The epilepsy gene *LGI1* encodes a secreted glycoprotein that binds to the cell surface.

Mª Salomé. Sirerol-Piquer[1, †], Ana Ayerdi-Izquierdo[1, †], José Manuel Morante-Redolat[1], Vicente Herranz-Pérez[1], Kristy Favell[2], Philip A. Barker[2], Jordi Pérez-Tur[1, *]

1. Unitat de Genètica Molecular. Departament de Genòmica i Proteòmica. Institut de Biomedicina de València-CSIC, València (Spain).
2. Montreal Neurological Institute, McGill University, Montreal, Quebec (Canada)

†: The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

*: To whom correspondence should be addressed at
Institut de Biomedicina de València-CSIC
Unitat de Genètica Molecular
C/ Jaume Roig, 11
E46010 València (Spain)
Tel. +34 96 339 1755
Fax: +34 96 339 3774
e-mail: jpereztur@ibv.csic.es

ABSTRACT.

Autosomal dominant lateral temporal epilepsy (ADTLE) is a partial epilepsy caused by mutations in *LGI1*, a multidomain protein of unknown function. To begin to understand the biological function of LGI1, we have determined its pattern of glycosylation, subcellular expression and capacity for secretion. LGI1 is expressed as two different isoforms in the brain and we show that the long isoform is a secreted protein whereas the short isoform is retained in an intracellular pool. ADLTE-related mutants of the long form are defective for secretion and are retained in the endoplasmic reticulum and Golgi complex. Finally, we show that normal secreted LGI1 specifically binds to the cell surface of differentiated PC12 cells. We propose that LGI1 is a secreted factor important for neuronal development and that ADTLE is a disease that results from the loss of regulation in the protein available either extracellular or intracellularly.

INTRODUCTION

Autosomal dominant lateral temporal epilepsy (ADLTE, OMIM #600512; also known by some authors as autosomal dominant epilepsy with auditory features or ADPEAF) is a neurological disorder characterized by auditory auras and focal seizures affecting the lateral temporal lobe of the brain. The disease was mapped to a 3cM locus on human chromosome 10 by linkage analysis (1, 2). In 2002, our group (3) and others (4) demonstrated that mutations in *LGI1* (Leucine-rich Glioma-Inactivated 1) cause ADLTE. Numerous mutations have been found throughout the protein coding regions of this gene but their effect on protein function is unknown. (3-11).

LGI1 was originally localized to the breakpoint of a balanced translocation between chromosomes 10 and 19 (t{10;19}{q24;13}) present in the glioblastoma cell line T98G (12). Because LGI1 showed low expression levels in human high grade glial tumor samples and glioblastoma cell lines compared to normal brain samples, it was proposed to act as a tumor suppressor (12). However, more recent studies have challenged this view and concluded that LGI1 loss of function is unlikely to play a role in glial tumor progression (13). The protein encoded by *LGI1* consists of a signal peptide and 3 leucine-rich repeats flanked by two cysteine-rich regions in the N-terminal part of the protein. Additionally, a novel domain comprised of seven tandem arrays of 50 amino acids, termed EPTP repeats, was found in the C-terminal part of the protein. These EPTP repeats likely form a seven-bladed beta-propeller structure (14). The EPTP repeat is also found in three paralogues of *LGI1* (*LGI2*, *LGI3* and *LGI4)* as well as in two otherwise unrelated genes *(TNEP1* and *VLGR1)* and together, these constitute the EPTP superfamily (14, 15).

The function of *LGI1* remains unclear. Recent studies from Senechal and coworkers have shown that showed that LGI1 is a secreted protein (16), consistent with our earlier prediction (3)

and some studies have suggested that LGI1 is involved in regulation of cell movement, growth and survival (17, 18). Interestingly, LGI4 has also been shown to be a secreted protein that may play a role in peripheral nerve myelination (19) whereas Schulte and colleagues have provided evidence that LGI1 is a membrane-associated protein that regulates the activity of the Kv1.1 voltage-dependent potassium channel subunit (20).

In this work we confirm that LGI1 is a secreted glycoprotein even in neuronal-like cells and show that a truncated naturally occurring spliced isoform is retained within cells. We show that the secretion of the full-length isoform depends on the integrity of each of its domains and that disease-causing ADLTE mutations block LGI1 secretion. Moreover, we provide evidence for an LGI1 receptor on cell surfaces. Together, our data indicate that manifestation of ADLTE is related to defects in LGI1 secretion.

RESULTS

The LGI1 isoforms are glycoproteins with different cellular fates.

Human LGI1-Flag^{LONG} and LGI1-Flag^{SHORT}, shown schematically in Figure 1A, were transiently transfected in HEK293T cells, the extracellular media was concentrated and the cell extracts were immunoprecipitated with anti-Flag monoclonal antibodies. LGI1-Flag^{LONG} was clearly present in both lysates and media whereas LGI1-Flag^{SHORT} was only detected in cell lysates, suggesting that only LGI1-Flag^{LONG} was capable of being secreted (Figure 1B). Similar experiments were performed on T98G cells, a glioblastoma-derived cell line lacking LGI1 expression (Figure 1B) and on COS7 and CHO cells. In all cases, LGI1-Flag^{LONG} accumulated in media and LGI1-Flag^{SHORT} did not, indicating that the secretion of LGI1- Flag^{LONG} does not show cell type specificity. In some instances, a minor band is observed in cell lysates over-expressing the LGI1-Flag^{LONG} or the LGI1-Flag^{SHORT} isoforms, the identity of such band has not been further investigated, therefore we cannot exclude the possibility that LGI1 is being post-transcriptionally processed at a position close to the C-terminus end of the protein which is common to both isoforms. Finally, we tested the ability of LGI1 to be secreted on differentiated PC12 cells, a neuronal-like cell type. As is shown on figure 1B, NGF-differentiated PC12 cells are also capable of secrete LGI1-Flag^{LONG} whereas retain LGI1-Flag^{SHORT} as well as an ADLTE-causing mutation (LGI1-Flag^{758delC}).

In addition to verify that the secretion was cell-line independent, we also tested the influence that the different tags used had on this process and found that the secretion of LGI1 was not altered by attaching a larger tag such as GFP, at its C-terminus (data not shown). To confirm that accumulation of LGI1 in media reflects active secretion from living cells, pulse-chase analyses were performed. Figure 1C shows that HEK 293T cells transfected with LGI1-Flag^{LONG} and, 24

hours later, labelled with ^{35}S-Cys/Met showed accumulation of radioactive LGI1 in media within 30 minutes. This time course suggests that LGI1 is actively secreted.

If LGI1 enters the secretory pathway, it is likely to undergo N-linked glycosylation. To test this, we performed PNGase F treatment of LGI1-Flag^{LONG} and LGI1-Flag^{SHORT}. Figure 2B shows that both isoforms undergo a substantial molecular weight shift when treated with PNGase F, suggesting that both become N-glycosylated in the endoplasmic reticulum. We introduced point mutations at three predicted N-glycosylation sites in the full length isoform (LGI1-Flag$^{LONG-N192Q/N277Q/N422Q}$) and found that the protein produced no longer underwent a molecular weight shift when treated with PNGase F, indicating that some or all of these sites are required for N-glycosylation of the protein. We also examined truncated forms of LGI1-Flag in this assay and found that a truncated protein lacking the LRR domain was sensitive to PNGase F treatment whereas a corresponding construct in which the glycosylation sites had been mutated to glutamic acid (LGI1-Flag$^{\Delta LRR-N277Q/N422Q}$) was not (Figure 2C). If glycosylation sites in this construct were mutated individually (ie. LGI1-Flag$^{\Delta LRR-N277Q}$ and LGI1-Flag$^{\Delta LRR-N422Q}$), the resulting proteins were sensitive to PNGase F treatment. We also examined a construct in which the EPTP domain was deleted and, using a similar approach, found that a single potential N-linked glycosylation residue in the resulting product (LGI1-Flag$^{\Delta EPTP-N192Q}$) conferred sensitivity to PNGase F treatment. Together, these data indicate that N197Q, N277Q, and N422Q are sites of N-linked glycosylation in LGI1.

We next assessed whether N-glycosylation of LGI1 was required for its secretion and evaluated the effect of the three potential N-glycosylation sites on the secretion of LGI1. For this, we compared secretion of the normal protein to the triple mutant and to each of the single glycosylation mutants (LGI1-Flag^{N197Q}, LGI1-Flag^{N277Q} and LGI1-Flag^{N422Q}). Figure 2D shows

that triple mutant (LGI1-Flag LGI1-Flag$^{LONG-N197Q/N277Q/N422Q}$)) was not secreted and that secretion of the N197Q mutant was severely attenuated.

Role of LGI1 domains in the secretion of the protein

To determine whether the LRR and EPTP domains were required for secretion, two artificial constructs, LGI1-Flag$^{\Delta EPTP}$ and LGI1-Flag$^{\Delta LRR}$ were transfected in HEK 293T cells and their accumulation in media was assessed. Figure 3B shows that LGI1 lacking its LRR domain is efficiently secreted, although this is in contrast with Senechal *et al* results, the constructs used in each work were not comparable. Whereas in this work, our construct deleted the full LRR-domain, including the flanking Cys-rich domains, in Senechal *et al* the construct used in this same experiments mantained the Cys-rich domains thus it is possible that this construct behaves as a pathogenic mutation rather than as a wild-type protein. On the other hand, LGI1 lacking its EPTP domain is retained in the transfected HEK293T cells. We attempted to rescue LGI1 secretion in the latter mutant by stepwise addition of each of the EPTP domains from one to six, using the scheme described in (15). Only the intact wild-type protein that contains all seven EPTP domains was secreted from cells.

Secretion of LGI1, but not N-glycosylation, is blocked by ADLTE-causing mutations.

We next assessed whether naturally occurring mutations in LGI1 that cause ADTLE alter the secretion or glycosylation of the protein. HEK293T cells were transfected with normal LGI1-Flag^{LONG} or with LGI1-Flag^{758delC}, LGI1-Flag^{R474X}, LGI1-Flag^{C46R}, LGI1-Flag^{S145R}, LGI1-Flag^{C200R}, LGI1-Flag^{F318C} and LGI1-Flag^{E383A}. All of these constructs resulted in robust LGI1 expression but only LGI1-Flag^{LONG} accumulated in media, indicating that the naturally occurring mutations in LGI1 that cause disease do not exit the cell, regardless of whether they were truncating or missense mutations (Figure 4B). We also examined the sensitivity of these proteins

to PNGase F treatment and found, despite these trafficking defects, all the LGI1 mutants tested were N-glycosylated (Figure 4C).

Subcellular localization of LGI1

To determine the subcellular localization of LGI1-GFP^{LONG}, LGI1-GFP^{SHORT} and ADLTE-related mutants LGI1-GFP^{758delC}, LGI1-GFP^{R474X}, LGI1-GFP^{F318C} and LGI1-GFP^{E383A}, COS7 cells were transiently transfected with each construct and transfected cells were immunostained with an antibody recognising calnexin, an endoplasmic reticulum resident protein, or labelled with the fluorescent Golgi marker Bodipy TR ceramide. Confocal analysis revealed that LGI1-GFP^{LONG} is enriched in the Golgi apparatus, whereas LGI1-GFP^{SHORT} and the mutants analyzed, excluding LGI1-GFP^{R474X}, accumulate in the ER chaperone. LGI1-GFP^{R474X} is present in both ER and Golgi (Figure 5). Colocalization of LGI1 and the ER was also demonstrated by co-immunoprecipitating the wild-type and the mutant constructs with an antibody that recognizes calnexin (data not shown).

LGI1 binds to the surface of PC12 cells.

Because LGI1 is secreted to the extracellular medium, we assessed whether LGI1 could specifically interact with cell surface proteins. For this, we prepared conditioned media containing either placental alkaline phosphatase alone or placental alkaline phosphatase fused to LGI1^{LONG} (AP-LGI^{LONG}) and compared these for their ability to bind NGF-differentiated PC12 cells as well as to the surface of non differentiated PC12 and COS7 cells (data not shown). Figure 6 shows highly specific, dose dependent and saturable binding of AP-LGI ^{LONG}, with half-maximal binding observed at a concentration of 4 nM, suggesting that specific LGI1 receptor complex is present on these cells.

Binding of AP-LGI1 to the cell surface results in reduction of activated ERK1/2

To determine the effect that AP-LGI1 binding had on the cells, we assessed the activation state of ERK1/2 as it has been shown that over-expression of LGI1 caused a decrease in their activated levels in T98G cells (Kunapuli *et al.*, 2004). Figure 7 shows that incubating NGF-differentiated PC12 cells with AP-LGI1^{LONG} results in a significant reduction in the level of activated ERK1/2 compared to AP treated cells.

DISCUSSION

We demonstrate here that LGI1 is a glycoprotein secreted to the extracellular media in several *in vitro* models including a neurona-like cell type. The shorter and much less prevalent spliced isoform is retained within the cell. This is true even though the short isoform is expressed at higher levels in our *in vitro* system. In some instances, usage of alternative splice sites within coding exons has been show to produce isoforms with different, even opposite, functions (24, 25) thus, the existence of two LGI1 isoforms with different cellular locations suggest that these proteins may have different functions.

LGI1 is the gene responsible for ADLTE and distinct mutations have been described in several families. Disease-causing *LGI1* mutations have been identified throughout the LGI1 open reading frame (3, 4, 6-11, 21) and in this study, we have tested the hypothesis that disease-causing mutations alter LGI1 secretion. We show here that each of the disease-causing forms of LGI1 are defective in secretion from transfected cells. We did not detect changes in the glycosylation of the mutant proteins but instead demonstrate that the mutant proteins are largely retained in the endoplasmic reticulum. The only exception was LGI1-Flag^{R474X}, which was present in both in the endoplasmic reticulum and the Golgi apparatus. This mutation produces a truncated protein lacking the C-terminal 83 amino acids and it is possible that it retains structural elements that allow it to partially mimic the normal protein and reach the Golgi apparatus. Using artificial mutations, we also show that the LRR domain is not required for secretion but that an intact EPTP domain with all seven tandem repeats is required for the protein to exit the cell and find that N-linked glycosylation of LGI1 is necessary for its secretion but individual mutation of any the three putative N-linked residues does not block its exit from the cell. We conclude that the disease-related missense mutations are exerting their effect by altering the tertiary structure of the protein, causing their retention and degradation through protein quality control mechanisms.

The secretion of the full-length isoform together with the retention of the spliced isoform raises also interesting questions regarding the function of LGI1. Kunapuli and coworkers showed that when T98G cells were forced to re-express the long isoform, the malignancy of this glioma cell line was severely reduced and this correlated with reduced ERK1/2 phosphorylation and reduced metalloproteinase production (26). Given that we have demonstrated that LGI1 binds in a dose-dependent manner to differentiated PC12 cells and that this binding has a functional effect in the cells, it is possible that forced overexpression of LGI1 in T98G cells may have activated a ligand-receptor autocrine loop in these cells that altered cellular signalling pathways.

Schulte and colleagues (2006) have recently shown that LGI1 is part of a Kv1 channel complex and provided evidence that LGI1 acts to prevent the inhibitory effect of the Kvβ1 channel subunit. They report that mutant forms of LGI1 assemble into Kv1 channel complexes yet are incapable of altering Kvβ1 channel inhibitor activity. They propose that the C-terminal domain of LGI1 lost in truncated mutants of the protein is required for Kvβ1 channel antagonizing activity. We show here that a major consequence of LGI1 mutation is ER retention and a failure to enter Golgi and the secretory pathway. Therefore, an equally plausible alternative explanation for the findings of Schulte *et al.* (2006) is that the mutant LGI1 assembles into Kv1 complexes in the ER and the resulting assembly remains trapped in this compartment. In this scenario, only Kv1 complexes lacking LGI1 would actually reach the cell surface.

Taken together, our data suggest a hypothesis for the molecular pathology of ADLTE based on the retention of LGI1 in intracellular compartments. This retention could have a gain of function with cell autonomous effects, such as on Kv1 channels, or could result in a lost of function from non-autonomous effects that include loss of a critical cell communication between cells that secrete LGI1 and cells that respond to it.

METHODS

Constructs.

Several constructs were prepared for this work as fusion proteins. Proteins fused to GFP were cloned into pEGFP-N1 (Clontech), proteins fused to AP were cloned into pc3.1AP6 whereas fusions to the Flag epitope were cloned into pSalo-Flag, a modified version of pEGFP-N1 with the GFP protein removed and substituted by a Flag epitope. For simplicity each construct is named by placing the name of the tag showing its position with respect to the cloned LGI1.

Both LGI1 isoforms, the full length (long, amino acids 1-557) and the splicing variant (short, amino acids 1-259), were cloned from a human cDNA library (Invitrogen). For this study we have selected mutations producing truncated proteins (c.758delC and p.R474X; (3)) and some missense mutations affecting different domains of the protein: the N-terminal cysteine rich domain (p.C46R;(21)), the third leucine rich repeat (p.S145R; (10)), the C-terminal cysteine rich domain (p.C200R; (8)) and the third and fourth EPTP repeats (p.F318C; (6) and p.E383A; (4) respectively). In addition to these, we have also introduced the mutations affecting the three potential N-glycosylation sites predicted by NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/), p.N192Q, p.N277Q and p.N422Q. Finally, we have created two deletion constructs; one lacks the EPTP repeat region (ΔEPTP, lacking amino acids 224-556) and one lacks the LRR region (ΔLRR) and contains the signal peptide (amino acids 1-34) fused to the EPTP-repeat region (amino acids 224-556). In addition to those, we have produced several chimeric forms lacking part or all of a particular domain as shown in figures 1 to 3. For the serial deletions of the EPTP repeats, different primers were used in order to obtain 7 constructs containing from 0 to 6 EPTP repeats tagged with FLAG; 0EPTPs (ending at amino acid 223), 1EPTP (ending at 267), 2EPTPs (ending at 314), 3EPTPs (ending at 364), 4EPTPs (ending at 415), 5EPTPs (ending at 462), 6EPTPs (ending at 506). To obtain the AP-LGI1 vector,

the cDNA encoding amino acids 35-557 was ligated into the pc3.1AP6 vector in frame with signal sequence and placental AP coding region. All mutagenesis were done with the Quick Change Mutagenesis kit (Stratagene) and all constructs generated by PCR were fully sequenced to ensure integrity of the cloned ORFs. All primers used as well as PCR conditions are available from authors on request.

Cell culture, transient transfection and media collection

CHO, COS7 and HEK 293T cells were grown in DMEM (Cambrex), supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS, Gibco) 2mM Glutamine (Gibco) and 2% (v/v) Penicillin/Streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin, Gibco) on 10 cm plates and maintained at 37ºC and 5% CO_2. T98G cells, a glioblastoma derived cell line that do not express LGI1 (12)**,** were grown in EMEM (Cambrex) 10% (v/v) FBS, 2 mM Glutamine, 1% Non Essential amino Acids (Gibco) and 1% Sodium Piruvate (Gibco). PC12 cells were grown on poly-D-lysine coated plates in DMEM supplemented with 2mM Glutamine, 2% (v/v) Penicillin/Streptomycin and 0.1% BSA. When required PC12 cells were differentiated with 50ng/mL NGF in complete medium for 48 hours.

T98G, COS7 and CHO cells were transiently transfected with liposomes (Fugene, Roche), while HEK 293T were transfected by calcium phosphate method. PC12 cells were transfected with lipofectamine and differentiated with NGF twenty-four hours post-transfection. For media production, HEK 293T cells were grown to approximately 60-70% confluence and were transiently transfected with 5µg DNA. After 16-20 hr of transfection the media were replaced with a reduced serum media (Optimem I, Gibco), and incubated for an additional 24 hr. Then, media were collected, supplemented with a protein inhibitor cocktail (Complete, Roche), filtrated through a 0,2 µm filter and concentrated 10-15 times to 300 µl using an Amicon Ultra15 device (MWCO: 30,000 KDa; Millipore) and analyzed by SDS-PAGE.

For binding experiments, after two days on serum-free DMEM, secreted AP-LGI1 and AP were first concentrated as before and then the amount of protein was estimated according to (22, 23) measuring the AP activity using p-nitro phenyl phosphate as substrate (Sigma). For the binding experiments, the concentrated media was diluted with PBS 1x medium to 5 - 40nM.

SDS-PAGE and Western-blotting

Equivalent volumes of concentrated media and immunoprecipitates were loaded onto 10% SDS-PAGE gels to resolve proteins. Following transfer, nitrocellulose membranes (Amersham) were blocked at room temperature for 1 hr with non-fat dry milk powder in Tris-HCl 20 mM pH 7.6, NaCl 140 mM, Tween-20 0.1% (v/v) (TBST) to reduce non specific binding. The Flag epitope was detected by incubation with either mouse anti-Flag antibody (1:500, in blocking solution; Sigma) or rabbit anti-Flag antibody (1:1000, in 3% BSA; Sigma) at 4ºC overnight. The membranes were washed in TBST prior to incubation with the HRP-conjugated anti-mouse IgG antibody or HRP-conjugated anti-rabbit IgG antibody (1:5000, in blocking solution, Jackson). The blots were further washed in TBST and subsequently incubated in ECL or ECL plus (Amersham).

Immunoprecipitation and pulse-chase analyses

HEK 293T cells were lysed and scraped in 1 ml NP40-containing lysis buffer (10 mM Tris HCl pH8, 150mM NaCl, 1% Nonidet-P40, 10% Glycerol, 1% Triton-X100 and protease inhibitors) for 30 min at 4ºC. Cell lysates were clarified by centrifugation at 13,000 rpm at 4ºC for 10 min and then the supernatants were incubated with protein G sepharose beads (Amersham) conjugated with M2 mouse anti-Flag antibody (Sigma) at 4ºC for 2 hr. The beads were washed three times with lysis buffer and then resuspended in 60µl 2x Laemmli sample buffer for SDS-PAGE. For pulse chase analyses, cells were transfected with LGI1 or with parental vector and 24 hours later, pre-incubated in cysteine- and methionine-free media for 30 minutes followed by

incubation for 30 minutes in labeling media consisting of 50 μCi/ml ^{35}S-Trans-label (ICN) and 3 mg/L unlabelled cysteine and methionine, respectively, for 30 minutes. Cells were then switched to normal media (30 mg/L cysteine and 30 mg/L methionine) for 10, 30 or 60 minutes. After lysis, immunoprecipitations were performed using monoclonal anti-Flag antibodies. Immunoprecipitates were separated by SDS-PAGE, gels were impregnated with Enhance (NEB) and dried, using the protocol provided by the manufacturer.

PNGase F digestion

Cell lysates and culture medium obtained as described from transiently transfected HEK 293T cells were immunoprecipitated as above and resuspended and denatured in 25 μl of denaturing buffer at 100ºC for 10 min and cooled to room temperature. The denatured samples were subjected to digestion with 4 mU of PNGase F (New England Biolabs) at 37ºC for 90 min. The samples were then analyzed by SDS-PAGE.

Subcellular localization of wild type and mutant LGI1

Twenty-four hours after transfection with the LGI1-GFP constructs using FuGene (Roche), COS7 cells grown on glass cover slips were fixed with 4% (w/v) paraformaldehyde in PBS a 37ºC for 30 minutes. Slides were blocked at room temperature for two hours with 5% BSA in PBS and then immunostained with the ER marker anti-Calnexin (1:500, Calbiochem) at 4ºC overnight. Slides were washed and incubated with Texas Red-conjugated anti-rabbit antibody (1:5000, Molecular Probes) at room temperature for 2 hr. Alternatively, COS7 cells were incubated with the Golgi marker Bodipy TR Ceramide (Molecular Probes) at a 3 μM concentration in culture media, for 30 min at 37ºC. Images were collected on a Leica TCS SL spectral confocal microscope coupled to an inverted microscope (Leica DMIRE2; Leica Microsystems) at room temperature with a 63x immersion objective (HCX PLAPO CS, 63x/1.40-

0.60 1.40-0.60. Images were captured on a computer system by the Leica Confocal Software v2.5 (Build 1347, Leica Microsystems).

Cell binding assays of AP fusion proteins

AP-tagged fusion proteins were produced by transient transfection of HEK 293T cells with either AP-LGI1 or empty vector producing secreted AP. PC-12 cells, $5x10^5$ cells/well, were plated in 6 well plates coated with poly-D-lysine and differentiated with NGF (50ng/mL) for 48 hours. Afterwards, plates were washed twice with PBS 1x and then cells were incubated with dilutions of either AP or AP-LGI1 for 90 min at 37ºC. Cultures were then washed three times with ice-cold Hanks balanced Salt solution containing 20mM sodium HEPES, 0.1% sodium azide and 0.5mg/mL BSA. Plates where the binding was quantified were processed with lysis buffer (20mM Tris-HCl pH 8.0, 0.1% Triton-X100 and protease inhibitors). Endogenous AP activity was heat inactivated at 65º for 15 minutes and bound AP activity was assessed using p-nitro phenyl phosphate as substrate measuring absorbance at 405nm. In order to normalize the results, total protein present in the lysates was measured by DC protein assay (Bio-Rad). Plates where pictures were taken were incubated at 65º for 90 minutes to heat inactivate the endogenous AP activity and the AP-LGI1 binding was revealed in the presence of NBT (nitro blue tetrazolium) and BCIP (5-Bromo-4-chloro-3-indolyl phosphate) (Sigma).

Determination of ERK1/2 levels.

Conditioned media from HEK 293T cells expressing either AP-$LGI1^{LONG}$ or the AP alone, were incubated with NGF-differentiated PC12 cells for the times shown in figure 7 at 37ºC normalising for the AP activity at 15nM. As the process of differentiation of the PC12 cells by NGF requires activation of the ERK1/2 pathway, and the incubation with conditioned media could affect the state of activation of ERK1/2 by itself, we pre-treated all cultures with an equivalent amount of conditioned media coming from mock-transfected HEK 293T for 2h prior

to AP-LGI1^{LONG} or AP treatment. After treatment, the cells were washed with PBS and harvested in RIPA buffer (1.5 M NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 1 % SDS, 50 mM Tris-HCl, pH 8.0) with a cocktail of protease inhibitors (Complete, Roche) supplemented with 1 mM sodium orthovanadate. Cell lysates were incubated on ice for 30 min prior to clarification at 4ºC and 16,000 g for 15 min. Fifteen micrograms of protein from each condition were assayed by Western blotting using an antibody that recognizes phosphorylated ERK1/2. After stripping of the blot, it was incubated with a phosphorylation-independent antibody that recognized total ERK1/2. In both cases, the Western was developed and an image was captured and the intensity of each band was measured with the help of the MultiGauge v2.1 software (Fujifilm). After this, we estimated the relative amount of phospho-ERK1/2 over total ERK1/2.

ACKNOWLEDGEMENTS

The authors thank two anonymous reviewers for their helpful criticism of the work presented herein. The authors are indebted to Silvia Aparicio-Domingo, Raquel Rodríguez-de Pablos and Benito Alarcón, for their technical support as well as to the Unidad de Genética y Medicina Molecular and Unidad de Biología Vascular of the Instituto de Biomedicina de Valencia-CSIC for technical assistance. JP-T is part of the Grupos de Excelencia of the Generalitat Valenciana (Grupos 03/015) and PAB is a CIHR Scientist. This work was supported by grants from the Ministerio de Educación y Ciencia (SAF2002-00060 and SAF2005-00136) to JP-T and from the Canadian Institute of Heath Research (PPP147918) to PAB. SS-P is funded by a fellowship of the Generalitat Valenciana (CTBPRB/2002/35), JMM-R is funded by an FPU and a Bancaixa fellowship. Support from the Ministerio de Educación y Ciencia (BES-2003-0243, to AA-I) and from the Ministerio de Sanidad y Consumo (BF03/00182, to VH-P) is also acknowledged. KF is funded by a Canadian NSERC award.

CONFLICTS OF INTEREST

The authors do not have any conflict of interest regarding the work reported in this manuscript.

REFERENCES

1. Ottman, R., Risch, N., Hauser, W.A., Pedley, T.A., Lee, J.H., Barker-Cummings, C., Lustenberger, A., Nagle, K.J., Lee, K.S., Scheuer, M.L. *et al.* (1995) Localization of a gene for partial epilepsy to chromosome 10q. *Nat. Genet.,* **10,** 56-60.
2. Poza, J.J., Saenz, A., Martinez-Gil, A., Cheron, N., Cobo, A.M., Urtasun, M., Marti-Masso, J.F., Grid, D., Beckmann, J.S., Prud'homme, J.F. *et al.* (1999) Autosomal dominant lateral temporal epilepsy: clinical and genetic study of a large Basque pedigree linked to chromosome 10q. *Ann. Neurol.,* **45,** 182-188.
3. Morante-Redolat, J.M., Gorostidi-Pagola, A., Piquer-Sirerol, S., Saenz, A., Poza, J.J., Galan, J., Gesk, S., Sarafidou, T., Mautner, V.F., Binelli, S. *et al.* (2002) Mutations in the LGI1 gene on 10q24 cause autosomal dominant lateral temporal epilepsy. *Hum. Mol. Genet.,* **11,** 1119-1128.
4. Kalachikov, S., Evgrafov, O., Ross, B., Winawer, M., Barker-Cummings, C., Martinelli Boneschi, F., Choi, C., Morozov, P., Das, K., Teplitskaya, E. *et al.* (2002) Mutations in LGI1 cause autosomal-dominant partial epilepsy with auditory features. *Nat. Genet.,* **30,** 335-341.
5. Gu, W., Brodtkorb, E. and Steinlein, O.K. (2002) LGI1 is mutated in familial temporal lobe epilepsy characterized by aphasic seizures. *Ann. Neurol.,* **52,** 364-367.
6. Fertig, E., Lincoln, A., Martinuzzi, A., Mattson, R.H. and Hisama, F.M. (2003) Novel LGI1 mutation in a family with autosomal dominant partial epilepsy with auditory features. *Neurology,* **60,** 1687-1690.
7. Kobayashi, E., Santos, N.F., Torres, F.R., Secolin, R., Sardinha, L.A., Lopez-Cendes, I. and Cendes, F. (2003) Magnetic resonance imaging abnormalities in familial temporal lobe epilepsy with auditory auras. *Arch. Neurol.,* **60,** 1546-1551.

8. Michelucci, R., Poza, J.J., Sofia, V., de Feo, M.R., Binelli, S., Bisulli, F., Scudellaro, E., Simionati, B., Zimbello, R., D'Orsi, G. *et al.* (2003) Autosomal dominant lateral temporal epilepsy: clinical spectrum, new epitempin mutations, and genetic heterogeneity in seven European families. *Epilepsia,* **44,** 1289-1297.

9. Berkovic, S.F., Izzillo, P., McMahon, J.M., Harkin, L.A., McIntosh, A.M., Phillips, H.A., Briellmann, R.S., Wallace, R.H., Mazarib, A., Neufeld, M.Y. *et al.* (2004) LGI1 mutations in temporal lobe epilepsies. *Neurology,* **62,** 1115-1119.

10. Hedera, P., Abou-Khalil, B., Crunk, A.E., Taylor, K.A., Haines, J.L. and Sutcliffe, J.S. (2004) Autosomal dominant lateral temporal epilepsy: two families with novel mutations in the LGI1 gene. *Epilepsia,* **45,** 218-222.

11. 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.

12. 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.

13. Piepoli, T., Jakupoglu, C., Gu, W., Lualdi, E., Suarez-Merino, B., Poliani, P.L., Cattaneo, M.G., Ortino, B., Goplen, D., Wang, J. *et al.* (2006) Expression studies in gliomas and glial cells do not support a tumor suppressor role for LGI1. *Neuro-oncol.,* **8,** 96-108.

14. 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 implicated in epileptic disorders. *Trends Biochem. Sci.,* **27,** 441-444.

15. Scheel, H., Tomiuk, S. and Hofmann, K. (2002) A common protein interaction domain links two recently identified epilepsy genes. *Hum. Mol. Genet.,* **11,** 1757-1762.

16. 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.

17. 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.

18. Gabellini, N., Masola, V., Quartesan, S., Oselladore, B., Nobile, C., Michelucci, R., Curtarello, M., Parolin, C. and Palu, G. (2006) Increased expression of LGI1 gene triggers growth inhibition and apoptosis of neuroblastoma cells. *J. Cell. Physiol.,* **207,** 711-721.

19. Bermingham, J.R., Shearin, H., Pennington, J., O'Moore, J., Jaegle, M., Driegen, S., van Zon, A., Darbas, A., Ozkaynak, E., Ryu, E.J. *et al.* (2006) The claw paw mutation reveals a role for Lgi4 in peripheral nerve development. *Nat. Neurosci.,* **9,** 76-84.

20. Schulte, U., Thumfart, J.O., Klocker, N., Sailer, C.A., Bildl, W., Biniossek, M., Dehn, D., Deller, T., Eble, S., Abbass, K. *et al.* (2006) The epilepsy-linked lgi1 protein assembles into presynaptic kv1 channels and inhibits inactivation by kvbeta1. *Neuron,* **49,** 697-706.

21. Gu, W., Wevers, A., Schroder, 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.

22. Flanagan, J.G. and Cheng, H.J. (2000) Alkaline phosphatase fusion proteins for molecular characterization and cloning of receptors and their ligands. *Methods Enzymol.,* **327,** 198-210.

23. Flanagan, J.G., Cheng, H.J., Feldheim, D.A., Hattori, M., Lu, Q. and Vanderhaeghen, P. (2000) Alkaline phosphatase fusions of ligands or receptors as in situ probes for staining of cells, tissues, and embryos. *Methods Enzymol.,* **327,** 19-35.

24. Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G. and Thompson, C.B. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell,* **74,** 597-608.

25. Roos, K.L. and Simmons, D.L. (2005) Cyclooxygenase variants: the role of alternative splicing. *Biochem. Biophys. Res. Commun.,* **338,** 62-69.

26. 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.

FIGURE LEGENDS

Figure 1. LGI1-Flag^{LONG} is secreted from HEK293T cells. (A) Schematic representation of the LGI1-Flag constructs with the discrete domains used The white box represents de signal peptide, ovals represent the N-term and C-term Cys-rich domains flanking the LRR domains (light grey rectangles) Dark grey rectangles represent the EPTP repeats. The pentagon indicates the position of the FLAG epitope. (B) Analysis of the secretion of LGI1-Flag^{LONG} and LGI1-Flag^{SHORT} in NGF-differentiated PC12 (left), HEK293T (middle) and T98G (right) cells. Cells were transfected with each construct and the lysates and the concentrated media were analyzed by Western blot using an anti-Flag antibody as detailed in the Methods section. Note the presence of an unspecific band close to the size of the LGI1-Flag^{SHORT} isoform in T98G cells. (C) HEK293 cells transfected with plasmid encoding LGI1-FLAG or control vector were incubated with ^{35}S-Translabel (ICN) for 30 minutes and then chased with media containing an excess of unlabelled cysteine and methionine for 10, 30 and 60 minutes, as indicated. LGI1-FLAG was immunoprecipitated using an anti-FLAG antibody and analyzed by SDS-PAGE/fluorography. LONG refers to the LGI1-Flag^{LONG} construct, SHORT refers to LGI1-Flag^{SHORT}.

Figure 2. LGI1-Flag is N-glycosylated at N192, N277 and N422. (A) Constructs used in these experiments showing the position of the predicted N-glycosylation sites. See figure 1 for details on the domains of the protein. (B) Both LGI1-Flag^{LONG} (LONG) and LGI1-Flag^{SHORT} (SHORT) were transfected in HEK293T cells and extracts treated in the absence (-) or presence (+) of PNGFase and subjected to SDS-PAGE followed by immunodetection with an anti-Flag antibody. (C) Analysis of the sites of glycosylation in the constructs. Each of the LGI1-Flag constructs shown with the N-glycosylation sites mutated was subjected to treatment without (-) or with (+) PNGFase. Whereas the LGI1-Flag^{LONG} isoform is glycosylated, the equivalent construct with all

three predicted glycosylation sites removed shows lack of glycosylation. Mutation of the N192, N277 or N422 residues clearly show a modification in the pattern of PNGFase-sensitive glycosylation. (2x: construct with both predicted sites mutated simultaneously; 3x: construct with all three predicted N-glycosylation sites mutated). Note the presence of bands resulting from incomplete digestion with PNGFase in some lanes. (D) Effect of abolishing the sites of N-glycosylation on the secretion of LGI1-Flag^{LONG}. Removing any individual N-glycosylation site does not block the secretion of LGI1-Flag^{LONG}; on the contrary, LGI1-Flag$^{N192Q/1277Q/N422Q}$ (first lane on the right) is not secreted (right panel) and gets retained within the cell (left panel).

Figure 3. LGI1 domains required for protein secretion. (A) Representation of the constructs employed in this experiment. See legend of figure 1 for details on the protein domains. (B) Analysis of the effect on the secretion of the two regions of the protein. As detailed in the Material and Methods section, HEK293T cells were transfected with the indicated constructs and, 48h post-transfection, cell lysates and concentrated culture media were analyzed by Western blotting with an anti-Flag antibody. (C) The requirement for a complete EPTP-repeat region was tested by transfecting HEK293T cells with constructs containing increasing numbers of EPTP repeats and analyzing both the cell extracts and concentrated culture media as before.

Figure 4. LGI1 mutations that cause ADTLE block secretion, but not the glycosylation, of LGI1-Flag. (A) Constructs employed in these experiments. See legend for figure 1 for an explanation of the different domains of the protein. (B). HEK293T cells were transfected with each construct and cell lysates (left panel) and concentrated culture media (right panel) were subjected to SDS-PAGE followed by immunoblotting with an anti-Flag antibody. (C) PNGFase treatment of ADLTE-causing mutations. HEK293T cells were transfected with each construct

and, 48h post-transfection, cell extracts were subjected to treatment without (-) or with (+) PNGFase prior to Western blotting.

Figure 5. Subcellular localization of disease-causing mutations of LGI1. COS7 cells were transfected with each GFP-fused protein as indicated and treated with either an anti-calnexin antibody to identify the endoplasmic reticulum or the Bodipy probe to detect the Golgi apparatus. Confocal images for each construct are shown together with the merged image. Bar: 8µm.

Figure 6. LGI1 binds to the cell surface of differentiated PC12 cells. AP-LGI1^{LONG} was obtained from the concentrated culture media of transfected HEK293T and incubated with NGF-differentiated PC12 cells. After 1h incubation, the reagents were removed and the cells washed and treated to (A) detect membrane-bound AP-LGI1^{LONG} (left) or membrane-bound AP as a control (right) or subjected to a quantitative analysis as detailed in the Methods section (B). Magnification of pictures: 200x

Figure 7. Binding of LGI1 to the surface of NGF-differentiated PC12 cells reduces the activation of ERK1/2. AP-LGI1^{LONG} was incubated with NGF-differentiated PC12 cells as in figure 6. After the times indicated, cells were harvested and analyzed to determine the level of phospho-ERK1/2 and total ERK1/2. (A) Western blot showing the reduction in phosphoERK1/2 at 5 and 15 minutes after AP-LGI1^{LONG} treatment compared to AP treatment. (B) Quantification of the relative levels of phosphoERK1/2 over total ERK1/2 expressed as percentage over the levels at t=0. * p=0.02 using a Student's t. Values are given as means±SEM for three independent experiments.

1

ABBREVIATIONS.

ADLTE: Autosomal dominant lateral temporal epilepsy

AP: Placental alkaline phosphatase

ER: Endoplasmic reticulum

LGI1: Leucine rich repeats glioma inactivated 1

LRR: Leucine rich repeat

PNGaseF: Peptide N-glycosidase F

Figure 1

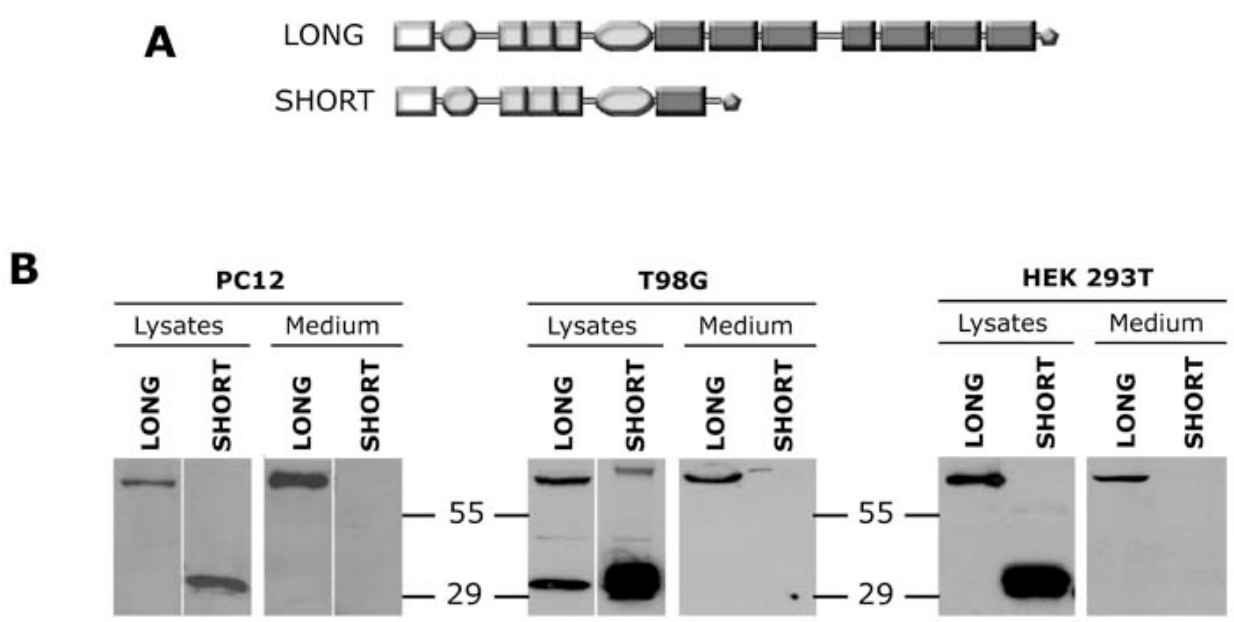

A
LONG
SHORT
B
PC12
T98G
HEK 293T
Lysates
Medium
LONG
SHORT
55
29
C
Vector
LONG
Time (min)
60
10
30
60
83
62
47

Figure 2

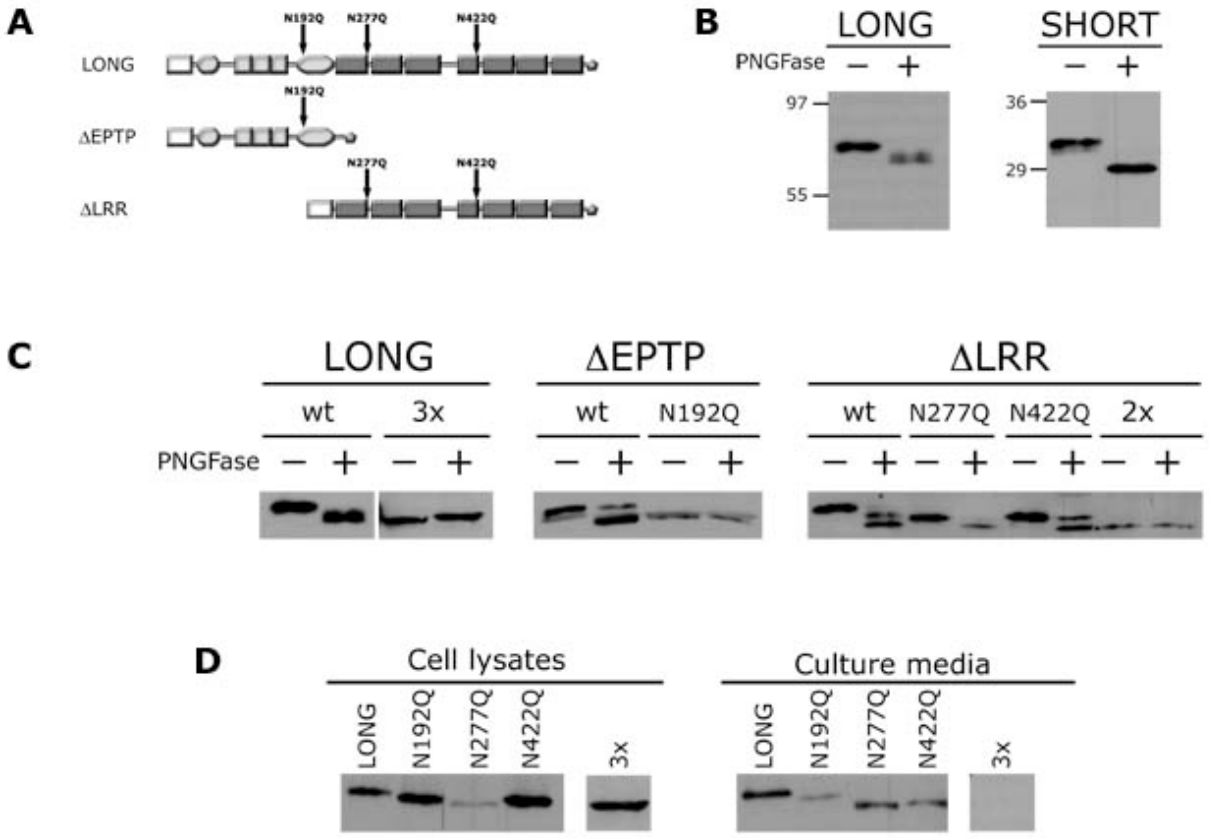

A
N192Q
N277Q
N422Q
LONG
ΔEPTP
ΔLRR
B
LONG
SHORT
PNGFase
97
55
36
29
C
LONG
ΔEPTP
ΔLRR
wt
3x
N192Q
N277Q
N422Q
2x
PNGFase
D
Cell lysates
Culture media
LONG
N192Q
N277Q
N422Q
3x

Figure 3

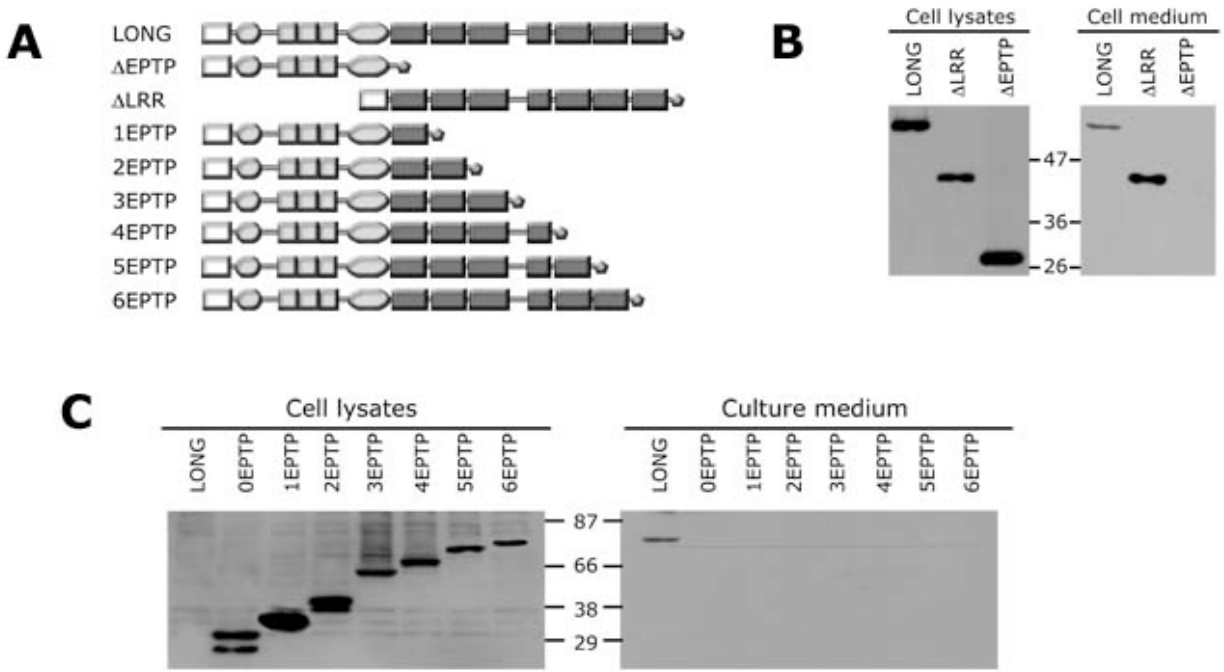
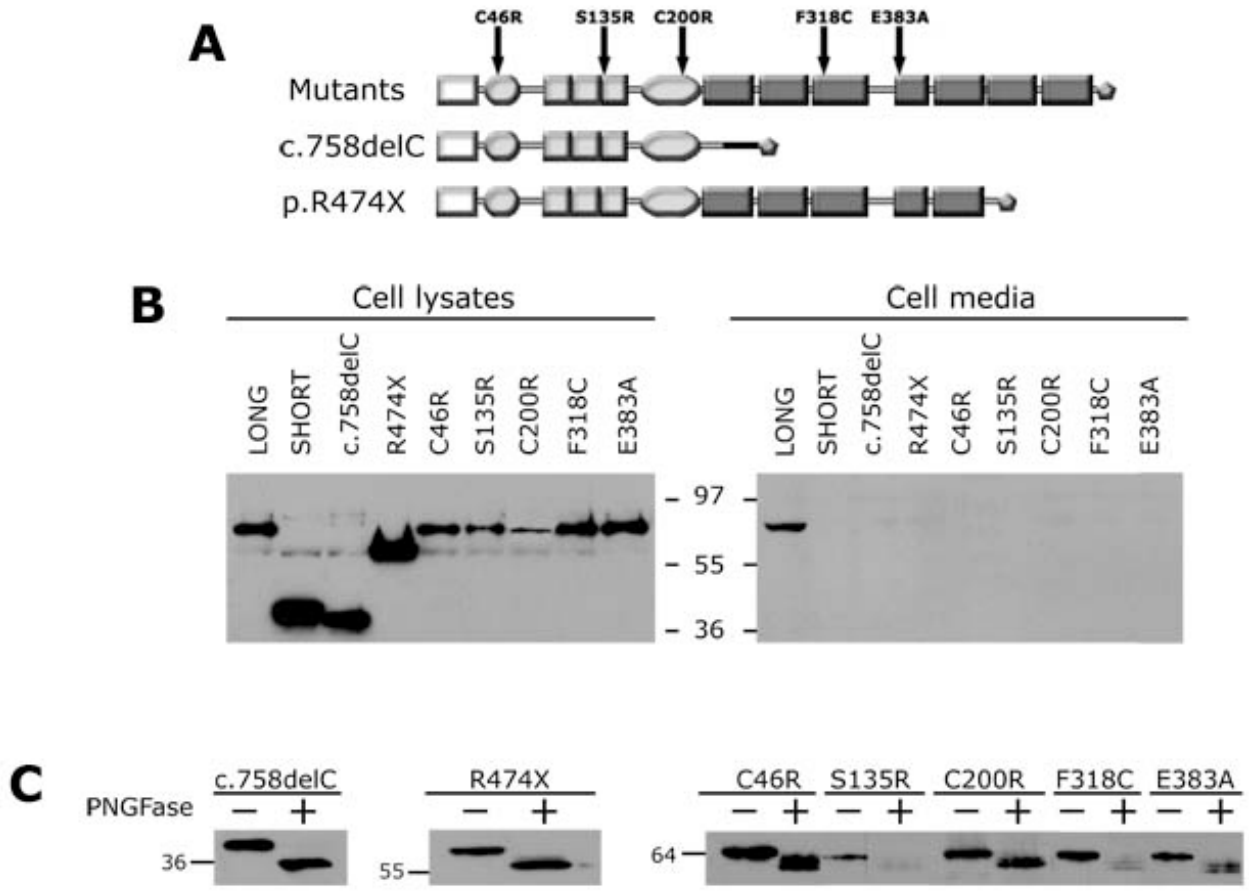
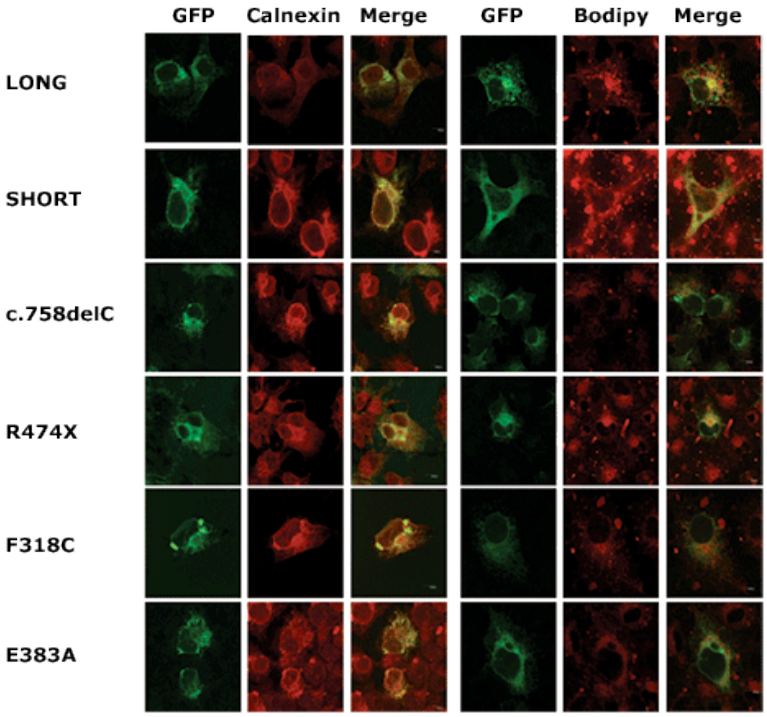

A
LONG
ΔEPTP
ΔLRR
1EPTP
2EPTP
3EPTP
4EPTP
5EPTP
6EPTP
B
Cell lysates
Cell medium
LONG
ΔLRR
ΔEPTP
47
36
26
C
Cell lysates
Culture medium
LONG
0EPTP
1EPTP
2EPTP
3EPTP
4EPTP
5EPTP
6EPTP
87
66
38
29

Figure 4


A
C46R
S135R
C200R
F318C
E383A
Mutants
c.758delC
p.R474X
B
Cell lysates
Cell media
LONG
SHORT
c.758delC
R474X
C46R
S135R
C200R
F318C
E383A
97
55
36
C
PNGFase
c.758delC
R474X
C46R
S135R
C200R
F318C
E383A
36
55
64

1 **Figure 5**


GFP
Calnexin
Merge
GFP
Bodipy
Merge
LONG
SHORT
c.758delC
R474X
F318C
E383A

2

Figure 6

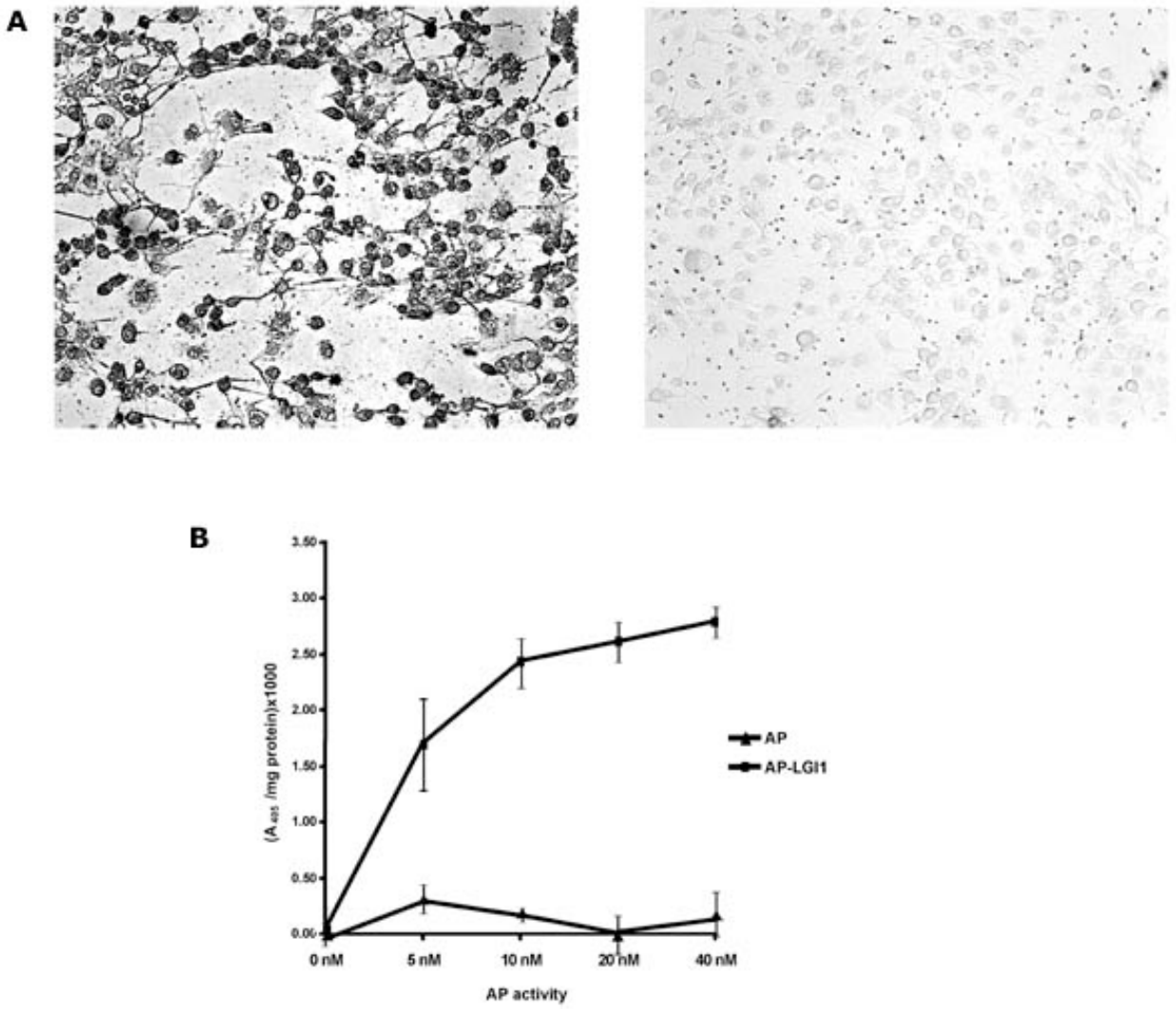
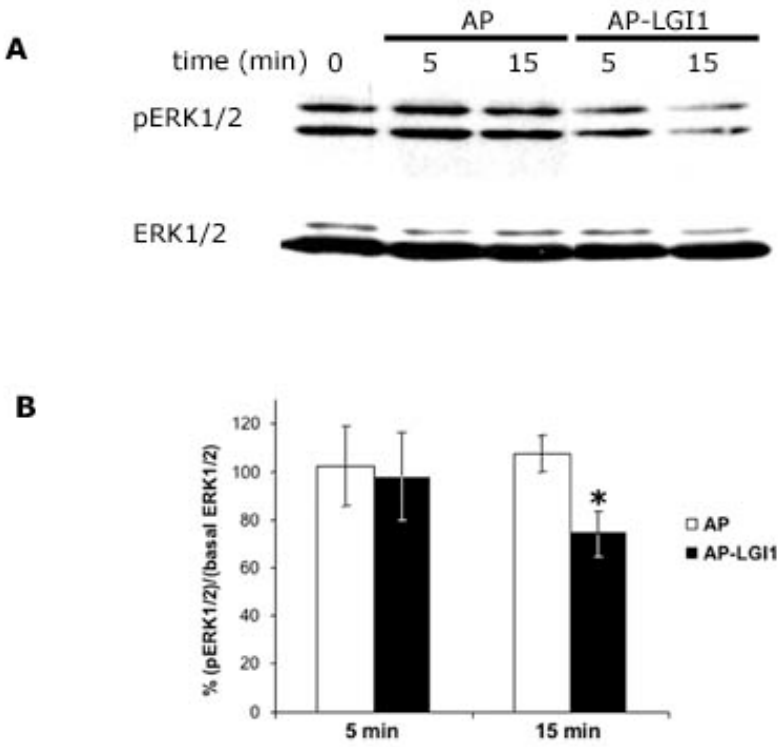

A
B
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
(A405 /mg protein)x1000
AP
AP-LGI1
0 nM
5 nM
10 nM
20 nM
40 nM
AP activity

Figure 7


A
AP
AP-LGI1
time (min) 0
5
15
5
15
pERK1/2
ERK1/2
B
% (pERK1/2)/(basal ERK1/2)
120
100
80
60
40
20
0
*
5 min
15 min
AP
AP-LGI1