Leucine-rich glioma inactivated 3 associates with syntaxin 1

Woo-Jae Park¹, Sang Eun Lee¹, Nyoun Soo Kwon, Kwang Jin Baek, Dong-Seok Kim, Hye-Young Yun∗

Department of Biochemistry, College of Medicine, Chung-Ang University, 221 Heuksuk-dong, Dongjak-koo, Seoul 156-756, Republic of Korea

Article history:
Received 27 June 2008
Received in revised form 6 August 2008
Accepted 8 August 2008

Abstract

Leucine-rich glioma inactivated 3 (LGI3) is a member of LGI/epitempin (EPTP) family. The biological function of LGI3 and its association with disease are not known. We previously reported that mouse LGI3 was highly expressed in brain in a developmentally and transcriptionally regulated manner. In this study, we identified syntaxin 1, a SNARE component in exocytosis, as a candidate functional target of LGI3. Western blot analysis of mouse brain extract with LGI3 antibodies detected multiple protein forms (75-, 60-, 35- and 25-kDa). Proteomic analysis, pull-down and coimmunoprecipitation experiments identified syntaxin 1 as an LGI3-associated protein. LGI3 colocalized with syntaxin 1 in processes of cortical neurons with punctate synaptic pattern and was enriched in synaptosomal fraction. Coimmunoprecipitation showed that LGI3-syntaxin 1 complex did not contain other SNARE components, SNAP25 and VAMP2. Recombinant LGI3 attenuated Ca2+-evoked glutamate release from digitonin-permeabilized synaptosomes and transfection of PC12 cells with LGI3 decreased K+-induced secretion of human growth hormone. Thus, LGI3 may play a regulatory role in neuronal exocytosis via its interaction with syntaxin 1.

Keywords:
Leucine-rich glioma inactivated 3
Syntaxin 1
Synaptosome
Syntaxin 1
Exocytosis

© 2008 Elsevier Ireland Ltd. All rights reserved.
Breeding Center, Korea) were housed at constant room temperature and on a 12-h light-dark cycle. Transient transfection of HEK293T cells with pcDNA3.1 plasmids encoding mouse LGI family members was performed as described previously [17]. Tissue or cell lysates were prepared in RIPA buffer (50 mM Tris, pH 7.5, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche, USA). Antibodies used were, rabbit polyclonal antibodies raised against bacterially expressed full-length mouse LG3-His6 (LGI3-FL), LG3-P1 peptide (amino acids 1–12; MACLRARRPGPR), LG3-P5 peptide (amino acids 329–340; PNDLEAFRDGD) and LG3-P6 peptide (amino acids 536–548; TLVYRHVVVDLSA). LG3-His6 was expressed in *Escherichia coli* BL21 (DE3) using pET28a(+) expression vector (Novagen) and chaperone system (pGro7) (Takara). The protein was purified by Ni-NTA agarose according to the manufacturer's protocol (Qiagen). Peptide immunogens were made by adding a cysteine at C-terminus of P1 and P5 peptides and at N-terminus of P6 peptide and then by cross-linking to maleimide-activated keyhole limpet hemocyanin (Pierce). Western blots were performed with primary antibodies (LGI3-FL antibody, 1:3000 dilution; LG3 peptide antibodies, 1:500 dilution) and with peroxidase-conjugated secondary antibody (1:20,000 dilution; Vector Labs) using chemiluminescence (Pierce) and LAS-1000 Plus detection system (Fujifilm).

For pull-down experiment, purified LG3-His6 (10 μg) was immobilized to Ni-NTA magnetic agarose (Qiagen). Mouse brain homogenate (5 mg protein) in interaction buffer (5 mM Tris, pH 7.4, 50 mM sodium phosphate, 10 mM imidazole, 10% glycerol, 100 mM NaCl, 1% Triton X-100) was incubated with LG3-His6-bound Ni-NTA magnetic agarose at 4 °C overnight. After extensive washes with the interaction buffer, bound proteins were eluted by 1 M imidazole in 50 mM sodium phosphate (pH 7.4), 10% glycerol, 0.5% Triton X-100, 100 mM NaCl. The eluates were dialyzed against 50 mM Tris, pH 6.8 containing 7 M urea, 2 M thiourea, 2% CHAPS, 60 mM dithiothreitol and 10% glycerol at room temperature for 2 h.

Two-dimensional electrophoresis was carried out using Ettan IPGPhor system according to the manufacturer's protocol (Amersham Biosciences). In-gel trypsin digestion after staining with silver stain kit (Amersham Biosciences) was performed as described [18]. Mass spectrometric analysis was performed using MALDI–TOF Voyager DE-RI mass spectrophotometer (PerSeptive Biosystems, USA). PeptIdent program from ExPASy was used for the peptide mass fingerprinting database search.

For immunoprecipitation, brains from 8-week old mice were homogenized (5 mg protein) in interaction buffer (5 mM Tris, pH 7.4, 50 mM sodium phosphate, 10 mM imidazole, 10% glycerol, 100 mM NaCl, 1% Triton X-100) and centrifuged at 10,000 × g for 15 min at 4 °C. Supernatants (100 μg protein) were immunoprecipitated by incubation with primary antibodies (1 μg) overnight at 4 °C followed by 1 h incubation with protein A- or G-agarose (20 μl). Immune complexes were washed five times with RIPA buffer, boiled in Laemmli sample buffer and analyzed by Western blot with indicated antibodies. All commercial antibodies were purchased from Santa Cruz Biotechnology.

Primary cortical neuronal cultures from E15.5 mouse embryos were maintained in Neurobasal medium (Gibco Invitrogen Corporation) containing 2% B27 supplement (Gibco Invitrogen Corporation), 1% antibiotics, 2 mM glutamine and 25 μM glutamate for 10 days. Immunocytochemical staining was performed with primary antibody (1:500 dilution) and goat anti-rabbit or mouse IgG-conjugated fluorescein isothiocyanate (FITC) or rhodamine (1:200 dilution). Images were collected by Zeiss confocal LSM510 meta imaging system. Subcellular fractionation of mouse brain homogenates and preparation of synaptosomes were performed as described previously [5]. Ca2+-evoked glutamate release from digitonin-permeabilized synaptosomes was measured by the procedure described previously [12,15]. Test samples were added to synaptosomes (100 μg protein) and incubated for 15 min prior to stimulation with CaCl2 (1.2 mM). Glutamate release was monitored by ELISA reader (Molecular Devices). Human growth hormone (hGH) release assay using transiently transfected PC12 cells was performed by hGH-ELISA kit (Roche) as described [21].

To verify the specificities of newly developed LGI3 antibodies (Fig. 1A for epitopes), we performed Western blot for each member of mouse LGI family expressed in HEK293T cells (Fig. 1B). All LGI3 antibodies recognized LGI3 protein without detectable cross-reactivity with other LGI members. Next, mouse brain extract was analyzed by Western blot with the LGI3 antibodies (Fig. 1C). Preimmune sera and preabsorbed immune sera did not detect nonspecific bands in the Western blot of brain extract (data not shown). LGI3-FL antibody detected 75- and 35-kDa proteins and minor bands with molecular masses of 60- and 25-kDa (Fig. 1C). The 60-, 35- and 25-kDa proteins were identical to the LGI3 protein forms detected in monkey brain [13] and the 75-kDa protein has not been previously reported. The 75- and 60-kDa proteins were recognized by all three anti-peptide antibodies (P1, P5, P6) (Fig. 1C). The 35- and 25-kDa proteins were recognized by P5 and P6 antibodies, respectively. These results indicate that the 75- and 60-kDa proteins represent full-length LGI3 proteins. Since the apparent molecular mass of the bacterially expressed LGI3 was 60-kDa (Fig. 1C, bottom), the 75-kDa protein may contain massive posttranslational modification(s). The 25-kDa protein is a C-terminal fragment of LGI3 protein of which its N-terminus begins downstream of P5 epitope located in the third unit of EPTP repeats. The 35-kDa protein has P5 epitope but lacks P1 and P6 epitopes, suggesting that this protein spans the middle part...
of LGI3 protein including at least the part of EPTP repeats. Whether these multiple protein forms are proteolytic fragments or products of RNA splicing variants remains to be addressed.

In order to explore the biological function of LGI3, we sought for LGI3-interacting proteins by employing LGI3-affinity purification and proteomic analysis of mouse brain extract. Two-dimensional gel electrophoresis of LGI3-His$_6$ affinity-purified proteins revealed a bait-specific spot at apparent isoelectric point and molecular mass of $\sim$5.3- and $\sim$34-kDa, respectively (Fig. 1D). MALDI–TOF and PeptIdent analyses identified the spot as syntaxin 1 (syntaxin 1A identified by MALDI mass mapping by PeptIdent analysis). Pull-down of brain extract with LGI3-His$_6$ resin and Western blot with syntaxin 1 antibody confirmed syntaxin 1 bound to LGI3 (Fig. 1E). LGI3 and syntaxin 1 were coimmunoprecipitated from mouse brain extract by syntaxin 1 and LGI3-FL antibodies respectively, showing the presence of LGI3-syntaxin 1 complex in vivo (Fig. 1F). Immunoprecipitation of brain extract with syntaxin 1 antibody followed by Western blot with LGI3-FL antibody detected the 35-kDa LGI3 protein and smaller amount of the 60-kDa protein, suggesting that the 35-kDa LGI3 protein is the major binding partner of syntaxin 1.

We next examined subcellular localization of LGI3 and syntaxin 1 by immunocytochemistry and subcellular fractionation (Fig. 2A and B). Immunocytochemical staining of mouse embryonic cortical neurons showed LGI3 immunoreactivity predominantly in neuronal processes with prominent punctate synaptic patterns (Fig. 2A). Double immunostaining of LGI3 and syntaxin 1 exhibited colocalization of two proteins mostly in neuronal processes and punctate synaptic structures (Fig. 2A). Next, we assessed
distribution of LGI3 in subcellular fractions of brain homogenate (Fig. 2B). LGI3 protein was found predominantly in membrane fractions (Fig. 2B, P3) comparing with smaller amount of the protein was detected in cytosol (Fig. 2B, lane C). LGI3 protein was enriched in synaptosomal membrane fraction and synaptic vesicle fraction (Fig. 2B, LP1, LP2). Both 75- and 35-kDa forms were detected in synaptic vesicle fraction that was enriched with syntaxin 1, SNAP25 and VAMP2 (Fig. 2B, LP2). Enrichment of LGI3 protein in synaptosomal membrane fraction and synaptic vesicle fraction (Fig. 2B, LP1 and LP2) paralleled with the distribution of the SNARE components (syntaxin 1, SNAP25 and VAMP2) (Fig. 2B).

Syntaxin 1, SNAP25 and VAMP2 proteins constitute the core SNARE complex required for exocytotic vesicle docking and fusion with plasma membrane. We next investigated whether LGI3-syntaxin 1 interaction involves other component of SNARE complex, SNAP25 and VAMP2. Common precipitation of brain extracts demonstrated that LGI3 was coimmunoprecipitated with neither SNAP25 nor VAMP2, while syntaxin 1 was coimmunoprecipitated with LGI3, SNAP25 and VAMP2 (Fig. 2C). These results indicate that LGI3 binds to syntaxin 1 unassociated with SNARE complex.

To test the effect of LGI3 on exocytosis, mouse brain synaptosomal preparation was permeabilized with digitonin to facilitate the entry of exogenously applied protein and the effect of recombinant LGI3 on Ca2+-evoked release of glutamate was measured (Fig. 3A). LGI3 markedly attenuated Ca2+-evoked glutamate release while nonspecific protein BSA had little effect. To further assess the effect of LGI3 on evoked secretion, we analyzed the effect of LGI3 overexpression in PC12 cells on K+-induced hGH secretion (Fig. 3B). Enrichment of LGI3 protein in 24 h-conditioned media was measured by ELISA. Each data set represents the mean of three experiments. Asterisk indicates values of <0.05 according to Student’s t test.

**Fig. 3.** Effect of LGI3 on evoked exocytosis. (A) Effect of LGI3 on synaptosomal glutamate release. Digitonin-permeabilized synaptosomal preparation was pretreated with Krebs-like solution (Krebs only) or test reagents [400 nM recombinant LGI3-His6 or bovine serum albumin (BSA); 2 mM EGTA as a baseline control] for 15 min prior to Ca2+-stimulation. The traces are the mean of three independent experiments. (B) Effect of LGI3 overexpression on hGH secretion from PC12 cells. PC12 cells were transiently transfected with pXGH5 for 24 h and were treated with 55 mM KCl for 15 min. Effect of cotransfection with pcDNA3.1/LGI3-myc on hGH level in 24 h-conditioned media was measured by ELISA. Each data set represents the mean of three experiments. Asterisk indicates P values of <0.05 according to Student’s t test.

**Acknowledgments**

We thank Drs. J.L. Noebels and Z. Sheng for providing LGI family member plasmids and pXGH5. This Research was supported by the Chung-Ang University Research Grants in 2008.

**References**


