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Ca^{2+}/calmodulin-dependent phosphorylation of endocytic scaffold ITSN1

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ITSN1 is an endocytic scaffold protein with a prominent function in synaptic transmission. It is known that Ca^{2+} signaling is crucial for the regulation of synaptic proteins functioning. ***Aim****. Checking the possibility of Ca^{2+}/calmodulin-dependent phosphorylation of ITSN1.* ***Methods****. Affinity chromatography, in vitro kinase reaction, Western blotting, gel staining with fluorescent stains.* ***Results****. We show that the fraction of calmodulin-binding proteins is able to phosphorylate the recombinant fragments encoding the coiled-coil region and the SH3 domain-containing region of ITSN1 in the presence of Ca^{2+} ions and calmodulin.* ***Conclusions****. The coiled-coil region and the SH3 domain-containing region of ITSN1 undergo Ca^{2+}/calmodulin-dependent phosphorylation in vitro, suggesting a possible regulation of ITSN1 by Ca signaling.*

Keywords: ITSN1, phosphorylation, calmodulin, Ca^{2+} signaling.

Introduction. ITSN1 is a scaffold protein involved in endocytosis and signal transduction. This protein possesses several domains that act as platforms for binding molecular partners (Fig. 1). To date ITSN1 has been shown to participate in numerous interactions with plenty of proteins [1–3]. The number of its known binding partners is much higher than the quantity of its modules for binding. Thus, it is reasonable to suggest that some regulatory mechanisms should exist in order to determine the sets of ITSN1-interacting partners in particular physiological contexts.

One of such mechanisms can be provided by the posttranslational modifications (PTM) which may alter binding properties of ITSN1 and thus adjust its function to current requirements. Phosphorylation is the most common and most studied type of PTM to date. The phosphorylation of ITSN1 was shown previously by large-scale mass spectrometry studies [4]. Moreover, recently the tyrosine phosphorylation of ITSN1 promoted by expression of LMP2A protein of Epstein-Barr virus has been reported [5]. However, neither the physiological background nor functional consequences of these phosphorylation events are known.

ITSN1 is an important player in synaptic vesicle cycling [6]. It is widely accepted that the majority of molecular events associated with the synaptic vesicle trafficking are triggered by changes in Ca^{2+} concentration and subsequent activation of the Ca^{2+}-dependent proteins, particularly kinases and phosphatases [7]. In this work we tested the hypothesis that ITSN1 may undergo the Ca^{2+}/calmodulin-dependent phosphorylation. We report that in *in vitro* conditions at least two structural components of ITSN1, namely the coiled-coil region and the SH3 domain-containing region, can be phosphorylated in the Ca^{2+}/calmodulin-dependent manner.

Materials and methods. *Expression constructs and antibodies*. GST-SH3A-E plasmid was described previously [5]. GST-CCR was obtained by cloning cDNA fragment encoding the coiled-coil region of ITSN1 (aa 323–726, accession number Q15811 in UniProtKB) in

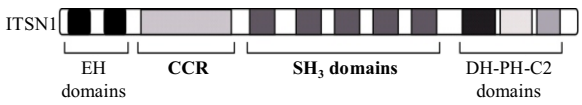

Fig. 1. Schematic representation of ITSN1 domain organization. Domains used for the experiments are shown in bold

pGEX-4T-3 vector («GE Healthcare», UK). The mouse monoclonal anti-STOP antibodies (MAB5524) and the secondary horseradish peroxidase-labeled (HRP) goat anti-mouse antibodies were purchased from «Millipore» (USA) and «Promega» (USA), respectively.

Isolation of calmodulin-binding proteins. Mouse brains were homogenized in MEM buffer (100 mM MOPS, pH 6.75, 400 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT and complete EDTA-free protease inhibitor cocktail («Roche», France)) and centrifuged for 20 min at 12,000 *g* at 4 °C. Supernatant was supplied by $CaCl_2$ to the final concentration of 2 mM and loaded to the column filled by calmodulin-agarose («Sigma-Aldrich», USA). After washing with the same buffer, bound proteins were eluted from the column by MEM buffer, which did not contain $CaCl_2$.

In vitro kinase reaction. Isolated calmodulin-binding proteins were dialyzed against kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT) using Spectra/Por 1 Dialysis Membrane («Spectrum Laboratories Inc.», USA). Recombinant GST and GST-fused proteins were expressed in *Escherichia coli* BL21 (DE3) and purified on glutathione sepharose 4B beads («GE Healthcare») according to manufacturer's instructions. After purification, proteins bound on sepharose beads were washed in kinase buffer and supplied by dialyzed calmodulin-binding protein fraction to the final volume of 50 µl in each reaction. All the reactions contained 0.4 mM EGTA and 0.5 µM microcystine. Additionally, some of the samples contained 100 µM ATP, 3 µM calmodulin, 2 mM $CaCl_2$ and 2 mM EGTA. Reactions were performed at 30 °C for 30 min and stopped by adding equal volume of Laemmli sample buffer (150 mM Tris-HCl, pH 6.8, 2.5 % glycerol, 10 % SDS, 3 % -mercaptoethanol and 0.5 % bromophenol blue). Then the samples were boiled, resolved by SDS-PAGE and either transferred to a nitrocellulose membrane for Western-blot analysis, or subjected to staining with Pro-Q Diamond or SYPRO Ruby protein stains («Molecular Probes», USA) according to manufacturer's instructions. Stained gels were analyzed on PharosFX Molecular Imager («BioRad», USA), images of Western-blots were acquired on ChemiDoc™ XRS+ system («BioRad»).

Results and discussion. In order to examine the possibility of Ca^{2+}/calmodulin-dependent ITSN1 phosphorylation we purified calmodulin-dependent kinases along with other calmodulin-binding proteins from the mouse brain protein lysate by affinity chromatography using calmodulin-agarose. The efficiency of purification was checked by protein staining with SYPRO Ruby stain and by Western blotting of calmodulin-binding protein STOP (stable tubule-only polypeptide) (Fig. 2, *A*). The obtained purified proteins were used as a source of the kinase activity for *in vitro* kinase assay with the GST-fused coiled-coil region (CCR) and the SH3-domain-containing fragment (SH3A-E) of ITSN1. Addition of Ca^{2+} ions and calmodulin to the reaction resulted in the phosphorylation of both GST-fused CCR and SH3A-E proteins visualized by Pro-Q Diamond Phosphoprotein Gel Stain (Fig. 2, *B*). No phosphorylation was observed without Ca and calmodulin. GST alone was equally stained in all the reactions, including a negative control, which indicates the absence of phosphorylation. Uniformity of protein loading was controlled by post-staining the gels with SYPRO Ruby (data not shown).

Our results for the first time demonstrate the Ca^{2+}/calmodulin-dependent phosphorylation of ITSN1, raising the question of its regulation by Ca^{2+} signaling. It has been shown previously that ITSN1 undergoes activity dependent shuttling between active and periactive zones in presynaptic termini [8, 9], but the way of conversion of electrical stimulation to such molecular event remains unknown. Taking into account the crucial role of Ca^{2+} in synaptic activity, the Ca^{2+}/calmodulin phosphorylation of ITSN1 may occur in response to synaptic stimulation and result in alteration of its interaction profile which may lead to the changes in its functioning. Furthermore, ITSN1 was shown to participate in dendritic spine development [10] that can also be regulated by Ca^{2+} signaling since the Ca^{2+}/calmodulin-dependent kinases are known to be the important players in neuronal de velopment and synaptic plasticity [11]. The precise sites of phosphorylation and its effect on the ITSN1 structure

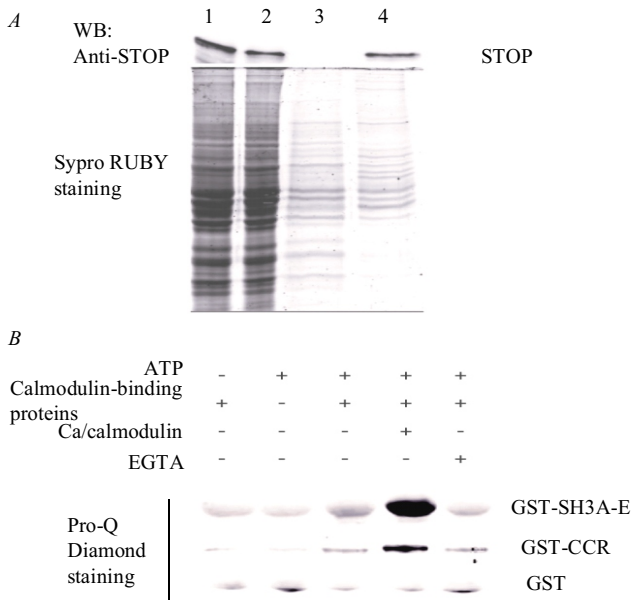

Fig. 2. CCR and SH3A-E regions of ITSN1 are phosphorylated *in vitro* by calmodulin-binding kinases in the presence of Ca/calmodulin: *A* – calmodulin-binding proteins were isolated from mouse brain lysate by affinity chromatography on calmodulin-agarose (bound proteins were visualized by Sypro RUBY staining, efficiency of purification was checked by the presence of STOP protein in the sample: *1* – brain lysate, *2* – flowthrough, *3* – wash, *4* – elution of bound proteins); *B* – recombinant GST-fused fragments of ITSN1 were used as substrates for *in vitro* kinase reaction with calmodulin-binding proteins. Phosphorylated proteins were visualized by Pro-Q Diamond staining

and functions are the focus of our further investigations.

Conclusions. The coiled-coil region and the SH3 domain-containing region of ITSN1 undergo the Ca^{2+}/calmodulin-dependent phosphorylation *in vitro*.

Кальцій/кальмодулін-залежне фосфорилювання ендоцитозного адаптора ITSN1

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ITSN1 – це ендоцитозний адапторний білок, що виконує значущі функції у синаптичному передаванні нервового імпульсу. Відомо, що кальцієва сигналізація є ключовим елементом регуляції функціонування синаптичних білків. ***Мета****. Дослідити можливість кальцій/кальмодулін-залежного фосфорилювання ITSN1.* ***Методи****. Афінна хроматографія, кіназна реакція in vitro, Вестерн блот-гібридизація, фарбування гелів флуоресцентними барвниками.* ***Результати****. Показано, що фракція кальмодулін-зв'язувальних білків здатна фосфорилювати рекомбінантні надспіралізовану та SH3 домен-вмісну ділянки ITSN1 in vitro за присутності іонів кальцію і кальмодуліну.* ***Висновки****. Надспіралізована та SH3 домен-вмісна ділянки ITSN1 підлягають кальцій/кальмодулін-залежному фосфорилюванню in vitro, що дозволяє припустити існування регуляції ITSN1 кальцієвою сигнальною системою.*

Ключові слова: ITSN1, фосфорилювання, кальмодулін, кальцієва сигналізація.

Кальций/кальмодулин-зависимое фосфорилирование эндоцитозного адаптора ITSN1

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ITSN1 – это эндоцитозный адапторный белок, выполняющий значительные функции в синаптической передаче нервного импульса. Известно, что кальциевая сигнализация является ключевым элементом регуляции функционирования синаптических белков. ***Цель****. Исследовать возможность кальций/кальмодулин-зависимого фосфорилирования ITSN1.* ***Методы****. Аффинная хроматография, киназная реакция in vitro, Вестерн блот-гибридизация, окрашивание белков флуоресцентными красителями.* ***Результаты****. Показано, что фракция кальмодулин-связывающих белков способна фосфорилировать рекомбинантные суперспирализованный и SH3 домен-содержащий участки ITSN1 in vitro в присутствии ионов кальция и кальмодулина.* ***Выводы****. Суперспирализованный и SH3 домен-содержащий участки ITSN1 подлежат кальций/кальмодулин-зависимому фосфорилированию in vitro, что позволяет предположить существование регуляции ITSN1 кальциевой сигнальной системой.*

Ключевые слова: ITSN1, фосфорилирование, кальмодулин, кальциевая сигнализация.

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