FEBS Letters xxx (2014) xxx–xxx

journal homepage: www.FEBSLetters.org

Synaptotagmin 11 interacts with components of the RNA-induced silencing complex RISC in clonal pancreatic β-cells

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ARTICLE INFO

Article history:
Received 13 April 2014
Revised 8 May 2014
Accepted 14 May 2014
Available online xxxx

Edited by Sandro Sonnino

Keywords:
Secretory pathway
Pancreatic Islets
ER-Golgi
RISC
Synaptotagmins
SNARE

ABSTRACT

Synaptotagmins are two C_2 domain-containing transmembrane proteins. The function of calcium-sensitive members in the regulation of post-Golgi traffic has been well established whereas little is known about the calcium-insensitive isoforms constituting half of the protein family. Novel binding partners of synaptotagmin 11 were identified in β-cells. A number of them had been assigned previously to ER/Golgi derived-vesicles or linked to RNA synthesis, translation and processing. Whereas the C2A domain interacted with the Q-SNARE Vti1a, the C2B domain of syt11 interacted with the SND1, Ago2 and FMRP, components of the RNA-induced silencing complex (RISC). Binding to SND was direct via its N-terminal tandem repeats. Our data indicate that syt11 may provide a link between gene regulation by microRNAs and membrane traffic.

Structured summary of protein interactions:
Syt11C2A physically interacts with **Vti1a** by pull down (View interaction)
Syt11C2B physically interacts with **SND1**, **PDIA6**, **Vti1b**, **Vti1a**, **Ago2** and **FMRP** by pull down (View interaction)
syt11C2B binds to **SND1** by filter binding (View interaction)
Syt11C2B physically interacts with **EIF3A**, **PDIA6**, **NPM1**, **EIF3B**, **NCL**, **RS3**, **RS3A**, **CBR1**, **ANP32B**, **LOC683961**, **SET**, **SND1**, **TBB2C**, **RS10** and **RS18** by pull down (View interaction)
SND1 physically interacts with **Ago2** by anti bait coip (View interaction)

1. Introduction

A number of transmembrane proteins are implicated in the transport, docking and fusion of vesicles during intracellular transport. Members of the synaptotagmin family (syts) have been shown to form calcium sensors in diverse trafficking pathways [1]. They share a common structure: a short intra luminal N-terminal domain, a single transmembrane domain and a cytoplasmic carboxy-terminal domain. In addition to this topology, synaptotagmins exhibit two C_2 domains in their cytoplasmic tail. Synaptotagmins are either Ca^{2+}-sensitive or -insensitive according to their capacity to bind the divalent ion and phospholipids via these C_2 domains, referred to as C_2A and C_2B. The C_2 domains may act as independent modules and their respective relevance and physiological targets are still not completely resolved. In addition to their role as Ca^{2+}-dependent trigger in membrane fusion, they may also participate in vesicle-membrane interactions [2].

The superfamily of synaptotagmins currently contains 17 isoforms in mammals which can be classified according to their biochemical properties or sequence alignments [1,3]. Syt1 to 3, 5 to 7, 9 and 10 are grouped in regard to their ability to bind phospholipids in a calcium-dependent manner suggesting an involvement of these isoforms in Ca^{2+}-regulated membrane fusion. On the contrary, syt4, syt8 and syt11 to 15 do not exhibit calcium-sensitivity [4–10]. The biochemical properties of syt16 and 17 are

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http://dx.doi.org/10.1016/j.febslet.2014.05.031
0014-5793/

still unknown but sequence comparison suggests calcium-independent functions. Thus, function of several Ca^{2+}-sensitive isoforms have been characterized in detail in post-Golgi trafficking, whereas the localization and the biological role of most other isoforms are still poorly understood. In the attempt to characterize the role of synaptotagmins in the exocytosis of large dense core vesicles, we and others have been able to assign a role to syt1, 2, 7 and 9 using endocrine β-cells as a model system [11–16].

In contrast, the role and distribution of the Ca^{2+}-insensitive forms are still largely unknown although they constitute about a half of this protein family. In this setting, syt11 is of particular interest. Altered expression patterns have been associated with type 2 diabetes and with Parkinson's disease [17,18]. This protein appears as a syt4 paralog in vertebrates [3] and Ca^{2+}-sensitivity has been lost due to mutation of key amino acids representing an evolutionary adaptation [4]. Thus, knowledge about this isoform may contribute to elucidate the panel of functions of synaptotagmins beyond calcium sensing in membrane fusion. In the absence of any known or predictable cellular function, we decided to investigate its binding partners in the well-established model of insulin-secreting β-cells.

2. Materials and methods

2.1. Materials

The following commercial monoclonal antibodies were employed: anti-Vti1b and anti-Vti1a (Transduction Laboratories); anti-insulin (Sigma–Aldrich); anti- βCOP (Sigma–Aldrich), anti-AP1-AP2 (Sigma–Aldrich); (Transduction Laboratories); anti-GST (Sigma–Aldrich); anti-glucagon (Sigma–Aldrich). The following commercial polyclonal antibodies were used: guinea pig anti-SND1 (Progen), guinea pig anti-insulin (Sigma–Aldrich), rabbit anti-Ago2 (Cell Signalling) and rabbit polyclonal anti-FMRP (Abcam). The following secondary antibodies were employed: HRP-conjugated secondary antibodies (GE Healthcare). Several antibodies were kindly donated: the monoclonal anti-FMRP antibody 1C3 (Dr. Mazroui, Université Laval, Québec, Canada); a rabbit polyclonal anti-CaBP1/PDIA6 (Dr. Nguyen Van, Universität Göttingen, Germany), a monoclonal anti-Ago2 (851) (Dr. Hobman, University of Alberta, Edmonton, Canada), a monoclonal anti-EEA1 (Dr. Grunberg, Genève, Switzerland), a monoclonal anti-LAMP1 (Dr. Malosio, Milano), a rabbit polyclonal anti-syntaxin5, anti-syntaxin18 and anti-D12/Use1p (Dr. Hatsuzawa, Fukushima Medical University, Japan). The rabbit polyclonal anti SND1/p102 had been described previously [19].

2.2. Plasmids

cDNA encoding mouse syt11 was generated by RT-PCR from total brain RNA and inserted in a pGEM-T cloning vector as described in [6]. Truncated syt11 were obtained by PCR on pGEM-T-Syt11 using the following primers: syt11C_2A (amino acids 150–264), sense primer: CGCGGATCCTCGCCAGAGGAAGAG and antisense primer: CCGGAATTCTTACAATGGCACCATGAC; syt11 C_2B (amino acids 265–430), sense primer: CGCGGATCCGCTGGAGTGGACCCC and antisense primer: CCGGAATTCTTAGTACTCGCTCAG. PCR fragments were then digested with BamHI and EcoRI and inserted in the pGEX-2T vector (GE Healthcare) allowing the expression of a N-terminal GST fusion protein. Plasmids encoding the cytoplasmic portions of synaptotagmins fused to GST were as described [6]. The plasmid pGEX-4T-1-p100-SN(1-4), containing the four N-terminal SN-like domains of SND1 (amino acids 1–639), were kindly provided by Dr. O. Silvennoinen (University of Tampere, Finland) [20].

2.3. Cell culture, fluorescence microscopy and immunoprecipitation

INS-1E were cultured as previously described [21]. Islets were isolated from adult male Wistar rats (Iffa Credo, Lyon, France) using collagenase digestion and separation on a Ficoll density gradient [22]. For immunofluorescence studies, isolated cells from islets were obtained after dissociation with 0.025% trypsin/0.27 mM EDTA and then cultured in complete RPMI-1640 medium containing 5.6 mM glucose for 1 week. Islet cells were seeded on poly-L-lysine (Sigma–Aldrich) coated Lab-Tek® Chamber Slide™ System. After fixation with 2% paraformaldehyde for 20 min, cells were permeabilised for 5 min in 0.1% Triton X-100 and blocked in 2% BSA. Stained cells were mounted in Citifluor (Citifluor LTD, London, UK) and observed with the BioRad confocal microscope equipped with an argon-krypton laser.

Postnuclear supernatants (PNS) from INS-1E cells were prepared as described in [23]. PNS were then centrifuged at 100,000*g*, 2 h, 4 °C to obtain the membrane fraction. Triton X-100 extracts were obtained as described previously [23,24] except that sonication was omitted. Immunoprecipitation was performed on solubilized fractions by adding the relevant antibody and a 50% suspension of protein A-Sepharose beads CL-4B (GE Healthcare) for 2 h at 4 °C. Beads were washed several times, resuspended in Laemmli buffer and supernatants subsequently analyzed by SDS–PAGE and immunoblots as published [12]. To test specificity, anti-syt11 antibodies were preabsorbed prior to immunoblot with antigenic peptide (10–50 μM) for 1 h at room temperature.

2.4. Purification of recombinant proteins, pull-down experiments and direct binding assay

GST-Syt fusion proteins were expressed as described [24]. Recombinant proteins were either eluted directly or after overnight cleavage at 4 °C from the GST-tag by 4 U/ml of thrombin (GE Healthcare). The pGEX-4T1-p100-SN (1–4) expression vector was kindly donated by Dr. Valineva (University of Tampere, Finland). This construct was expressed in Rosetta host strain (Novagen) under the same conditions as for other GST-tagged proteins. Purified recombinant proteins were analyzed by Coomassie blue stained SDS–PAGE.

25 μg of GST fusion-proteins were immobilized onto gluthatione-Sepharose beads in buffer A (20 mM MOPS, 250 mM sucrose, 5 mM $MgCl_2$, pH 7). Beads were washed 4 times with buffer A/1 M NaCl and once without buffer A alone. Immobilized fusion proteins were subsequently treated for 10 min at 30 °C with micrococcal nuclease (Calbiochem) at a final concentration of 33 U/ml. Beads were washed again in Buffer A/0.01% Triton X-100. 500 μg of a freshly solubilized PNS was mixed with 25 μg of immobilized GST fusion proteins. After incubation at 4 °C for 2 h, beads were washed twice with buffer A/0.1% Triton X-100, twice with buffer A/300 mM KCl and finally with buffer A. Bound proteins were eluted by adding Laemmli's buffer. The eluted samples were resolved by SDS–PAGE followed by spectrometric mass analysis or by Western blot detection with the relevant antibodies.

Purified recombinant syt11C_2A and syt11C_2B (1 μg) were directly spotted onto PVDF membranes, which were subsequently washed in TBS (50 mM Tris, 190 mM NaCl, pH 8)-0.1% Tween and blocked in TBS-1% BSA prior to incubation in 10 μg of purified GST or GST-SN (N-terminal four staphylococcus nuclease domains of SND1 fused to GST). Membranes were washed with TBS-Tween and TBS, blocked in 5% milk TBS-Tween and incubated overnight with a guinea pig anti-SND1 antibody (1/2000). After three washes in TBS-Tween, blots were incubated for 1 h at room temperature with HRP-conjugated anti-guinea pig IgG secondary antibody. Binding of antibodies was visualized by chemiluminescence.

Please cite this article in press as: Milochau, A., et al. Synaptotagmin 11 interacts with components of the RNA-induced silencing complex RISC in clonal pancreatic β-cells. FEBS Lett. (2014), http://dx.doi.org/10.1016/j.febslet.2014.05.031