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Identification of Ca^{2+}/calmodulin-dependent phosphorylation sites of endocytic scaffold ITSN1 by tandem mass spectrometry

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ITSN1 is a scaffold protein involved in endocytosis, signal transduction and cytoskeleton regulation. It has been previously shown that ITSN1 undergoes Ca^{2+}/calmodulin-dependent phosphorylation *in vitro*. **Aim.** We intend to identify these phosphorylation sites. **Methods.** In vitro kinase reaction; liquid chromatography-tandem mass spectrometry (LC/MS/MS). **Results.** We identified five sites of Ca^{2+}/calmodulin-dependent phosphorylation in the recombinant fragments of ITSN1. **Conclusions.** We have shown that the ITSN1 coiled-coil region (CCR) and the interdomain linkers between EH2 and CCR, SH3A and SH3B, SH3B and SH3C domains were phosphorylated in a Ca^{2+}/calmodulin-dependent manner *in vitro*.

Keywords: ITSN1, Ca^{2+}, phosphorylation, LC/MS/MS.

Introduction

ITSN1 is a scaffold protein implicated in various cellular processes including the endocytosis, signal propagation through a number of signaling pathways, actin cytoskeleton regulation, *etc* [1]. Accumulating evidence connects ITSN1 misregulation with the neurodevelopmental and neurodegenerative disorders, such as Down syndrome, Alzheimer's disease and Huntington's disease [2–4]. It is believed that ITSN1 operates in a cell by interacting with the partner molecules, thereby promoting an assembly of macromolecular complexes. As for today, dozens of ITSN1 interactors are already known and their number continues to increase [5]. Such complexity implies the existence of precise regulatory mechanisms which control the dynamics of interaction and the selection of partner molecules for ITSN1 binding. The post-translational modifications of ITSN1 can potentially be involved in these mechanisms.

Phosphorylation is one of the most widespread and well studied post-translational modifications of proteins. It results in the covalent attachment of phosphate group to the serine, threonine or tyrosine residues, which can lead to an alteration in the protein conformation and subsequent change of its properties. Therefore, phosphorylation is a common mechanism for triggering the protein activation state. ITSN1 phosphorylation has been already revealed in several large-scale studies of phosphoproteome in different tissues [6–9]. Additionally, the tyrosine phosphorylation of ITSN1 in response to the overexpression of Epstein-Barr virus protein LMP2A and tyrosine kinase Syk in HEK293 cells has been reported [10]. However, it is unclear which intracellular pathways

B

CCR

312_R**SGSGISVISSTSVDQRLPEEPVLEDE*QQQLEKK*** *LPVTFEDKKRENFERGNLELE*KRR**QALLEQQR**
KEQERLAQLERAEQERKERERQEQERKRQLELEKQLEKQRELERQREEERRKEIERREAAKRELERQR**QLEWER**NRR**RQ**
ELLNQRNKEQEDIVVLKAKK*KTLEFELEALNDKKHQLE**GKLQDIR**CRLTTQRQEIESTNKSRELR**IAE*ITHL
QQQLQESQQML*GRLIPEK**QILNDQLKQVQQNSLHRDSLVTLKR*ALEAKE*LAR*QHLRDQLDEVEKETRS
KLQEIDIFNNQLK*ELREIHNKQQLQKQKSMEAERLKQKE*QER**KIIELEK**QKEEAQRRAQE*RDKQWLEHVQ***
***QEDE*HQRPR**KLHEEEKLKREESVKKKDGEEKGKQEAQDKLGRLFHQHQEP_715

SH3A-E

718_PAVQAPWSTAE***KGPLTISAQEN*VK**VVYYR**ALYPFESR**SHDEITIQPGDIVMVKGEWVDE*SQTGEPGWLGGE***
LKGKTGWFPANYAEKIPENEVPAPVKPVTDSTSAPAPKLALRETPAPLAVTSSEPSTTPNNWADFSSTWPTST
NEKPETDNWDAWAAQPSLTVPSAGQLR**QRSAFTPATATGSSPSPVLGQGEK**VEGLQAQALYPWRAK**KDNHLNF**
NKNDVITVLE*QQDMWWFGEVQGQKGWFPKSYVK**LISGPIRKSTSMDSGSSE*SPASLKR*VASPAAKPV***
VSGEEFIAMYTYESSEQGDLTFQQGDVILVTK**KDGDWWTGTVGDKAGVFPSNYVR**LKDSEGSGTAGKTGSLGK**
KPEIAQVIASYTATGPEQLTLAPGQLILIRKKNPGGWWEGELQARGKK**RQIGWFPANYVKLLSPGTSKI**
TPTEPPKSTALAAVCQVIGMYDYTAQNDDELAFNKGQIINVLNKEDPDWWKGEVNGQVGLFPSNYV
KLTTDMDPSQQ_1220

Fig. 1. *A* – Schematic representation of ITSN1 domain structure. Domains studied in this work are underlined. *B* – Aminoacid sequences of studied fragments of ITSN1. Peptides identified by mass spectrometry are enlarged and underlined. Peptides identified after treatment with trypsin are highlighted in bold. Peptides identified after treatment with Glu-C are in italics

drive these phosphorylation events. Recently, we have demonstrated that the CCR and SH3A-E fragments of ITSN1, containing the coiled-coil region and SH3 domains, respectively (Fig. 1A), can be phosphorylated in the Ca^{2+}/calmodulin-dependent manner, suggesting the possibility of ITSN1 phosphorylation in response to the elevation of the intracellular Ca^{2+} concentration [11]. Here we report the identification of Ca^{2+}/calmodulin-dependent phosphorylation sites in ITSN1 using tandem mass spectrometry combined with liquid chromatography (LC/MS/MS).

Materials and Methods

Plasmid constructions

The plasmid encoding GST-fused CCR fragment of ITSN1 was described previously [12]. Nucleotide sequence encoding SH3A-E fragment of ITSN1 was PCR amplified and cloned in pGEX-4T-2 vector (GE Healthcare, USA).

Recombinant protein expression and purification.

Recombinant GST-fused proteins were expressed using *Escherichia coli* BL21(DE3) strain. Obtained proteins were affinity-purified using glutathione-sepharose 4B (GE Healthcare, USA) according to manufacturer's instruction.

In vitro kinase reaction

The reaction was performed as described previously [11]. Briefly, calmodulin-binding proteins were purified from mouse brain lysate on calmodulin-agarose beads (Sigma-Aldrich, USA), dialyzed against kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2 mM DTT) and then mixed with purified recombinant ITSN1 fragments. The reaction was supplied by 0.4 mM EGTA, 0.5 μM microcystin, 100 μM ATP, 3 μM calmodulin, 2 mM $CaCl_2$, and incubated at 30 ˚C for 30 min. Then the reaction was stopped by addition of an equal volume of Laemmli sample buffer (150 mM Tris–HCl pH 6.8, 2.5 % glycerol, 10% SDS, 3 % β-mercaptoethanol and 0.5 % bromophenol blue). After boiling the samples were resolved by SDS-PAGE and stained by Coomassie Brilliant Blue.

LC/MS/MS

In-gel digestion of proteins was performed at 37°C overnight with trypsin or Glu-C proteases (Roche, Switzerland) (1:50 protease : protein ratio). Next day the peptides were extracted from the gel using Oasis C18 kit. Then, the peptides were dissolved to a final concentration of 4 % in formic acid before analysis on LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fischer Scientific, USA) equipped with a

nano spray Flex ion source (Thermo Fischer Scientific, USA), coupled to a Dionex Ultimate NCS-3000 LC system (Thermo Fischer Scientific, USA). Fragmentation of primary ions was performed by HCD (high-energy collision dissociation) technique. Tandem mass spectra were extracted by Proteome Discoverer™ software (Thermo Scientific, USA). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 1.3.0.339). Mascot was set up to search Mascot5_SwissProt_Homo sapiens (human) (16369 entries) assuming the digestion enzyme trypsin or V8. Mascot was searched with a fragment ion mass tolerance of 0.05 Da and a parent ion tolerance of 15 PPM. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine, acetylation of the N-terminus and phosphorylation of serine and threonine were specified in Mascot as variable modifications. The phosphopeptides with reliably identified phosphorylation site were selected by the value of Mascot Delta Ion Score, using value 13 as a threshold [13]. Their spectra were also manually revised using Scaffold software version 3.6.5 (Proteome Software) [14].

Results and Discussion

In order to identify the sites of Ca^{2+}/calmodulin-dependent phosphorylation in the ITSN1 recombinant fragments we repeated our previously described assay for *in vitro* Ca^{2+}/calmodulin-dependent phosphorylation [11] and subsequently analyzed the samples by LC/MS/MS. To increase the sequence coverage and probability of identification for all possible phosphosites, the samples were divided and digested independently by two distinct endoproteases: trypsin and Glu-C. As a result of the mass spectrometric peptide detection we obtained 48.7 % coverage for CCR sequence and 59.4 % coverage for SH3A-E sequence using trypsin, whereas for Glu-C the corresponding values were 39.1 % and 23.3 %, respectively. In summary we obtained 61.6 % and 67.4 % sequence coverage for CCR and SH3A-E fragments, respectively (Fig.1*B*).

For each digestion variant except the SH3A-E fragment digested by Glu-C we were able to identify a number of phosphopeptides (Supplementary Table 1). The obtained phosphopeptides were sorted according to their calculated Mascot Delta Ion Score value to select those with the most confidently located phosphorylation sites. In this way we identified five phosphopeptides containing the phosphorylation sites corresponding to positions T349, T567, S624, S904 and S978 in ITSN1 sequence (Q15811 in UniProtKB database) (Table 1). We also carefully checked the MS/MS spectra of selected peptides manually for the presence of phosphospecific secondary ions to confirm a reliable location of the phosphorylated sites (Fig. 2).

Table 1. **Identification of Phosphorylation Sites in ITSN1 Fragments by LC/MS/MS**

Peptide Sequence	Position of Phosphosit-ein ITSN1 (Q15811)	Fragment	Protease Used	Peptide Identification Probability	Mascot Ion Score	Mascot Identity Score	Mascot Delta Ion Score	Modifications
(R)DSLVtLKR(A)	567	CCR	Trypsin	95 %	57.93	28.5248	19.68	Phospho (+80)
(K)KLPVtFEDK(K)	349	CCR	Trypsin	95 %	35.35	25.0	35.35	Phospho (+80)
(E)IHNKQQLQKQKsMEAERLKQKE(Q)	624	CCR	Glu-C	95 %	35.18	26.374897	35.18	Phospho (+80)
(R)SAFTPATATGSSPsPVLGQGEK(V)	904	SH3A-E	Trypsin	95 %	86.77	25.0	15.0099945	Phospho (+80)
(K)STsMDSGSSESPASLKR(V)	978	SH3A-E	Trypsin	95 %	117.05	29.604706	16.410004	Phospho (+80)

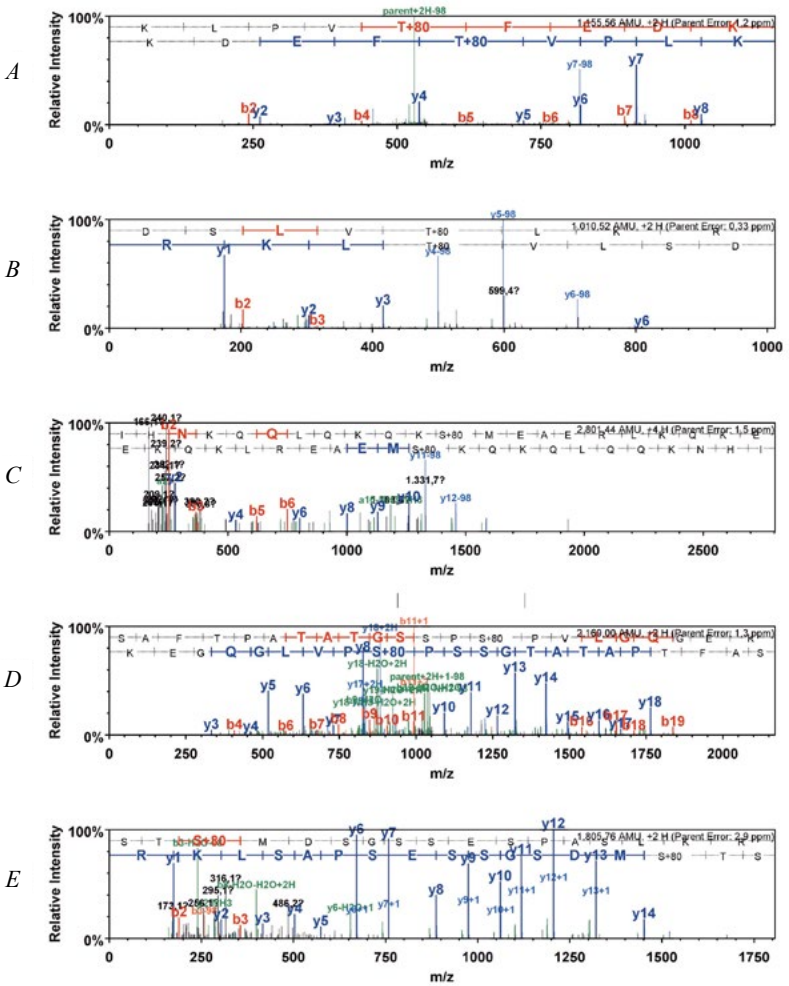

Fig. 2. HCD MS/MS spectra of identified phosphopeptides. *A* – HCD spectrum of (K) KLPVpTFEDK(K) (residues 345–353). The most intensive peak corresponds to the neutral loss of H_3PO_4 from parental ion (–98), indicating phosphorylation of the peptide. *B* – HCD spectrum of (R)DSLVpTLKR(A) (residues 563–570). Phosphorylation of T567 is confirmed by the presence of y4, y5 and y6 ions with the neutral losses of H_3PO_4 (–98). *C* – HCD spectrum of (E)IHNKQQ-LQKQKpSMEAERLKQKE(Q) (residues 613–634). Phosphorylation of S624 is indicated by y11 and y12 ions with the neutral losses of H3PO4 (–98). *D* – HCD spectrum of(R)SAFTPATATGSSPpSPVLGQGEK(V) (residues 891–912). Phosphorylation of the peptide is indicated by parental ion with the neutral loss of H_3PO_4 (-98). Assignment of phosphorylation to S904 site is justified by the presence of y10 ion. *E* – HCD spectrum of (K)STpSMDSGSSESPASLKR(V) (residues 976–992). Phosphorylation of S978 [is] indicated by b2 and b3 ions, as well as by the presence of b3 ions containing losses of H_3PO_4 (-98) and H_2O^+ H_3PO_4 (–116)

B

740_KVVYYRALYPFESRSHDEITIQPGDIVMVKGEWVDESQTGEPGWLGGELKG

SH3A DOMAIN

KTGWFPANYAEKIPENEVPAPVKPVTDSTSAPAPKLALRETPAPLAVTSSEPSTTPN

NWADFSSTWPTSTNEKPETDNWDAWAAQPSLTVPSAGQLRQRSAFTPATATGSS

PSPVLGQGEKVEGLQAQALYPWRAKKDNHLNFNKNDVITVLEQQDMWWFGEVQ

SH3B DOMAIN

GQKGWFPKSYVKLISGPIRKSTSMDSGSSESPASLKRVASPAAKPVVSGEEFIAMY

TYESSEQGDLTFQQGDVILVTKKDGDWWTGTVGDKAGVFPSNYVRLKDS_1060

SH3C DOMAIN

Fig. 3. *A* – Location of phosphorylation sites within the fragments of ITSN1. *B* – Partial sequence of SH3A-E between SH3A and SH3C domains. Domain sequences are shown in grey. Serine/threonine residues within domains are shown in blue and within interdomain linkers – in red. Frequency of occurrence of serine/threonine residues in the interdomain linkers is higher than inside domains

Among the identified phosphorylation sites, only S624 and S978 match the R/K-X-X-S/T canonical recognition motif for Ca^{2+}/calmodulin-dependent kinases [15]. Amino acid sequences of other sites do not resemble the known kinase recognition motifs, thus making a kinase responsible for these phosphorylation events difficult to predict. However, it is known that the absence of full match with the consensus motif does not mean the inability of a particular kinase to phosphorylate a given substrate. For instance, it has been shown that cyclin-dependent linase CDK1 under certain condition can phosphorylate the minimal S/T-P recognition motif instead of the canonical S/T-P-X-K/R motif [16, 17]. Interestingly, the identified S904 site corresponds to this minimal motif. Additionally, the S904 phosphorylation site is flanked by proline residues, indicating its possible phosphorylation by so called proline-directed kinases, which include the CDK, MAPK, JNK and GSK kinase families [18]. Interestingly, CDK4 and CDK5 can interact with calmodulin and be activated by either Ca^{2+} or Ca^{2+}-dependent phosphorylation [19–22], suggesting the possibility of their presence in our purified calmodulin-binding fraction and the activation in Ca^{2+}-dependent manner. Notably, the S904 phosphorylation has already been detected in the large-scale studies of phosphoproteome [6–9].

For S349 and T567 it is hard to predict a kinase that could phosphorylate these residues. Interestingly, both of them are surrounded by hydrophobic amino acids (at positions –2, –1 and +1), suggesting their modification by the same kinase.

A functional role of the identified phosphorylation sites is unclear and needs to be studied in future experiments. The T567 and S624 sites are located in the coiled-coil region of ITSN1 (Fig.3*A*). This domain mediates its oligomerization or interactions with other proteins containing similar domains [23]. It has been shown that phosphorylation of the threonine residues within such regions can destabilize helices, whereas phosphorylation of the serine residues can lead to either their stabilization or destabilization [24, 25]. It can be expected that the phosphorylation events within the coiled-coil region can affect its ability to oligomerize or interact with other proteins. T349, S904 and S904 are located in the interdomain linkers that are predicted to be intrinsically disordered (Fig. 3A). It has been demonstrated that phosphorylation of the serine and threonine residues most often occurs in such regions [26–28]. Moreover, the S904 and S978 sites are located within the regions enriched in the serine/threonine residues (Fig.3*B*). Phoshorylation of several residues in these clusters has been identified [6–8]. Interestingly, these two clusters are located at both sides and in close proximity to the SH3B domain. Therefore, we suggest that phosphorylation of the serine and threonine residues belonging to these clusters can affect the SH3B domain ability to mediate protein-protein interactions. We believe that examination of the role of identified phosphorylation sites in the ITSN1 function is a promising direction for further investigations.

Conclusions

We showed that ITSN1 coiled-coil region (CCR) and interdomain linkers between the EH2 and CCR, SH3A and SH3B, SH3B and SH3C domains were phosphorylated in Ca^{2+}/calmodulin-dependent manner *in vitro*.

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Ідентифікація сайтів Ca^{2+}/кальмодулін-залежного фосфорилювання скаффолдного білка ендоцитозу ITSN1 за допомогою тандемної мас-спектрометрії.

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ITSN1 – це скафолдний білок, задіяний у процесах ендоцитозу, сигнальної трансдукції та регуляції цитоскелету. Раніше було показано, що ITSN1 підлягає Ca^{2+}/кальмодулін-залежному фосфорилюванню *in vitro*. **Мета** Ідентифікувати сайти цього фосфорилювання. **Методи.** In vitro кіназна реакція; рідинна хроматографія, поєднана з тандемною мас-спектрометрією (LC/MS/MS). **Результати.** Ми ідентифікували 5 сайтів Ca^{2+}/кальмодулін-залежного фосфорилювання у рекомбінантних фрагментах ITSN1. **Висновки.** Було показано, що надспіралізована ділянка (CCR) та міждоменні лінкери між EH2 та CCR,

SH3A та SH3B, а також між SH3B та SH3C доменами ITSN1 підлягають Ca^{2+}/кальмодулін-залежному фосфорилюванню.

Ключові слова: ITSN1, Ca^{2+}, фосфорилювання, LC/MS/MS.

Идентификация сайтов Ca^{2+}/кальмодулин-зависимого фосфорилирования скаффолдного белка эндоцитоза ITSN1.

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ITSN1 является скаффолдным белком, задействованным в процессах эндоцитоза, сигнальной трансдукции и регуляции цитоскелета. Ранее было показано, что ITSN1 подлежит Ca^{2+}/кальмодулин-зависимому фосфорилированию *in vitro*. **Цель** Идентифицировать сайты Ca^{2+}/кальмодулин-зависимого фосфорилирования ITSN1. **Методы.** In vitro киназная реакция, жидкостная хроматография в сочетании с тандемной масс-спектрометрией (LC/MS/MS). **Результаты.** Мы идентифицировали 5 сайтов Ca^{2+}/кальмодулин-зависимого фосфорилирования в рекомбинантных фрагментах ITSN1. **Выводы.** Было показано, что суперспирализированый участок (CCR) и междоменные линкеры между EH2 и CCR, SH3A и SH3B, а также между SH3B и SH3C доменами ITSN1 подвергаются Ca^{2+}/кальмодулин-зависимому фосфорилированию.

Ключевые слова: ITSN1, Ca^{2+}, фосфорилирование, LC/MS/MS.

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