Neuroscience Letters 444 (2008) 240–244

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Leucine-rich glioma inactivated 3 associates with syntaxin 1

Woo-Jae Park[1], Sang Eun Lee[1], 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 INFO

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

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

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 Ca^{2+}-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.

Leucine-rich glioma inactivated (LGI) family consists of four members (LGI1, 2, 3 and 4) [4]. The first member of the family, LGI1/epitempin, was discovered in T98G glioblastoma cell line, in which it was rearranged as a result of a balanced translocation [2,9]. Members of LGI family share amino acid sequence homologies of 60–70%. Among these members, LGI1 and LGI3 are predominantly expressed in brain [4,10]. LGI family members have leucine-rich repeats (LRR) and belong to the LRR protein superfamily [8]. LGI3 and other LGI family members also belong to epitempin (EPTP)(or epilepsy-associated repeat, EAR) family. Similar to other members of LGI family, LGI3 protein contains seven EPTP repeats in its C-terminal half region [16,20]. EPTP repeats consist of the consensus unit of ~40 amino acid motif and tend to be present in seven tandem repeats which are predicted to constitute seven-fold β-sheet repeats that fold into a β-propeller structure [20]. Since EPTP repeats were found in epilepsy-associated genes such as very large G-protein-coupled receptors/monogenic audiogenic seizure susceptibility 1 (VLGR/MASS1), thrombospondin-N domain-epilepsy-associated repeat (TSPEAR) and LGI1, this motif was proposed to be associated with pathogenesis of epilepsy [16]. LGI1 was found to be mutated in families with autosomal dominant partial epilepsy with auditory features (ADPEAF) [3,6,11]. LGI4 was reported to be associated with claw paw (clp) phenotype in mice and was implicated in Schwann cell signaling that controls myelination [1]. Both LGI1 and LGI4 are secreted proteins and alteration of extracellular release was proposed to be critical for their role in these diseases [1,19]. LGI3 was also shown to be secreted from transfected HEK293T cells, however, its biological role is unknown [17].

Human LGI3 gene is localized to chromosome 8p21-q22 that includes torsion dystonia susceptibility region [16]. However, its potential association with neurological disorders is not known. We previously reported that LGI3 was expressed in broad areas with regional variation in adult mouse brain and its expression was highly upregulated during development [10]. Furthermore, we found that transcriptional regulation of mouse LGI3 gene was mediated by neuronal restrictive silencer and AP-2 and proposed their roles in neuron-specific expression and developmental regulation of LGI3 gene, respectively [10]. Recent studies showed that amyloid β upregulated and colocalized with LGI3 in astrocytes and that LGI3 accumulated in aged monkey brain [7,13]. Although the previous studies implicated the roles of LGI3 in neural development and aging, the molecular target of LGI3 remains obscure. In this study, we report that LGI3 interacts with syntaxin 1, a core component of SNARE complex, and may participate in the regulation of neuronal exocytosis.

All the animal protocols have been approved by the Institutional Animal Care and Use Committee and followed the National Institutes of Health guidelines. Mice (C57BL/6, Samtako Animal

* Corresponding author. Tel.: +82 2 820 5684/5651; fax: +82 2 817 9866.
E-mail address: hyyunoffice@gmail.com (H.-Y. Yun).
[1] These authors contributed equally to this work.

0304-3940/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2008.08.044

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 LGI3-His_6 (LGI3-FL), LGI3-P1 peptide (amino acids 1–12; MAGLRARRGPGR), LGI3-P5 peptide (amino acids 329–340; PNDLEAFRIDGD) and LGI3-P6 peptide (amino acids 536–548; TLVYRHVVVDLSA). LGI3-His_6 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; LGI3 peptide antibodies, 1:500 dilution) and with peroxidase-conjugated secondary antibody (1:20,000 dilution; Vector Labs) using chemiluminescence substrates (Pierce) and LAS-1000 Plus detection system (Fujifilmn).

For pull-down experiment, purified LGI3-His_6 (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 LGI3-His_6-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-RP 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 (Ultra-Turrax, Janke & Kunkel) in ice-cold RIPA buffer with protease inhibitor cocktail (Roche) 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]. Ca^{2+}-evoked glutamate release from digitonin-permeabilized synaptosomes was measured by the procedure described previously [12,15]. Test samples were added

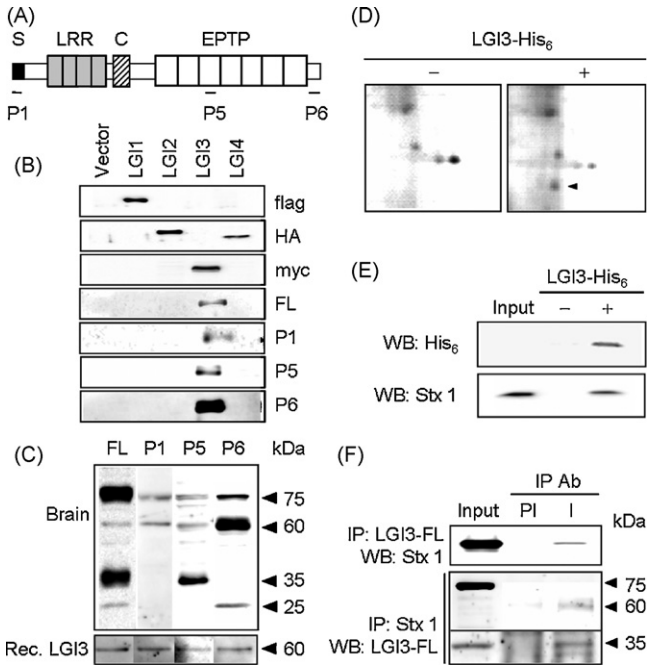

Fig. 1. Identification of syntaxin 1 as an LGI3-associated protein. (A) Diagram depicting domain structure of LGI3 protein. S, putative signal peptide; LRR, leucine-rich repeat; C, cysteine-rich flanking domain; EPTP, epitempin repeat. (B) The specificities of antibodies. Western blot of HEK293T conditioned media collected after transfection with each LGI family members tagged with indicated epitopes. FL, antibody to full-length LGI3. (C) Western blot of mouse brain extract (20 μg protein/lane). Rec. LGI3, recombinant LGI3-His_6 (10 ng protein/lane). (D) Two-dimensional gel electrophoresis and silver staining of LGI3-His_6 affinity-purified proteins from mouse brain extract. Arrowhead indicates syntaxin 1 identified by MALDI–TOF. (E) Western blot of the samples from pull-down experiment using LGI3-His_6 affinity resin from mouse brain extract. IP, immunoprecipitation. WB, Western blot. Stx 1, syntaxin 1. (F) Coimmunoprecipitation and Western blot of mouse brain extract with indicated antibodies. PI, preimmune IgG. I, immune antibody.

to synaptosomes (100 μg protein) and incubated for 15 min prior to stimulation with $CaCl_2$ (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