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# Proteomic analysis of proteins associating with Nck-α

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Nck proteins are a family of SH2/SH3 domain-containing adaptor proteins. Both Nck-α and Nck-β play a major role in regulating a wide variety of cellular processes, including cytoskeletal rearrangement, cell morphology, gene expression and protein translation. We have analysed proteins interacting with SH3 domains of Nck-α. The proteins were separated using two-dimensional electrophoresis, subjected to in-gel digestion with trypsin and identified by mass spectrometry using MALDI-TOF mass fingerprinting. Here we report novel potential binding partners for Nck-α such as 14-3-3γ protein, eukaryotic translation initiation factor 4E and ras-related protein Rab-30. Our results provide additional information presumably confirming Nck-α role in actin cytoskeleton rearrangement regulation and the modulation of the eukaryotic translation initiation. Data also suggest that Nck-α might be involved in the control of Golgi functioning.

**Key words**: Nck, proteomics, mass spectrometry, SH3 domains

## INTRODUCTION

The Nck family belongs to a group of Src homology (SH) 2/SH3 adaptors, i. e. proteins composed almost entirely of protein–protein interaction mediating SH2 and SH3 domains but lacking any intrinsic enzymatic abilities. The family consists of Nck-α and Nck-β proteins. They have three consecutive SH3 and one SH2 domains which share 68% of amino acid sequence homology between both Nck, although they appear to have distinct functional assignments in the same cells [1, 2].

SH2 domains associate with specific phosphotyrosine-containing sites. SH3 domains bind proline-rich motives, and generally these interactions are phosphorylation-independent, unless phosphorylation changes the protein conformation. Adaptor proteins through their SH3 domains can associate with a number of signalling proteins and upon cell stimulation can recruit them to tyrosine-phosphorylated cytoplasmic or membrane-attached partners [1]. Multiple SH3 domains of Nck allow it to bind two or more partners simultaneously. Another function of multiple SH3 domains is to modulate the efficiency and specificity of Nck interactions. It is also possible that binding two or more SH3 domains can stabilize the conformation of Nck-binding partners [3]. The presense of three consecutive SH3 domains is a unique feature of Nck proteins and may be responsible for diverse Nck functions in the cellular processes.

Nck proteins play an important role in mediating receptor tyrosine kinase signalling to the actin cytoskeleton [4, 5]. In T cells Nck-α helps to mediate the phosphotyrosine-dependent assembly of a complex containing WASP and Vav, which provides localized actin polymerization following T cell receptor stimulation [3]. Nck-β is implicated in actin rearrangements downstream of PDGF receptor stimulation [6]. Nck proteins are also involved in cell proliferation, adhesion, migration, protein translation, alternative splicing regulation [1, 5].

In this study, we performed a proteomic analysis of Nck-α SH3 domain-binding proteins and identified three novel potential Nck-interacting proteins: 14-3-3γ protein, eukaryotic translation initiation factor 4E and ras-related protein Rab-30.

## MATERIALS AND METHODS

**Cell culture and preparation of cell lysates**. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% foetal bovine serum (GibcoBRL, UK). HepG2 cells were grown to 70–80% confluence and made quiescent by culturing in serum-free DMEM overnight. Cells were washed with ice-cold PBS and lysed in $EB^{++}$ lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM PMSF, 2 mM $NaVO_4$). Lysates were cleared by centrifugation at 20.000×g for 15 min (0 °C).

**Precipitation assay with GST-3SH3 protein**. DNA fragment encoding three consecutive SH3 domains of Nck-α (called 3SH3, amino acids 5-251) was generated by PCR and subcloned into the pGex2T bacterial expression (GE Healthcare). Glutathione S-transferase (GST) fusion proteins were generated and purified as

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described earlier [7, 8]. Precipitation experiments were carried out using 10 mg of GST-3SH3 fusion protein or GST alone immobilised on Glutathione Sepharose 4B beads per sample. The beads were incubated with cellular lysates for 1 h at 4 °C mixing continuously and washed five times with $EB^{++}$ buffer. The GST or GST--3SH3 fusion proteins with co-precipitated proteins from cellular lysates were eluted from the beads with rehydration buffer (8 M urea, 0.5% Triton X-100, 0.2% DTT, Pharmalyte 4-7).

**2D electrophoresis**. Immobiline DryStrips (pH 4–7) (GE Healthcare) were reswollen overnight in 200 µl of protein eluate. The isoelectrofocusing was carried out up to a total of 30 kVh. Prior to second dimension electrophoresis gel stripes were incubated in a solution of 0.5% dithiotriethol in equilibration buffer and subsequently in a solution of 4.5% iodoacetamide in the same buffer for 10 min. SDS-PAGE was performed in 8–18% gradient ExcelGel. Gels were stained with 0.1% $AgNo_3$.

**In-Gel tryptic protein digestion**. After staining, the spots of interest were cut from the gel and destained with the solution of 25 mM ammonium bicarbonate / 50% acetonitrile. The proteins were digested in gel with 100 ng trypsin at 37 °C for 16 h. Peptides were extracted from the gel with 1% trifluoroacetic (TFA) and vacuum-dried.

**Mass spectrometric analysis**. Samples were dissolved in 3 ml of 0.1% TFA. Aliquots of 0.8 µl were mixed with an equal amount of matrix (saturated solution of α-cyano-4-hydroxy cinnamic acid) and applied to a sample plate. Spectra were obtained using a Voyager MALDI-TOF mass spectrometer (Applied Biosystems, USA). For interpretation of the spectra of peptides we used Voyager Data Explorer software and PeptIdent program available on the ExPaSy server (http://us.expasy.org/).

## RESULTS

**Precipitation assay and 2D electrophoresis**. HepG2 cells were made quiescent by serum starvation and lysed. Postnuclear lysates were used in the precipitation assay with sepharose-immobilized recombinant GST-3SH3 protein containing three SH3 domains of Nck-α as a bait (Figure A). Simultaneously, control experiments with Sepharose-immobilized GST-3SH3 protein incubated in lysis buffer (Figure B) and with Sepharose-immobilized GST incubated in lysate (Figure C) or lysis buffer (Figure D) were performed. The protein complexes were separated by two-dimensional (2D) electrophoresis. The separation by pI was limited to the range of 4–7 for a better resolution of sample proteins. We observed about 20 proteins that have specifically associated with SH3 domains of Nck-α.

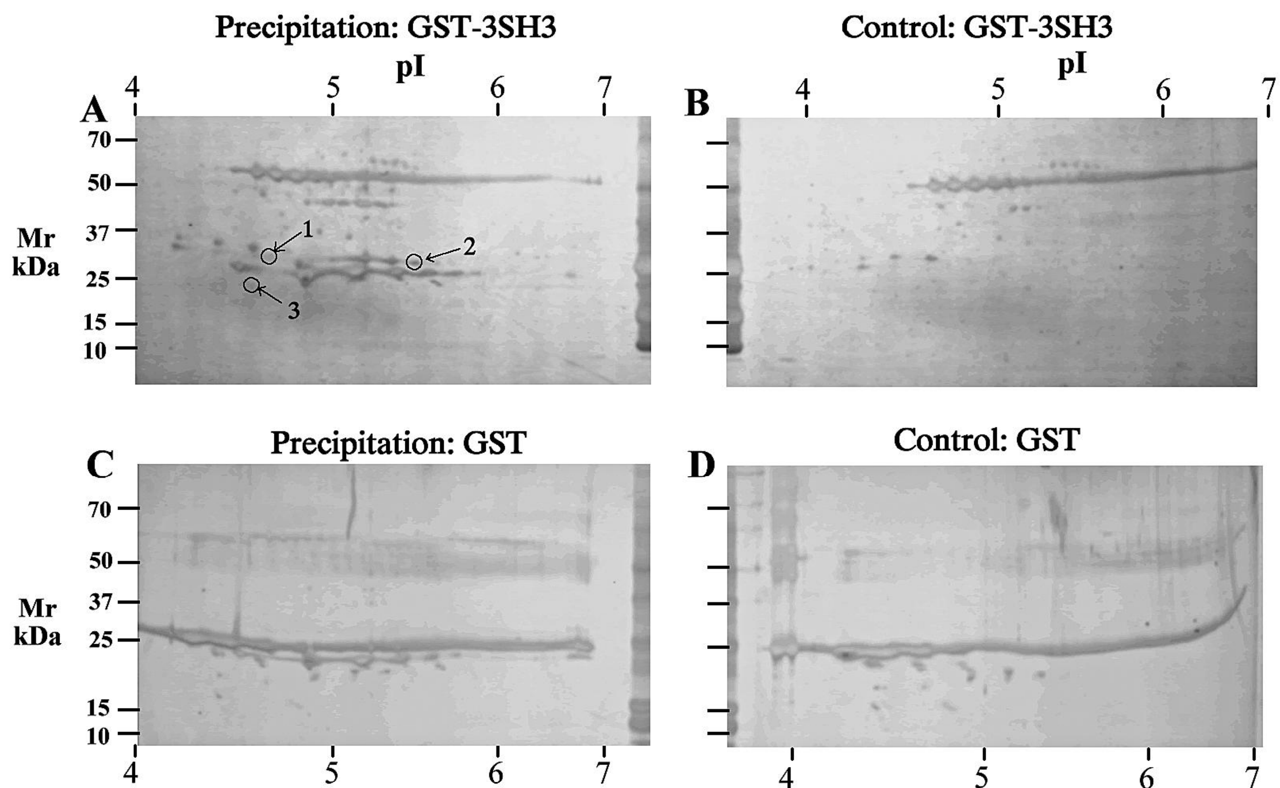


**Figure**. Nck-α SH3 domains-associated proteins
Quiescent cultures of HepG2 cells were lysed and cellular lysates were precipitated with Sepharose-immobilized GST-fused Nck-α SH3 domains (A) or GST only as a controle (C). Simultaneously, sepharose coupled with GST-3SH3 (B) or GST (D) was incubated in lysis buffer. Protein complexes were separated by 2D electroforesis and stained with silver nitrate.

**Protein analysis by MALDI-TOF mass spectrometry**. Due to the limited sensitivity of the mass spectrometer, we have analysed strong protein spots containing approximately no less than 1 pmol of a protein. Protein spots were cut out from the gel and subjected to in-gel tryptic digestion. Mass fingerprints of the peptide mixtures were obtained by MALDI-TOF mass spectrometry.

Peptide mass spectra were processed using Voyager Data Explorer software and analysed by PeptIdent program against Swiss-Prot protein database. Searches were performed using a maximum ±0.4 Da mass tolerance, no trypsin missed cleavages and allowing reduced cystein modification. For identification of a protein sequence coverage was set for at least 15% of the entire protein sequence. This led to the identification of three novel Nck-α-binding proteins: 14-3-3 protein gamma (14-3-3γ) (Figure A, spot 1), eukaryotic translation initiation factor 4E (eIF4E) (Figure A, spot 2) and ras-related protein Rab-30 (Figure A, spot 3). The major characteristics of newly identified proteins are presented in Table.

## DISCUSSION

Up to date, there are about 40 proteins known to associate with SH3 domains of Nck-α, which are involved in a wide variety of cellular functions, and their number is still growing. In this work, for the first time we have applied the proteomic approach to identify proteins interacting with Nck. The precipitation of Nck-binding proteins with recombinant Nck-α, unlike co-immunoprecipitation, makes it possible to avoid the problem of low antibody specificity and allows to obtain larger quantities of Nck-interacting proteins. However, the major problem of this assay is contamination with a recombinant bait protein, which can be eliminated by performing proper control experiments. In this work we have identified three novel Nck-associated proteins: 14-3-3γ protein, eukaryotic translation initiation factor 4E and ras-related protein Rab-30.

The family of 14-3-3 proteins consists of abundant and conserved polypeptides that are characterized by their ability to bind specific peptide motives phosphorylated on serine or threonine. The most common role for 14-3-3 proteins is allosteric regulation by stabilizing their binding partners in a particular conformation. Alternatively, they can restrict the subcellular localization of their binding partners [9]. Nck-α is known to be phosphorylated on both serine and threonine residues [10] and thus it has potential sites for binding 14-3-3 proteins. On the other hand, analysis by Scansite software (http://scansite.mit.edu/) shows that 14-3-3γ protein has a proline-rich motive which is potentially able to recruit Nck-α via its SH3 domains. Furthermore, 14-3-3γ isoform is known to interact with numerous proteins involved in the regulation of the actin cytoskeleton and Nck is also known for its major role in actin cytoskeleton, remodelling as well as linking cytoskeleton with cell surface receptors [9]. Several proteins identified as potential 14-3-3γ protein partners are also known to associate with Nck-α, for example, the Nck-associated protein NAP125 which is implicated in indirect activation of Arp2/3 complex by Nck [11], the SH3 adaptor protein SPIN90 participating in downstream signalling triggered by integrin β1A [12], and adaptor protein Cbl [13] acting as E3 ubiquitin ligase and a negative regulator of tyrosine kinases [14]. Thus, Nck-α may interact with 14-3-3γ directly or in a ternary complex which would include another protein as a mediator. Presumably, the Nck-α and 14-3-3γ complex is involved in actin cytoskeleton regulation.

The eukaryotic translation initiation factor 4E (eIF4E) is part of the eIF4F cap binding complex. Together with other subunits of eIF4F, the heterotrimeric factor eIF4E provides an efficient ribosomal attachment to capped mRNA [15]. Nck-α is known to associate with the other

Table. **Description of Nck-α-associated proteins identified by mass spectrometry**

| Spot | Identified Protein | Swiss-Prot Access Nr. | Mass, Da | | pI | | Number of peptides | % of sequence |
|---|---|---|---|---|---|---|---|---|
| | | | Expected | Measured | Expected | Measured | | |
| 1. | 14-3-3 protein gamma | P35214 | 28171 | 30000 | 4.8 | 4.7 | 5 | 18.3 |
| 2. | Eukaryotic translation initiation factor 4E | P06730 | 25097 | 27000 | 5.8 | 5.4 | 3 | 16.6 |
| 3. | Ras-related protein Rab-30 | Q15771 | 23000 | 24000 | 5.0 | 4.6 | 9 | 30.3 |

Spot – conventional number of the protein spot in question; identified protein – name of the protein identified; Swiss-Prot access No. – Swiss-Prot access number of the protein identified; mass expected – theoretical mass of the identified protein, mass identified – mass of the protein in question, identified by PAGE; pI expected – theoretical pI of the identified protein, pI identified – pI of the protein in question identified by PAGE; number of peptides – number of peptides of the protein in question identified by mass spectrometry as peptides belonging to the sequence of identified protein; % of the sequence – part of the sequence of identified protein covered by the above-mentioned peptides.

protein translation machinery component, eIF2 complex subunit, and is involved in the modulation of protein β translation [16]. We cannot exlude the possibility that interaction between eIF4E and Nck-α is indirect and occurs due to the 48S ribosomal complex formation. Nevertheless, if confirmed by other methods, the direct association between eIF4E and Nck-α will surggest a new molecular mechanism of the role of Nck-α in the modulation of protein translation.

Rab family small GTPases regulate vesicular protein transport and vesicule fusion in endocytosis, traficking, endosome fusion and exocytosis. There are 60 different Rab GTPases and each Rab has a distinct intracellular localization and function [17]. Rab 30 is associated with Golgi stacks, and the exact function of this GTPase is still to be investigated [18]. Our data showing an association between Nck-α and Rab-30 is the first evidence of the possible role of Nck in Golgi-mediated secretion.

Altogether we have identified three novel potential Nck-associated proteins: 14-3-3γ protein, eukaryotic translation initiation factor 4E and ras-related protein Rab-30. Although the further elucidation of the identified potential protein–protein interactions by biochemical methods is nessesary, the proteomic approach described in this study has proven to be suitable and efficient for identification of multiple proteins involved in diverse Nck-dependent celullar processes.

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### SU NCK-α BALTYMU SĄVEIKAUJANČIŲ BALTYMŲ PROTEOMINĖ ANALIZĖ

Santrauka

Į Nck baltymų šeimą įeina du SH2 ir SH3 domenus turintys adaptoriniai baltymai – Nck-α ir Nck-β, kurie yra svarbūs įvairiems ląstelės procesams: citoskeleto persitvarkymui, ląstelės morfologijos kaitai, genų ekspresijai bei baltymų transliacijos reguliavimui. Šiame darbe buvo atlikta su Nck-α SH3 domenais sąveikaujančių baltymų paieška. Baltymai buvo išskirstyti dvikrypte elektroforeze ir suskaldyti tripsinu iki atskirų peptidų, kurių masės buvo nustatytos MALDI-TOF masių spektrometrija, ir identifikuoti pagal peptidų mases. Aptikti nauji potencialūs, su Nck-α sąveikaujantys partneriai: 14-3-3γ baltymas, eukariotų baltymų transliacijos inicijavimo veiksnys 4E ir į ras GTPazę panašus baltymas Rab-30. Gauti duomenys patvirtina, kad Nck-α reguliuoja aktino citoskeleto persitvarkymo bei eukariotų baltymų transliacijos inicijavimą. Nck-α sąveika su Rab-30 rodo, kad Nck-α gali būti susijęs su Goldžio aparato veiklos reguliavimu.