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SHORT COMMUNICATIONS

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Ca^{2+} does not affect the binding properties of ITSN1 EH domains

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ITSN1 is an endocytic scaffold protein implicated in synaptic functioning. Ca^{2+} is known to be important for endocytosis in both pre- and post-synaptic terminals. ITSN1 contains two EH (Eps15 homology) domains which possess putative Ca^{2+}-binding EF-hand motifs. ***Aim****. To test the effect of Ca^{2+} on the EH domain binding properties.* ***Methods****. His-tag pulldown, Western blotting.* ***Results****. Addition of 1.5 mM Ca^{2+} does not affect the binding of the ITSN1 EH domains to the C-terminal fragment of the endocytic protein Epsin 1.* ***Conclusions****. The data obtained indicate that Ca^{2+} has no effect on the binding properties of the ITSN1 EH domains.*

Keywords: ITSN1, Ca^{2+}, EH domains.

Introduction. ITSN1 is a scaffold protein required for the formation of large multimolecular complexes involved in endocytosis and cellular signaling [1]. It participates in both constitutive endocytosis (*i. e*. transferin receptor internalization) and regulated forms of endocytosis (such as internalization of activated receptor tyrosine kinases or synaptic vesicles) [2–4]. In the latter case a signal must be produced by a cell to trigger the endocytic process. Usually it is the post-translational modification of a cargo that leads to its inclusion in forming endocytic structures. Additionally, the action of endocytosis-inducing stimuli often leads to modifications of certain components of the endocytic machinery themselves, resulting in increased endocytic efficiency [5].

ITSN1 is enriched in the nervous system and was shown to participate in endocytosis in both pre- and post-synaptic terminals [4, 6]. Endocytosis in synaptic compartments is tightly connected with the synaptic activity that is expressed by rapid changes in the intracellular concentration of ions. It is widely accepted that the changes in concentration of Ca^{2+} play a central role in the regulation of various cellular events, including endocytosis, within a synapse [7]. For instance, the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, was shown to dephosphorylate a number of endocytic proteins in response to the electrical stimuli that result in facilitating synaptic vesicle endocytosis [8].

Most ITSN1 isoforms contain two N-terminal EH domains (Fig. 1), except for a putative isoform transcribed from an alternative promoter that is predicted to have one EH domain [9]. These domains interact with NPF (asparagine-proline-phenylalanine) motifs within the protein partners. In particular, EH domains mediate interaction of ITSN1 with Epsin 1 – an auxiliary endocytic protein involved in generating membrane curvatu-

Fig. 1. Schematic representation of structure of ITSN1 molecule. Positions of Ca^{2+}-binding EF-hand motifs are highlighted by asterisk

re, clathrin-coated pit formation and endocytic cargo selection [10, 11]. EH domains are common of a variety of proteins implicated in the membrane trafficking and consist of two helix-loop-helix motifs, that often contain canonical amino acid sequences inherent in the EF-hand motifs [12]. These motifs are known to bind Ca^{2+} [13]. The ability of the ITSN1 EH1 domain to bind Ca^{2+} has been already confirmed by X-ray crystallography (Vorobiev *et al*., unpublished data). In the present study, we tested the effect of Ca^{2+} on binding the ITSN1 EH domains to the C-terminal fragment of Epsin 1.

Materials and methods. *Plasmid constructs and antibodies*. To obtain the plasmid construct for bacterial expression of the C-terminal fragment of Epsin 1 fused to the His-tag (His-Epsin1-C), the cDNA fragment encoding amino acid residues 457–576 (accession number Q9Y6I3 in UniProtKB) was PCR amplified and cloned into the pET28c vector («EMD Biosciences», USA). The construct encoding the GST-fused EH domains of ITSN1 (GST-EH1-EH2) was kindly provided by Dr. Dergai. Rabbit polyclonal antibodies against ITSN1 were described previously [14].

Cell culture. HEK293 cells were cultivated at temperature 37 °C in the atmosphere containing 5 % CO_2 in Dulbecco modified Eagle's medium (DMEM) containing 10 % fetal bovine serum («HyClone», USA), 4.6 g/l of glucose, 10 μg/ml of penicillin and 0.25 μg/ml of streptomycin. For the preparation of cell lysates, the cells were washed in PBS and incubated in IP solution (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 % NP-40, 1 mM PMSF) for 15 min on ice. The solution with lysed cells was then centrifuged at 12,000 rpm for 30 min and the supernatant was collected and frozen at –80 °C.

His-tag pulldown assay. Proteins were expressed in *Escherichia coli* BL21(DE3) cells. After 4 h of induced protein expression, the cells were sonicated in lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% NP-40, 10 mM imidazole, 1 mM PMSF). The lysates were then centrifuged at 12,000 rpm for 30 min and the supernatants were collected and stored at –80 °C. For His-tag pulldown assays, lysates containing His-Epsin1-C were mixed with lysates containing GST-EH1-EH2 or with lysates of HEK293 cells and 20 μl of Ni-NTA agarose («Qiagen», USA) were added for each reaction. All the reactions were supplied with 0.5 mM EGTA and some also with 1.5 mM $MgCl_2$ or 1.5 mM $CaCl_2$ when indicated. The pulldown mixture was incubated at 4 °C in an orbital rotator for 1 h. After 3-time washing with wash buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 % NP-40, 20 mM imidazole), the samples were mixed with an equal volume of Laemmli sample buffer (150 mM Tris–HCl, pH 6.8, 2.5 % glycerol, 10 % SDS, 3 % β-mercaptoethanol, 0.5 % bromophenol blue), boiled for 10 min and analyzed by SDS-PAGE and Western blotting.

Densitometry measurements and statistical analyses. All experiments were repeated 8 times. Images of SDS-PAGE and Western blots were acquired using the ChemiDocTM XRS+ visualization system and the Image LabTM software («Bio-Rad», USA). All densitometry measurements were performed using the Image Lab™ software. Statistical significance of differences was evaluated with the non-parametric Mann-Witney U-test using the Origin Pro v9.0.0 software («Origin Lab Corporation», USA). The P-values above 0.05 were considered statistically insignificant.

Results and discussion. To evaluate the effect of Ca^{2+} on the functioning of the ITSN1 EH domains, a set of *in vitro* binding assays was performed. The recombinant His-tagged C-terminal fragment of the endocytic protein Epsin 1 (His-Epsin1-C) served as a bait since it is known to interact with the ITSN1 EH domains [10]. His-Epsin1-C was expressed in bacteria and used for His-tag-pulldown assays with recombinant GST-EH1-EH2. All the experiments were performed in 3 variants: with addition of Ca^{2+} or Mg^{2+} and without these bivalent cations. The bound proteins were then resolved by SDS-PAGE. For each variant the amount of bound protein was established as a ratio between the signals from GST-EH1-EH2 and His-Epsin1-C. Addition of bivalent cations resulted in a slight increase in binding, which was even more pronounced when adding Mg^{2+} than Ca^{2+}. Nonetheless, we did not observe significant differences in binding GST-EH1-EH2 to His-Epsin1-C between all the variants tested (Fig. 2, *A*). To exclude a possibility of the effect of improper folding of bacterially-expressed ITSN1 EH domains, the His-tag pulldown assays were performed between His-Epsin1-C and cell lysate of HEK293 cells followed by Western blotting using anti-ITSN1 antibodies. This experiment confirmed that addition of Ca^{2+} or Mg^{2+} does not significantly change the level of His-Epsin1-C binding by ITSN1 (Fig. 2, *B*).

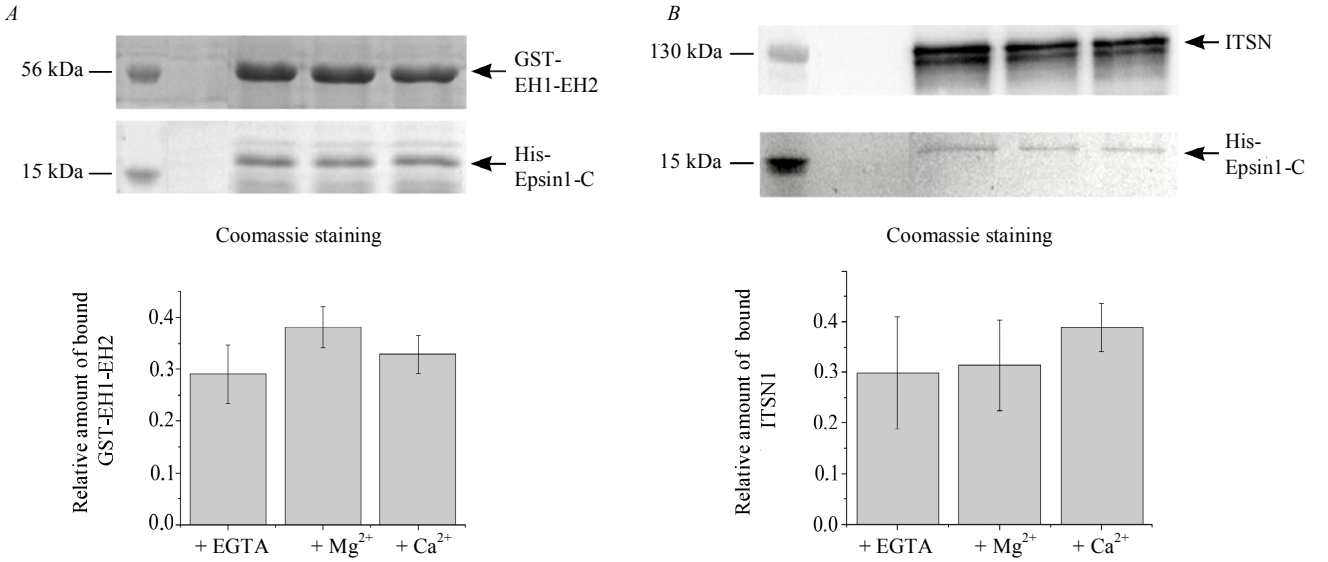

Fig. 2. Ca^{2+} does not affect binding of ITSN1 EH domains to the endocytic protein Epsin1: *A* – Ni-NTA agarose was loaded with His-Epsin1-C and GST-EH1-EH2, and incubated in the presence of either 0.5 mM EGTA alone or additionally with 1.5 mM $MgCl_2$ or $CaCl_2$ (bound proteins were resolved by SDS-PAGE; the amount of bound GST-EH1-EH2 was normalized to the amount of His-Epsin1-C; the values obtained for all the variants within one repeat were summarized and the relative portion from the sum was established for each variant; the statistical significance of differences was evaluated with the non-parametric Mann-Witney U-test using the Origin Pro v9.0.0 software; the data are presented as mean ± ± SD); *B* – Ni-NTA agarose was loaded with His-Epsin1-C and lysate of HEK293 cells and incubated in the same conditions as in *A* (the amount of bound ITSN1 was established by Western blotting; calculations were performed as in *A*)

In contrast to our results, Kelly and Phillips have previously shown that 1 mM EGTA reduces the binding of the EH domains of ITSN1 *Drosophila* ortholog Dap 160 to the synaptic protein stoned-B by about 25 % compared to the binding in the presence of 100 μM Ca^{2+} [15]. Despite this fact, the authors postulated that Ca^{2+} had no important effect on this interaction. The discrepancy between their and our results could be explained by differences in the Ca^{2+} concentrations used. However, in the preliminary experiments we tested different Ca^{2+} concentrations ranging from 200 nM to 2 mM (without adding EGTA to the mixture) and did not observe any effect on the interaction between the proteins studied (data not shown). Nevertheless, taking into account these results, we cannot ignore the possibility that Ca^{2+} can affect the interaction of ITSN1 with the proteins that we did not study. On the other hand, there can also be interspecies differences between the ITSN1 EH domains of different organisms. To summarize, the data we have to date indicate the absence of a prominent regulatory role of the EF-hand motifs within the ITSN1 EH domains, but it is still possible that binding Ca^{2+} by these motifs could shift the binding equilibrium of certain EH domain-mediated interactions in some species, thus demonstrating the «fine-tuning» of these interactions.

Although the ITSN1 EH domains seem not to be subjected to Ca^{2+}-dependent regulation, Ca^{2+} signaling remains one of the likely instruments in the regulation of the function of ITSN1. Recently we have shown that ITSN1 can potentially undergo Ca^{2+}/calmodulin-dependent phosphorylation [16]. This finding demonstrates another possible mode of regulation of the ITSN1 function by Ca^{2+} the role of which remains to be determined. Taking into account the prominent role of ITSN1 in the synaptic functioning, the study of the Ca^{2+}-dependent regulation of ITSN1 functions is a promising research direction.

Conclusions. The data reported indicate the absence of an effect of Ca^{2+} on the binding properties of the ITSN1 EH domains.

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Іони Ca^{2+} не впливають на зв'язувальні властивості ЕН-доменів ITSN1

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Резюме

ITSN1 – адаптерний білок ендоцитозу, залучений до функціонування синапсів. Відомо, що іони Ca^{2+} є важливими для ендоцитозу

у пре- та постсинаптичних закінченнях. ITSN1 має два ЕН-домени, гомологічних Eps15, які містять передбачені Ca^{2+}-зв'язувальні мотиви EF-hand. ***Мета****. Перевірити ефект іонів Ca^{2+} на зв'язувальні властивості доменів ЕН.* ***Методи****. Преципітація білків, злитих з His-тагом, Вестерн-блот-гібридизація.* ***Результати****. Показано, що додавання 1,5 мМ Ca^{2+} не впливає на зв'язування ЕН-доменами ITSN1 С-кінцевого фрагмента білка ендоцитозу Епсину 1.* ***Висновки****. Отримані дані свідчать про відсутність ефекту іонів Ca^{2+} на зв'язувальні властивості ЕН-доменів ITSN1.*

Ключові слова: ITSN1, іони Ca^{2+}, ЕН-домени.

Ионы Ca^{2+} не влияют на связывающие свойства ЕН-доменов ITSN1

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Резюме

ITSN1 – адапторный белок эндоцитоза, вовлеченный в функционирование синапсов. Известно, что ионы Ca^{2+} важны для эндоцитоза в пре- и постсинаптических окончаниях. ITSN1 имеет два ЕН-домена, гомологичных Eps15, которые содержат предсказанные Ca^{2+}-связывающие мотивы EF-hand. ***Цель****. Проверить эффект ионов Ca^{2+} на связывающие свойства доменов ЕН.* ***Методы****. Преципитация белков, слитых с His-тагом, Вестерн-блот-гибридизация.* ***Результаты****. Показано, что добавление 1,5 мМ Ca^{2+} не влияет на связывание ЕН-доменами ITSN1 С-концевого фрагмента белка эндоцитоза Эпсина 1.* ***Выводы****. Полученные данные свидетельствуют об отсутствии эффекта ионов Ca^{2+} на связывающие свойства ЕН-доменов ITSN1.*

Ключевые слова: ITSN1, ионы Ca^{2+}, ЕН-домены.

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