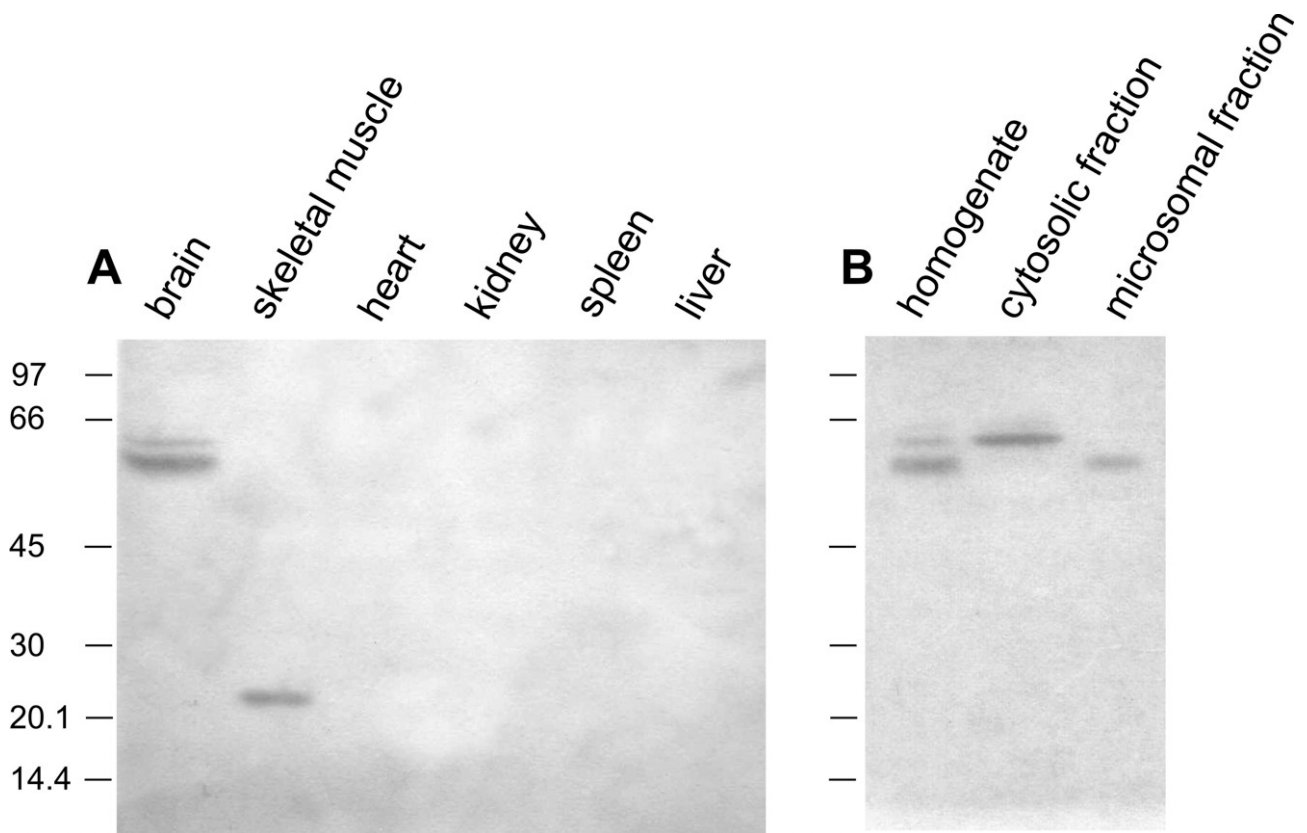


Fig. 1. A: Immunoblot analysis of mouse tissues with the anti-LGI1 antibody sc-9583. The tissues investigated are indicated on top. Molecular weight markers (in kDa) are indicated on the left. **B:** Immunoblot analysis of fractionated mouse brain tissue extract. Cytosolic and microsomal fractions obtained by ultracentrifugation of mouse brain homogenate were analyzed with sc-9583.

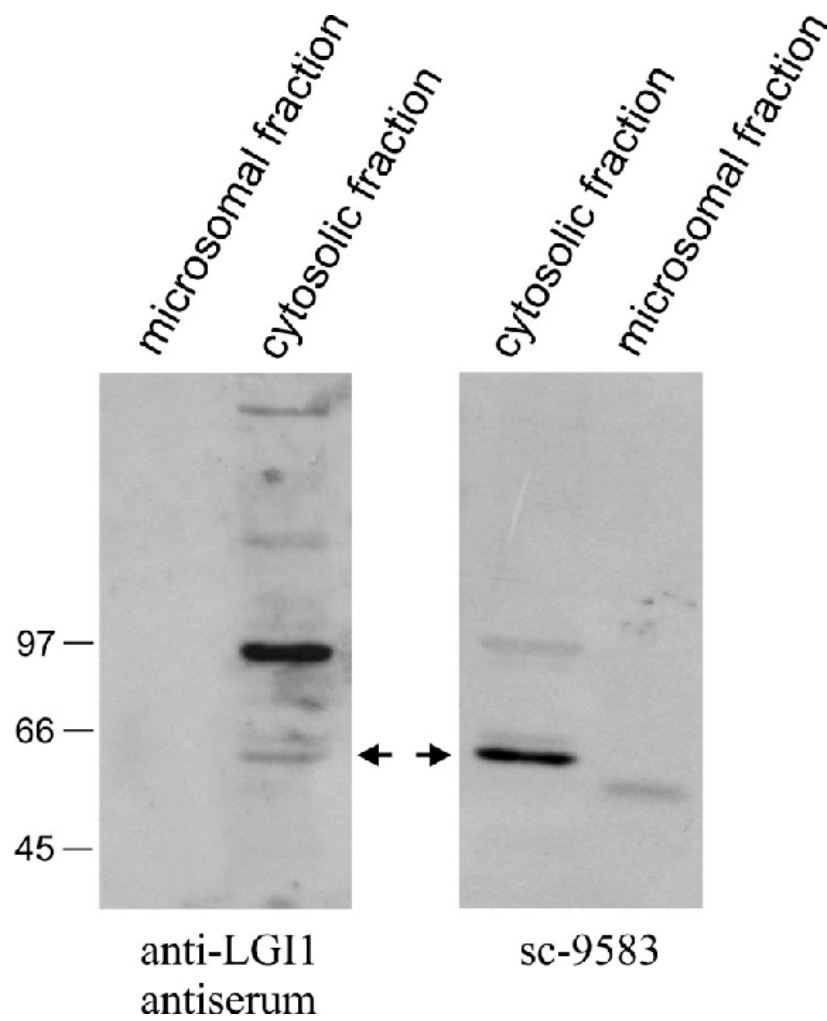


Fig. 2. Immunoblot analysis of mouse brain protein fractions. Microsomal and cytosolic fractions prepared as in Fig. 1 were probed with the sc-9583 antibody (right panel) or the anti-LGI1 antiserum developed by Kunapuli and co-workers (2003) (left panel). The arrows point to the common 65-kDa band. Molecular mass markers are indicated on the left.

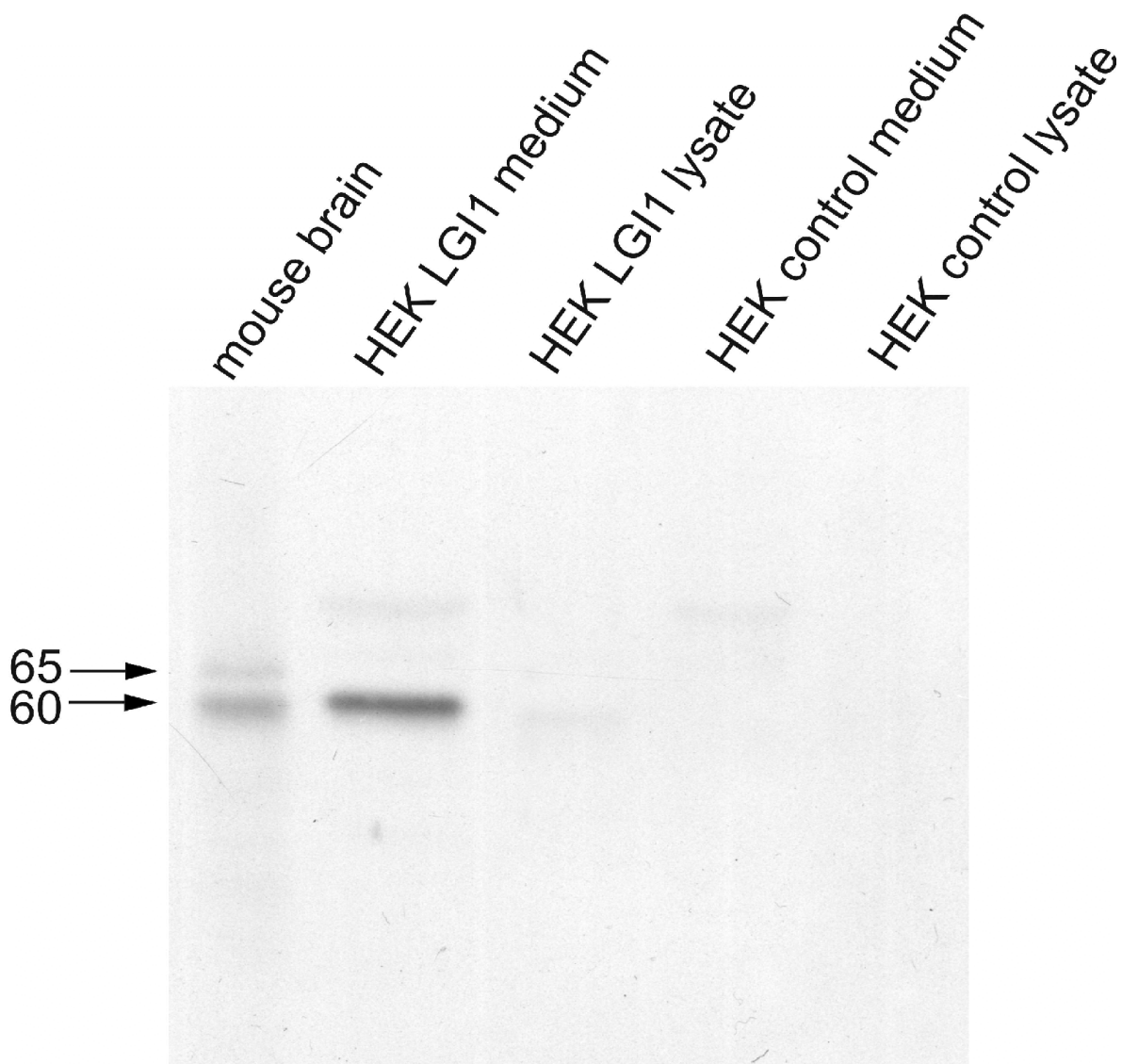


Fig. 3. Immunoblot analysis of transfected HEK293 cells. Cell lysate and concentrated (about 40x) medium of HEK293 cells transfected with an LGI1/Epitempin expression construct (HEK LGI1) or with a GFP expression construct (HEK control) were analyzed with the anti-LGI1 antibody sc-9583 together with a mouse brain homogenate sample. Arrows on the left indicate the LGI1/Epitempin 60- and 65-kDa proteins detected in whole mouse brain homogenate.

A

```

1 MESESSRRMGNACIPLKRIAYFLCLFSVLLTEGKKPAKPKCPAVCTCSK
51 DNALCENARSIPTVPPDVISLSFVRSFGFTEISEGSFLFTPSLQLLLFTS
101 NSFVISDDAFIGLPHLEYLFIENNNIKSISRHTFRGLKSLIHLSLANNN
151 LQTLPKDIFKGLDSLNTVDLRGNAFNCDCCLKWLVWLGHTNATVEDIYC
201 EGPPEYKKRKINSLSPKDFDCIITEFAKSQDLQSLSDTFSYLNDEYV
251 VIAQPFTGKCIFLEWDHVEKTFRNYDNITGTSTVVCKPIVIDTQLYVIVA
301 QLFGGSHIYKRDGFANKFIKIQDIEVLKIRKPNDIETFKIEDNWFVVD
351 SSKAGFTTIYKWNNGGFYSHQSLHAWYRDTDVEYLEIARPPLALRTPHLI
401 LSSSSQRPVIYQWSKATQLFTNQTDIPNMEDVYAVKHFSVKGDVYICLTR
451 FIGDSKVMKWGGSSFQDIQRMPSRGSVMFQPLQINNYQYAILGSDYSFTQ
501 VYNWDAEKAKFVKFQELNVQAPRSFTHVSINKRNFLFASSFKGNTQIYKH
551 VIVDLSA

```

B

```

1 MESESSRRMGNACIPLKRIAYFLCLFSVLLTEGKKPAKPKCPAVCTCSK
51 DNALCENARSIPTVPPDVISLSFVRSFGFTEISEGSFLFTPSLQLLLFTS
101 NSFVISDDAFIGLPHLEYLFIENNNIKSISRHTFRGLKSLIHLSLANNN
151 LQTLPKDIFKGLDSLNTVDLRGNAFNCDCCLKWLVWLGHTNATVEDIYC
201 EGPPEYKKRKINSLSPKDFDCIITEFAKSQDLQSLSDTFSYLNDEYV
251 VIAQPFTGKCIFLEWDHVEKTFRNYDNITGTSTVVCKPIVIDTQLYVIVA
301 QLFGGSHIYKRDGFANKFIKIQDIEVLKIRKPNDIETFKIEDNWFVVD
351 SSKAGFTTIYKWNNGGFYSHQSLHAWYRDTDVEYLEIARPPLALRTPHLI
401 LSSSSQRPVIYQWSKATQLFTNQTDIPNMEDVYAVKHFSVKGDVYICLTR
451 FIGDSKVMKWGGSSFQDIQRMPSRGSVMFQPLQINNYQYAILGSDYSFTQ
501 VYNWDAEKAKFVKFQELNVQAPRSFTHVSINKRNFLFASSFKGNTQIYKH
551 VIVDLSA

```

Fig. 4. LGI1 peptides identified by mass spectrometry analysis of mouse brain protein samples. Grey boxes along the mouse LGI1 amino acid sequence delimit the peptides from the microsomal (A) and cytosolic (B) protein forms.

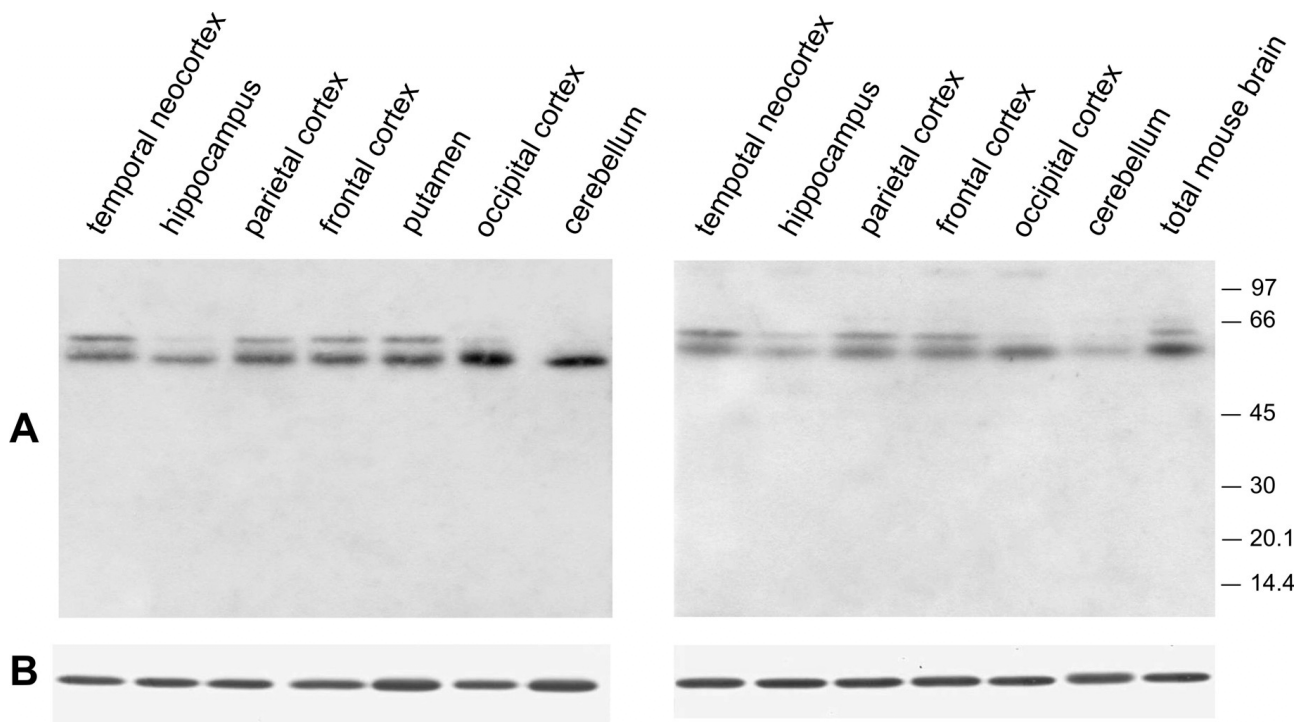


Fig. 5. A: Immunoblot analysis of human brain tissue samples from two different individuals. Comparable amounts of whole cell protein extracts from samples taken from the brain regions indicated were analyzed with the anti-LGI1 antibody sc-9583. Left panel, subject 1; right panel, subject 2. The rightmost sample on the right panel is whole mouse brain. Molecular weight markers are indicated on the right. **B:** Immunoblot analysis of the same tissue samples as in A with an antibody to GAPDH.