VILNIUS UNIVERSITETY
INSTITUTE OF BIOCHEMISTRY

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# THE MECHANISM AND THE ROLE OF ADAPTOR PROTEIN NCK INTERACTION WITH P120 RAS GTPASE-ACTIVATING PROTEIN IN RAS REGULATION

Summary of doctoral thesis
Physical sciences, biochemistry (04 P)

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VILNIAUS UNIVERSITETAS
BIOCHEMIJOS INSTITUTAS

Marija Ger

# NCK ADAPTORINIO BALTYMO IR P120 RAS GTPAZĘ AKTYVINANČIO BALTYMO SĄVEIKOS MECHANIZMAS BEI VAIDMUO RAS AKTYVUMO REGULIAVIME

Daktaro disertacijos santrauka
Fiziniai mokslai, biochemija (04 P)

Vilnius, 2009

Disertacija rengta 2003-2009 metais Biochemijos institute, Vystymosi biologijos skyriuje
Disertacija ginama eksternu.

Mokslinis konsultantas:
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## Introduction

Communication between cells is the basic condition of the very existence of multicellular organisms. Normally cells undergo proliferation, differentiation or apoptosis only in response to respective biochemical stimuli, usually protein growth factors. The family of intracellular peripheral membrane small G-proteins of Ras are the crucial intracellular regulators of growth factor signaling. Ras proteins switches between two states: GTP-bound, which is the active state, and GDP-bound, inactive state. The effects of Ras are highly dependent on the dynamics of Ras activity. That is why the regulation, both activation and inactivation of Ras are very important processes. Ras is activated when Ras-bound GDP is exchanged to GTP. This process is regulated by Ras guanine nucleotide exchange factors (RasGEFs). Ras proteins possess GTPase domain and are able to hydrolyze GTP to GDP on their own. But the internal GTPase activity of Ras is very weak. Ras GTPase-activating proteins (RasGAPs) interact with active Ras and by enhancing intrinsic Ras GTPase activity promote Ras inactivation. RasGEFs and RasGAPs are regulated by various modifications, cofactors and interacting proteins.

The main negative regulator of Ras is p120 RasGAP. Though p120 RasGAP is the first of the RasGAPs discovered little is known about its regulation.

Nck is the family of two adaptor proteins, Nck1 and Nck2. Nck proteins consist of three consecutive Src homology 3 (SH3) domains followed by C-terminal SH2 domain. SH2 domains associate with specific phosphotyrosine-containing sites. SH3 domains bind proline-rich motives, and generally these interactions are phosphorylation-independent. The adaptor proteins are best known to promote protein-protein interaction but recent studies suggest that the role of adaptor proteins may be far more complicated.

## The aim of the dissertation work

The main aim of the current research was to elucidate the mechanism of the complex formation between adaptor protein Nck and p120 Ras GTPase-activating protein (RasGAP) and to determine the role of this interaction in the regulation of Ras activity. The following tasks were formulated:

1. Investigate the association between Nck proteins and RasGAP in different cell lines.

2. Study the colocalization of the adaptor protein Nck1 and RasGAP in the cell.

3. Elucidate the mechanism of complex formation between adaptor protein Nck1 and RasGAP.

4. Investigate the effect of cell detachment on the association between Nck1 and RasGAP.

5. Evaluate the role of Nck and RasGAP complex formation on RasGAP activity in vitro and H-Ras activity in the cell

**Scientific novelty**

Ras activity is strictly controlled in time and space through the modulation of positive and negative Ras regulators. Therefore, Ras regulators are potential targets for oncogenic Ras inhibition. There are different mechanisms of GAPs and GEFs activity regulation including protein translocation, protein-protein interactions, second messengers and posttranslational modifications (reviewed by Bos JL et al, 2007).

Though p120 RasGAP is the first of the RasGAPs discovered little is known about its regulation. p120 RasGAP is involved not only in the regulation of Ras activity but also is the possible effector of Ras (Cailliau K et al, 2001). RasGAP is involved in regulation of apoptosis (Yang JY et al, 2005) and cell migration (Kulkarni S et al, 2000). There is a variety of proteins interacting with p120 RasGAP (reviewed by Pamonsinlapatham P, 2009). p120 RasGAP is known to be inhibited by interaction with tyrosine-phosphorylated $p62^{DOK}$ (Kashige N. et al, 2000) or with tyrosine-phosphorylated p190 RhoGAP (Moran M, 1991). Also RasGAP function is regulated by membrane translocation via protein-protein interactions, for example with annexin 6 (Grewal T. et al, 2006). The positive contribution of N-terminal p120 RasGAP domains for its catalytic activity has been demonstrated (Bryant S. et al, 1996) suggesting conformational impact of the N-terminal domains or positive regulation via protein-protein interactions.

We have shown that adaptor protein Nck1 interacts with p120 RasGAP (Ger M. et al, 2003) and increases RasGAP activity. On the other hand, Nck family proteins have been shown to interact with Ras positive regulator Sos and activate Ras-dependent gene

transcription (Hu Q. et al, 1995). Altogether, these facts suggest the new dual role of Nck in Ras activity regulation.

To sum up, the current research elucidates the mechanism of complex formation between p120 RasGAP and Nck1. The data reveals a new role of Nck-1 adaptor protein as the modulator of Ras activity.

**The defensive statements**

1. Nck family proteins form stable complex with p120 Ras GTPase-activating protein (p120 RasGAP).
2. Adaptor protein Nck1 and RasGAP colocalize within the cell.
3. The association between adaptor protein Nck1 and RasGAP is mediated most by the first and the third SH3 domains of Nck1 and the N-terminal proline-rich sequences of RasGAP. Nck1 SH3 domains associate with RasGAP directly *in vitro*.
4. The complex between adaptor protein Nck and RasGAP is dependent on the cell adhesion to the substrate.
5. Nck1 association with RasGAP enhances the catalytic activity of RasGAP.

**Dissertation contents.** The dissertation is written in Lithuanian and contains the following parts: Introduction, Literature Review, Materials and Methods, Results, Discussion, Conclusions, List of References (217 positions), List of Publications and Figures (27). Total 116 pages.

## MATERIALS AND METHODS

**Cell culture and preparation of cell lysates**. The HepG2 is the cell line of human hepatocellular carcinoma origin, HeLa is cervical carcinoma-derived cell line, NIH3T3 is mouse embryonic cell line, and Myo26 cells are rabbit myogenic cell line.
All cell lines 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 (Sigma). HepG2 cell line, devoid of endogenous PDGFR-β, was used for stable expression of PDGFR-β using the retrovirus expression vector pLXSn as described

previously (Van Lint, Ni et al. 1998). Cells expressing wild type PDGF receptor were named H-WT and the cells transfected with an empty pLXSn vector were named H-N. Cells were grown to 70-80% confluence and made quiescent by culturing in serum-free DMEM overnight. 50 ng/ml of PDGF-BB (Amgene) at 37 ºC for 5 min were used for PDGF stimulation. 2 mg/ml trypsin solution with 1 mM of EDTA for 5 min at room temperature was used for cell detachment. 25 $cm^2$ flasks coated with 1 µg/ml fibronectin (Sigma) were used to re-plate cells after detachment. After the respective treatment cells were washed three times with ice-cold PBS and lysed in $EB^{++}$ buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM PMSF, aprotinin 20 nM, 2 mM $NaVO_4$). Lysates were cleared by centrifugation at 20.000×g for 15 min at 0°C.

**Expression constructs, antibodies**. pRK5-HA-Nck1 expression vectors containing wild type Nck1 cDNA or Nck1 cDNA with inactivating point mutations in SH3 or SH2 domains: Nck-SH3-I- (K38 mutation; impaired SH3-I domain), Nck-SH3-II- (K143; impaired SH3-II domain), Nck-SH3-III- (K229; impaired SH3-III domain), Nck-SH2- (K308; impaired SH2 domain) and Nck-$(SH3)_3$ (K38,143,229; impaired all SH3 domains) mutants (Fig. 1A), and anti-Nck2 antibody were provided by W. Li (2000, W Chen et al). Other pRK5-HA-Nck1 constructs: Nck-SH3-I+ (K143,229,308; only SH3-I domain left functional), Nck-SH3-II+ (K38,229,308; only SH3-II domain left functional), Nck-SH3-III+ (K38,143,308; only SH3-III domain left functional) and Nck-0 (all domains impaired) were made in our laboratory by cloning the restriction fragments between pRK5-HA-Nck1-SH2- and respective SH3 domain mutants (Fig. 1B). pGex2T-Nck1-SH2- and pGex2T-Nck1- Nck-$(SH3)_3$ vectors were constructed by cloning the restriction fragments of pRK5-HA-Nck1 respective vectors into pGex2T-Nck1-WT plasmid. pGex2T-RBD and pGEX2T-Nck2 plasmid was obtained from L.A. Quilliam . The Nck, GST and RasGAP rabbit polyclonal antisera were raised in our laboratory. Nck1 and HA monoclonal antibodies were purchased from Santa Cruz Biotechnology. Nck and Ras monoclonal antibodies were purchased from BD Transduction Laboratories.

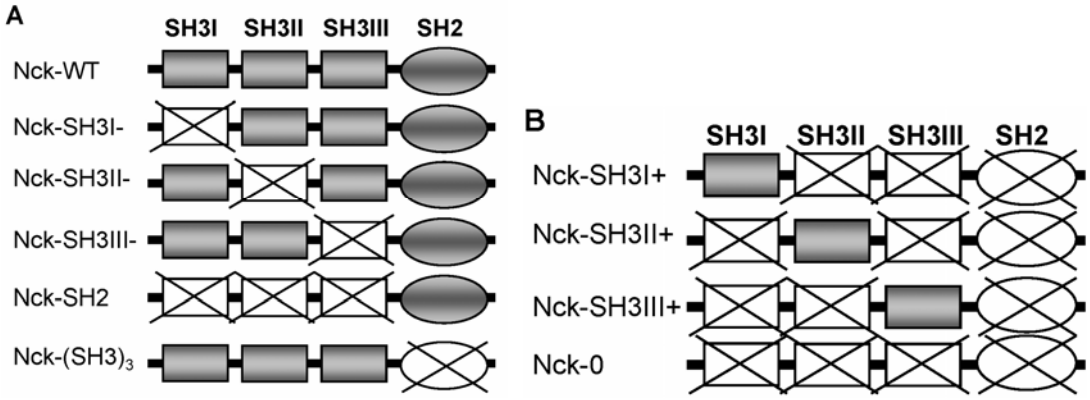


Fig. 1. **The schemes and the nomenclature of Nck1 point mutants**. A. Nck1 point mutants obtained from W. Li in pRK5-HA vector. B. Nck1 point mutants constructed in our laboratory.

**Immunoprecipitation and Western blot analysis**. The titers of antibodies used in immunoprecipitation experiments were: Nck1 – 1:50, Nck2 – 1:100, HA – 1:250, RasGAP – 1:100. Postnuclear lysates were incubated with antibody indicated in

experiment for 2 h at 4 °C. The immune complexes were collected with protein A Sepharose (GE Healthcare) for rabbit antibodies or protein G Sepharose (GE Healthcare) for mouse antibodies for 1 h at 4 °C mixing continuously. The immunoprecipitates representing about $10^6$ cells were washed five times with lysis buffer, boiled in 50 µl of 1X Sample buffer and fractionated by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad), blocked in Blotto (0.9% NaCl, 8mM Tris HCl, 2mM Tris, 1% skimmed milk, 0.025% Tween-20, 0.05% NaN3), blotted for 2 h with primary antibody and for 0.5 h with alkaline phosphatase-conjugated secondary antibody (Sigma). Blots were developed using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate tolidium salt (Roth).

**Generation of GST-fusion proteins and precipitation assay**. GST-fused proteins were expressed in BL21 strain of *E. coli* and purified using Glutathione Sepharose 4B (GE Healthcare) according the manufacturer's instructions. For RasGAP activation assay GST was cleaved from GST-Nck proteins by Thrombin protease (GE Healthcare) and removed by incubation with Glutathione Sepharose 4B for 2 hours. GST-Nck precipitation assays were carried out using 10 µg of GST or GST-fusion Nck proteins immobilised on Glutathione Sepharose 4B beads per sample. Beads with immobilised GST-fusion proteins or GST were incubated with postnuclear lysates for 1 h at 4 ºC mixing continuously. Then the beads were washed five times with $EB^{++}$ buffer, boiled in 50 µl of 1X Sample buffer and fractionated by SDS-PAGE. RasGAP co-precipitated with GST-Nck fusion proteins was assayed by Western blot as described above.

**Transient transfection**. H-WT cells in 25 $cm^2$ flasks were transfected with 10 µg of the indicated vectors using standard calcium phosphate precipitation method. 24 h after the transfection cells were starved in serum-free DME medium overnight and lysed. Postnuclear lysates were used for further analysis.

**Far Western blot**. Lysates from resting H-WT cells were immunoprecipitated with RasGAP antibodies. Immunoprecipitates were separated by SDS-PAGE electrophoresis, transferred to PVDF membrane and blotted for 3 hours with 10 µg/ml of GST or GST-fused Nck1 wild type or mutant proteins in Far Western buffer (22 mM HEPES pH7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl, 1% BSA, 0.05% NP-40, 1 mM DTT). The membrane was incubated with GST antibody for 1.5 hours and probed with alkaline phosphatase-conjugated secondary antibody for 0.5 hour (Sigma, USA). Blots were developed using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate tolidium salt (Roth).

**RasGAP activity assay**. Lysates from resting H-WT cells were immunoprecipitated with RasGAP antibodies as described above. Immunoprecipitates were washed three times with $EB^{++}$ buffer and incubated or not with recombinant wild-type Nck1 without GST for 1 hour. Recombinant H-Ras protein (Sigma) was preloaded with 0.1 mM GTP in Ras loading buffer (20 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1mM DTT, 50µg/ml BSA, 100 mM NaCl) for 45 min and incubated with or without RasGAP immunoprecipitates. The level of the active H-Ras was evaluated by precipitating H-Ras with 20 µg of GST-fused Ras-binding domain of Raf1 (GST-RBD) and analysing GST-RBD-bound H-Ras by Western blot with antibodies against H-Ras as described above.

**Over-expressed HA-H-Ras activity assay**. H-WT cells were transfected with an empty pcDNA3 vector or pcDNA3 vector expressing HA tagged H-Ras (HA-H-Ras). Cells were made quiescent by culturing in serum-free DMEM overnight. After the 48 hours of transfection cells were left adherent, or detached, or treated with 50 ng/ml of PDGF-BB

as indicated and lysed. The postnuclear lysates were used for precipitation with 20 µg of GST-RBD. GST-RBD-bound HA-H-Ras was analysed by Western blot with antibodies against HA epitope.

All the experiments described in this study have been repeated at least three times with similar results.

## RESULTS

**The association of adaptor proteins Nck with RasGAP**. We have previously shown that the recombinant protein Nck1 is able to associate with RasGAP (Ger M. et al, 2003). This association was independent of the cell stimulation with growth factors. We tested if endogenous Nck1 and Nck2 proteins form complex with RasGAP in cells. Nck1 and Nck2 were immunoprecipitated from quiescent cultures of resting or stimulated for 5 min with 50 ng/ml PDGF H-WT cells. The immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane, and the appropriate portion of the membrane was subjected to anti-RasGAP or anti-Nck Western blot (Fig. 2). The RasGAP Western blot showed that both Nck1 (lanes 2-3) and Nck2 (lanes 4-5) in resting and PDGF-stimulated cells recruited a similar amount of RasGAP. The Nck blot showed that there was a comparable level of Nck immunoprecipitated from all the samples. No Nck or RasGAP were detected in the control sample without Nck antibodies (lane 1).

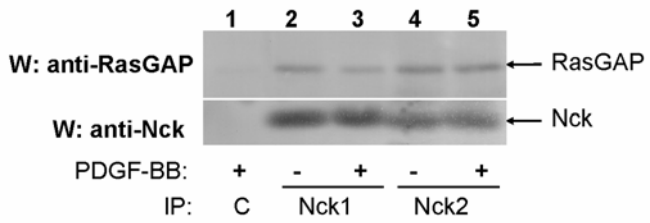


Fig. 2. **The adaptor proteins Nck form complex with RasGAP**. Quiescent cultures of H-WT cells were left resting or stimulated for 5 min at 37°C with 50 ng of PDGF-BB per ml and then lysed. The lysates were immunoprecipitated with anti-Nck1 (lanes 2-3) or anti-Nck2 (lanes 4-5) antibodies, immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was subjected to Western blot analysis with the antibodies indicated in the left-hand margin. No Nck or RasGAP were detected in control immunoprecipitates (C; lane 1).

In addition we checked if both recombinant GST-fused Nck (GST-Nck) proteins bound the similar amount of RasGAP. GST-Nck1 and GST-Nck2 were coupled to glutathione sepharose beads and used in precipitation assay from resting or PDGF-stimulated HepG2 cells transfected with an empty pLXSN vector (H-N) or pLXSN

vector expressing wild type PDGF receptor-β (H-WT). Co-precipitated proteins were resolved by SDS-PAGE, and RasGAP was detected by Western Blot assay (Fig. 3). RasGAP was present in similar amounts in precipitates from both GST-Nck1 (lanes 3-6) and GST-Nck2 (lanes 7-10) independently of PDGF receptor expression or stimulation with PDGF-BB. No RasGAP were detected in GST alone precipitates (lanes 1-2). The amounts of GST, GST-Nck1 and GST-Nck2 stained by Coomasie blue were similar (data not shown).

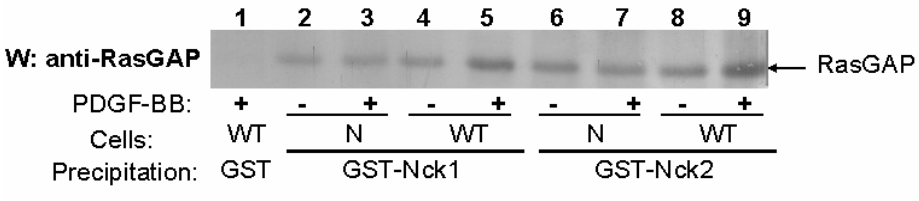


Fig. 3. **The recombinant Nck proteins bind to RasGAP**. H-N or H-WT cells were either treated or not with PDGF and the postnuclear lysates were subjected to precipitation assay with glutathion sepharose-immobilised GST (lanes 1-2), or GST-Nck1 (lanes 3-6), or Nck2 (lanes 7-10). Bead-bound proteins were eluted in electroforesis sample buffer and analysed by SDS-PAGE electroforesis and subsequent Western imunoblotting using antibodies against RasGAP.

We have shown that Nck1 and Nck2 interact with RasGAP similarly and concentrated further research on Nck1. To determine whether the Nck1 and RasGAP association is not HepG2 cell-specific phenomenon we immunoprecipitated Nck1 from other cell lines (Fig. 4). Resting HepG2 (lanes 1-2), HeLa (lanes 3-4), Myo26 (lanes 5-6) or NIH3T3 (lanes 7-8) cells were lysed and Nck1 was immunoprecipitated.

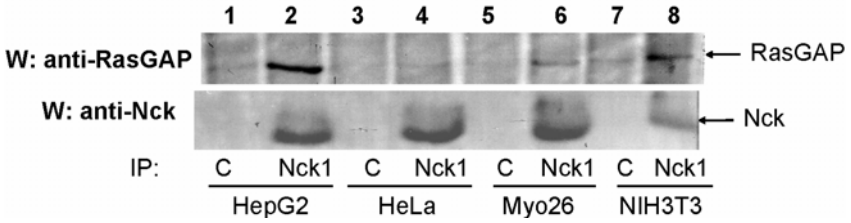


Fig. 4. **Nck1 and RasGAP form complex in different cell lines**. Quiescent cultures of H-WT (lanes 1-2), HeLa (lanes 3-4), Myo26 (lanes 5-6), NIH3T3 (lanes 7-8) cells were used for immunoprecipitation with antibody against Nck1 (Nck1) or without (C). Immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF membrane and the membrane was subjected to Western blot analysis with the antibodies indicated in the left-hand margin.

Simultaneously the control immunoprecipitation without Nck1 antibody was performed. The immunoprecipitates were subjected to anti-RasGAP or anti-Nck Western blot analysis. RasGAP was present in immunoprecipitates from all cell lines. The

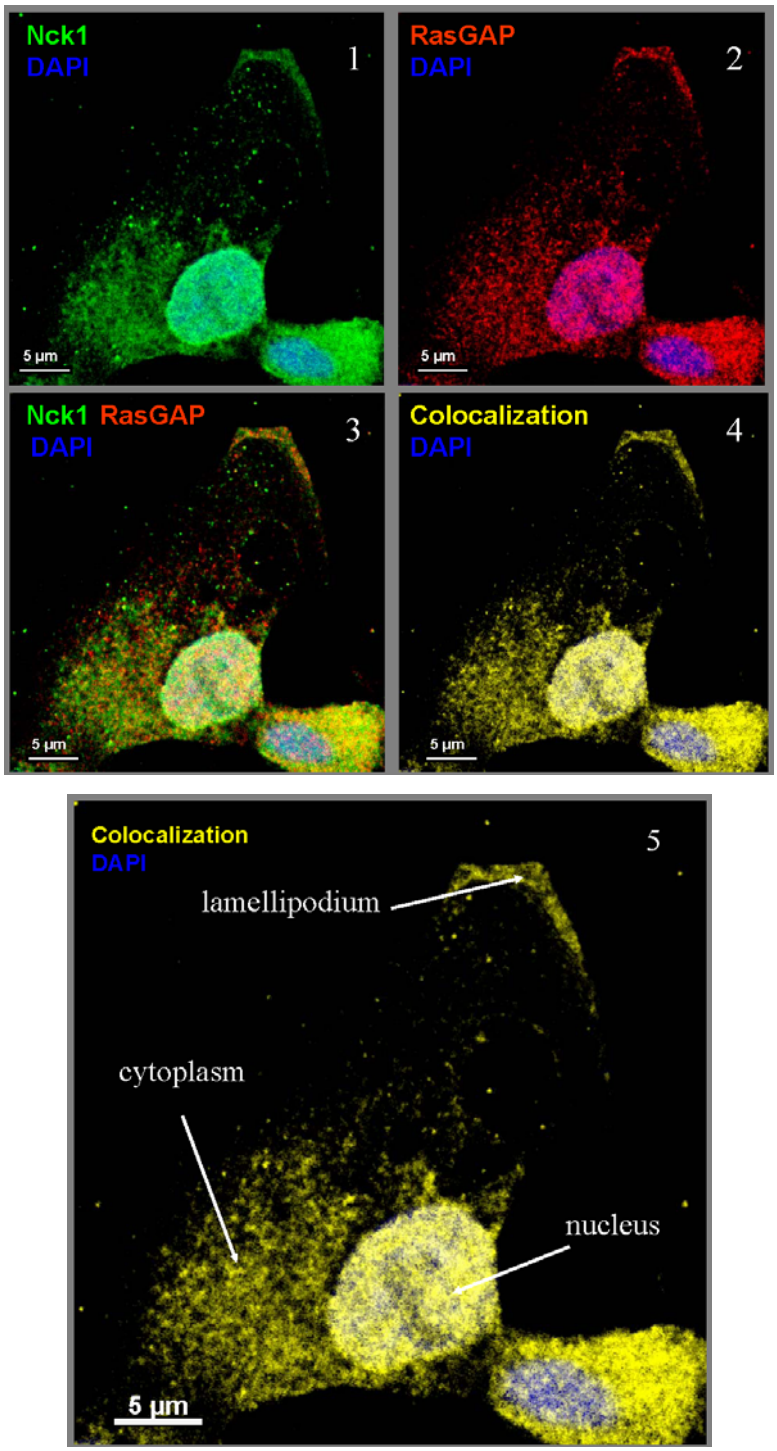


Fig. 5. **Nck1 and RasGAP colocalize within the cell**. Growing H-WT cells have been fixed and stained with specific antibodies against Nck1 (image 1, green) and RasGAP (image 2, red), also DNA stained with DAPI (blue). Samples have been scanned with laser scanning microscope Nikon C1. Image 3 represents the staining patterns of both Nck1 and RasGAP. Colocalization pattern of Nck1 and RasGAP generated by Imaris image analysis program is displayed in yellow (image 4). Nck1 and RasGAP are colocalised in cytoplasm, nucleus and lamellipodia (image 5). The scale size is 5 µm.

differences in RasGAP amount in the immunoprecipitates were due to different Nck1 and RasGAP expression levels in the cell lines (data not shown).

**Colocalization of Nck1 and RasGAP proteins**. The interaction between two proteins in the cell is possible only if the proteins are localized in the same compartment of the cell. To establish the possibility of interaction between Nck1 and RasGAP we analysed the colocalization of Nck1 and RasGAP in the cell. H-WT cells were fixed, Nck1 and RasGAP proteins were detected with specific monoclonal antibodies. Antibodies against Nck1 were conjugated with Alexa 488 green fluorescent dye, antibodies against RasGAP were conjugated with Alexa 594 red fluorescent dye. The nuclei were stained with DNA specific blue fluorescent dye 4,6-diamidin-2-fenilindol (DAPI). Stained samples were visualized with laser scanning confocal microscope Nikon C1.

Data in the Fig.5 indicate that both Nck1 (image 1, green) and RasGAP (image 2, red) proteins are present in cell cytoplasm and in the nucleus. The patterns of both Nck1 and RasGAP are shown in the image 3. Image 4 displays the colocalization pattern of Nck1 and RasGAP calculated by image analysis software Imaris.

Nck1 and RasGAP are co-localized in the nucleus, cytoplasm (no exact cell structure were identified) and in the lamellipodia (image 5).

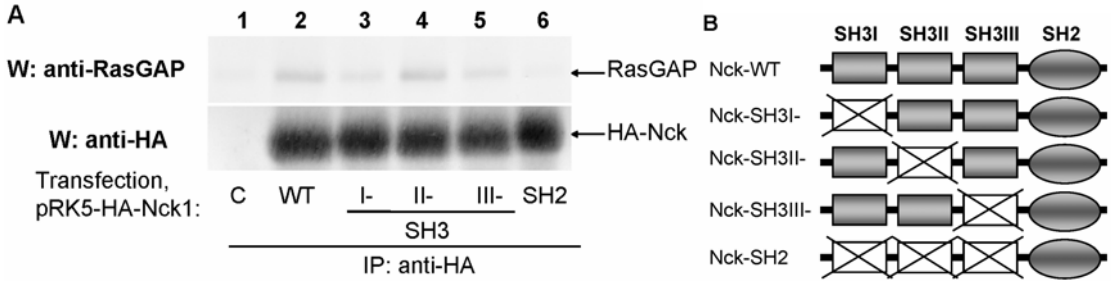


Fig. 6. **The mutations of the first and of the third domains of Nck1 diminish Nck1 association with RasGAP**. H-WT cells were mock-transfected (mock; lane1) or transfected with pRK5 vector expressing HA-tagged wild type Nck1 (WT; lane 2), or Nck1 with inactivating point mutations the first (I-; lane 3), or the second (II-; lane 4), or the third (III-; lane 5) SH3 domains, or Nck1 with inactivating point mutations in all three SH3 domains (SH2; lane 6). HA-tagged Nck1 proteins immunoprecipitated with anti-HA antibody. Immunoprecipitates were analysed by Western blot with the antibodies indicated in the left-hand margin.

**The first and the third SH3 domains of Nck1 are responsible for the interaction with RasGAP**. To elucidate the mechanism by which Nck1 interacts with RasGAP we have compared the ability of various Nck1 functional mutants to form

complexes with RasGAP. Nck1 has three SH3 domains and one SH2 domain. To test which domain or domains are responsible for the association with RasGAP we expressed HA-Nck1 with point mutations in the first, or the second, or the third, or all SH3 domains in the H-WT cells (Fig. 6B). The amount of RasGAP in HA-Nck1 immunoprecipitates was tested by the Western blot (Fig. 6A). Similar amount of RasGAP was detected in wild type HA-Nck1 immunoprecipitates (lane 2) and in the immunoprecipitates of HA-Nck-1 with mutated second SH3 domain (lane 4). HA-Nck1 with mutated first (lane 3) or third (lane 5) SH3 domain bound reduced amount of RasGAP compared to wild type HA-Nck1. HA-Nck1 mutant with impaired all three SH3 domains (lane 6) did not interact with RasGAP. No HA-Nck1 or RasGAP were detected in immunoprecipitates from mock-transfected cells (lane 1).

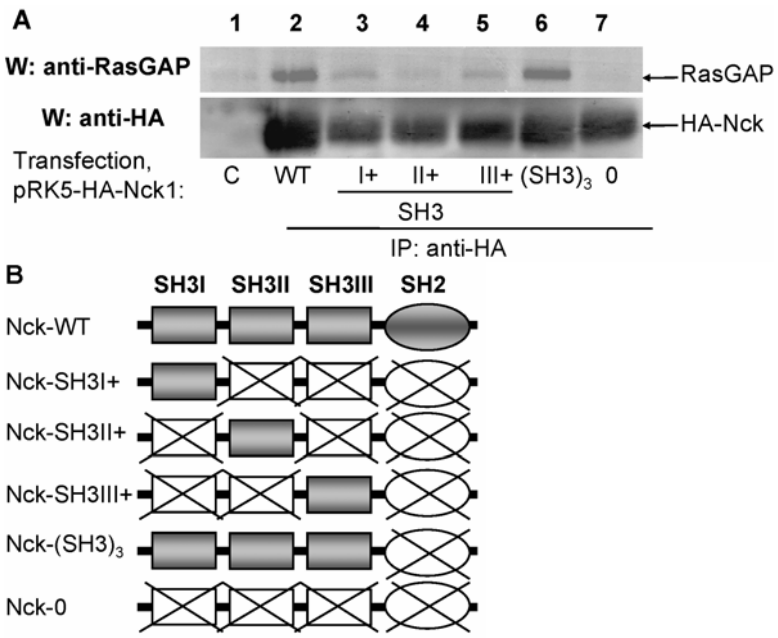


Fig. 7. **The first and the third domains of Nck1 associate with RasGAP.** H-WT cells were mock-transfected (mock; lane1) or transfected with pRK5 vector expressing HA-tagged wild type Nck1 (WT; lane 2), or Nck1 with inactivating point mutations in SH2 and all SH3 domains except the first (I+; lane 3), or the second (II+; lane 4), or the third (III+; lane 5) SH3 domains, or Nck1 with inactivating point mutations in SH2 domain ($(SH3)_3$; lane 6), or in all four SH2 and SH3 domains (0; lane 7). HA-tagged Nck-1 proteins immunoprecipitated with anti-HA antibody. Immunoprecipitates were analysed by Western blot with the antibodies indicated in the left-hand margin.

After determining which Nck1 mutations reduce Nck-1 association with RasGAP we tested which SH3 domains on their own are capable to interact with RasGAP. We expressed in H-WT cells HA-Nck1 with point mutations in all domains except for the first, second or the third SH3 domains, respectively, or all four Nck domains impaired

(Fig. 7B). The amount of RasGAP in HA-Nck1 immunoprecipitates was tested by the Western blot (Fig. 7A). The HA-Nck1 with functional first (lane 3) or third (lane 5) SH3 domains were capable to bind RasGAP though to a lesser extent than HA-Nck1 with all three SH3 domains intact (lane 6) or wild type HA-Nck1 (lane 2). Only traces of RasGAP were detectable in immunoprecipitates of HA-Nck1 with the second intact SH3 domain (lane 4) or with all domains impaired (lane 7). No HA-Nck1 or RasGAP were detected in immunoprecipitates from mock-transfected cells (lane 1).

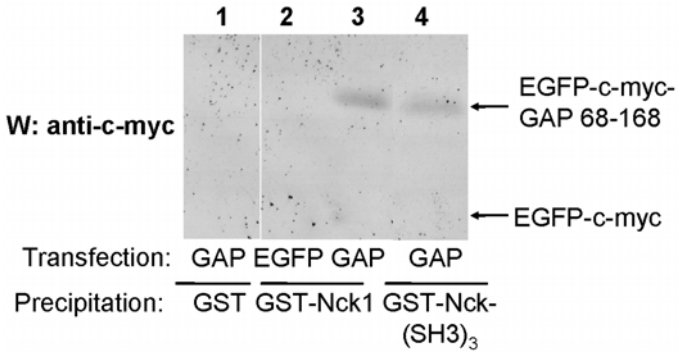


Fig. 8. **RasGAP N-terminal proline-rich sequence binds SH3 domains of Nck1.**
EGFP-c-myc and EGFP-c-myc RasGAP 68-168 proteins were expressed in H-WT cells and precipitated with GST, wild-type GST-Nck1 or GST-Nck-$(SH3)_3$ recombinant proteins. The amount of EGFP-c-myc or EGFP-c-myc-RasGAP 68-168 on precipitates was tested by Western blot against c-myc epitope. Lane 1 – precipitation with GST, lanes 2-3 – precipitation with GST-Nck1-WT, lane 4 – precipitation with GST-Nck-$(SH3)_3$. EGFP – precipitation from EGFP-c-myc expressing cells; GAP – precipitation from EGFP-c-myc-RasGAP 68-168 expressing cells.

SH3 domains are known to bind specific proline-rich motives. RasGAP has several proline-rich motives between amino acids 135 and 145. We cloned the sequence of RasGAP coding amino acids 68-168 (named RasGAP N-terminal proline-rich sequence) into the pEGFP-C2 eukaryotic transfection vector with c-myc epitope. The pEGFP-c-myc-RasGAP 68-168 plasmid was transfected into H-WT cells, the cells were lysed and the lysates used for the precipitation with GST, GST-Nck1-WT or GST-Nck-$(SH3)_3$ proteins. C-myc tagged proteins in the precipitates were analysed by Western blot with antibody against c-myc epitope (Fig. 8). Both Nck1, Nck1-WT (lane 3) and Nck1 mutant with all SH3 domains left intact (lane 4) have bound to EGFP-c-myc-RasGAP 68-168 protein. The EGFP-c-myc protein has not associated with Nck1-WT (lane 2). GST alone did not precipitate EGFP-c-myc-RasGAP 68-168 protein. Therefore, data show that the N-terminal proline rich sequence of RasGAP associates with SH3 domains of Nck1.

In summary, data show that the association between adaptor protein Nck1 and RasGAP is mediated most by the first and the third SH3 domains of Nck1 and RasGAP N-terminal proline-rich sequences.

**Nck1 and RasGAP associate directly *in vitro***. To determine whether SH2 domain or SH3 domains of Nck1 are responsible for the direct association with RasGAP we immunoprecipitated RasGAP from resting H-WT cells and subjected immunoprecipitates to Far Western assay with wild type GST-Nck1 or with GST-Nck1 mutants with intact SH2 or intact all three SH3 domains (Fig. 9). Wild type Nck1 (lane 2) and Nck1 with intact SH3 domains (lane 3), but not Nck-1 with only SH2 domain left intact (lane 4), were able to bind RasGAP. GST alone did not bind RasGAP (lane 1).

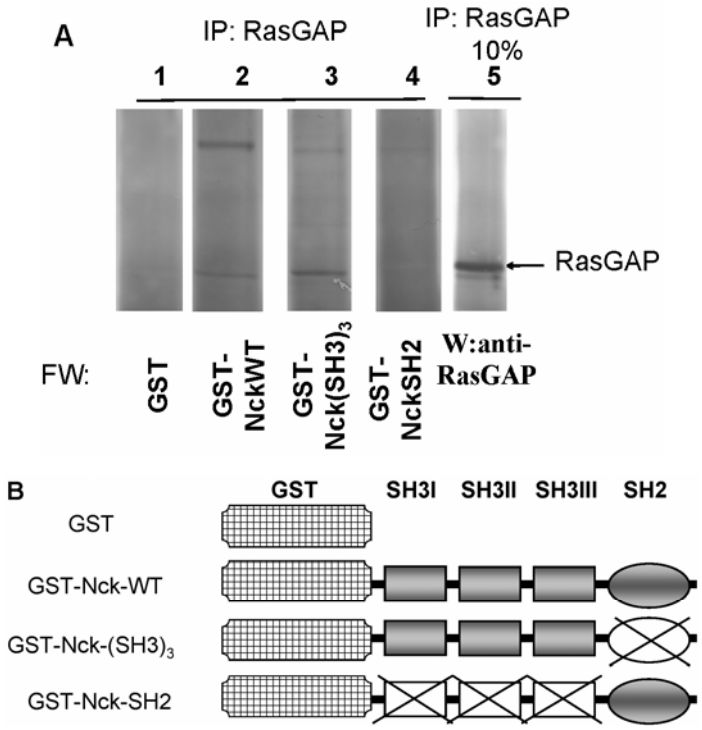


Fig. 9. **SH3 domains of Nck1 associate with RasGAP directly**. The lysates of H-WT cells were used for immunoprecipitation with anti-RasGAP antibody. The immunoprecipitates were subjected to Far Western blotting using recombinant glutathion-S-transferase (GST; lane 1) or GST-fused wild type Nck1 (GST-NckWT; lane 2) or Nck1 with inactivating point mutations in SH2 domain (GST-Nck$(SH3)_3$; lane 3) or in all three SH3 domains (GST-NckSH2; lane 4). Ten percent of the immunoprecipitate were subjected to Western blot with anti-RasGAP (antiGAP; lane 5) for a positional control

**Nck1 association with RasGAP depends on cell adhesion to the substrate**. Both Nck1 (Buday, Wunderlich et al. 2002) and RasGAP (Kulkarni, Gish et al. 2000) proteins are known to take part in the regulation of the actin skeleton rearrangement. Cell

adhesion to the substrate is one of the major actin cytoskeleton-associated events. To test whether the cell adhesion changes the amount of Nck1-bound RasGAP we immunoprecipitated Nck1 from adherent H-WT cells (Fig. 10, lane 2) or cells detached from the substrate with trypsin/EDTA solution (lane 3), as well as H-WT cells detached and re-plated on fibronectin for 0.5 (lane 4), 1 (lane 5), 2 (lane 6), 3 (lane 7), 4 (lane 8), or 5 (lane 9) hours. The immunoprecipitates of Nck1 were subjected to anti-RasGAP or anti-Nck Western blot analysis. Only the traces of RasGAP in complex with Nck1 (upper panel) were detectable after the cell detachment (lane 3), compared to undetached adherent cells (lane 2). Notably, the level of Nck1 and RasGAP complex did not increase (lanes 4-8) until the fifth hour of re-plating (lane 9). The amount of Nck1 in all the immunoprecipitates was similar (lower panel). No Nck or very little of RasGAP were detected in the control sample without Nck antibodies (lane 1).

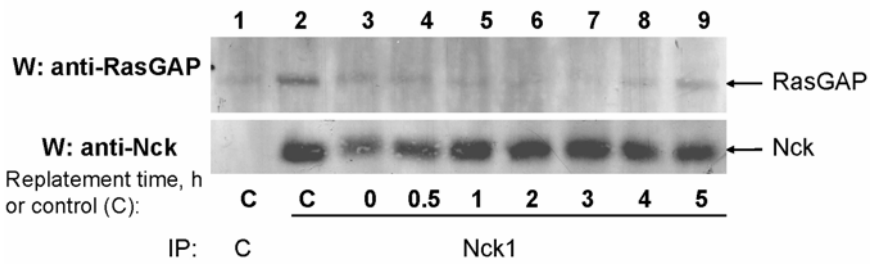


Fig. 10. **Nck1 association with RasGAP depends on cell adhesion to the substrate.** H-WT cells were left attached (lane 2) or detached with trypsin-EDTA solution (lane 3), and replated for 0.5 (lane 4), 1 (lane 5), 2 (lane 6), 3 (lane 7), 4 (lane 8) or 5 (lane 9) hours, and lysed. The lysates were immunoprecipitated with anti-Nck1 antibody, immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was subjected to Western blot analysis with the antibodies indicated in the left-hand margin. No Nck or RasGAP were detected in control immunoprecipitates (C; lane 1)

The next step was to elucidate which one of the proteins, Nck1 or RasGAP or both, is (are) responsible for the complex dissociation in detached cells.

First, we tested the capability of RasGAP in adherent and detached cells to bind recombinant wild-type GST-Nck1. GST-Nck1 was coupled to glutathione Sepharose beads and was used to precipitate RasGAP from adherent or detached H-WT cell lysates, as well as from cell lysates obtained from detached and subsequently re-plated on fibronectin for 4 or 7 hours cells. Co-precipitated proteins were resolved by SDS-PAGE, and RasGAP was detected by Western Blot assay. The RasGAP associated with GST-Nck1 in adherent (Fig. 11, lane 2) but not in detached (lane 3) or re-plated for 4 h (lane 4)

cell lysates. RasGAP association with GST-Nck1 was partly restored after the 7 h of re-plating (lane 5) that correlates with Nck1 and RasGAP complex dynamics in re-plated cells tested by Nck1 immunoprecipitation (Fig. 11). No RasGAP were detected in GST alone precipitates (lane 1). The amounts of GST-Nck1 in all the samples stained by Coomasie blue were similar (lower panel).

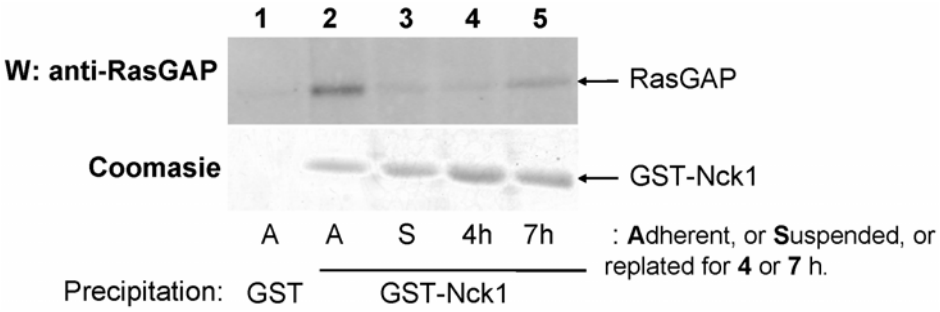


Fig. 11. **RasGAP after the cell detachment from the substrate does not bind to Nck1.** H-WT cells were were left attached (lanes 1-2) or detached with trypsin-EDTA solution (lane 3), and allowed to reattach for 4 (lane 4) or 7 (lane 5) hours, and lysed. The postnuclear lysates were subjected to precipitation assay with glutathion sepharose-immobilised GST (lane 1), or GST-Nck1 (lanes 2-5). Bead-bound proteins were eluted in electroforesis sample buffer and analysed by SDS-PAGE electroforesis and subsequent Western imunoblotting using antibodies against RasGAP. The GST-Nck1 was visualized with Coomasie Blue dye.

Next, we tested if Nck from detached cells was capable of association with RasGAP from adherent cells. We immunoprecipitated Nck1 from adherent or detached H-WT cell lysates. The Nck1 immunoprecipitates from adherent cells were incubated with the lysate of adherent cells (Fig. 12, lane 2). The Nck1 immunoprecipitates from detached cells were incubated with lysates of detached (lane 3) or adherent H-WT cells (lane 4). After the incubation the immunoprecipitates were subjected to anti-RasGAP and anti-Nck Western blot analysis. Data show that Nck1 from detached cells is still capable to bind RasGAP from adherent cell lysate (lane 4) but not from detached cell lysate (lane 3). The amount of RasGAP bound by Nck1 from detached cells in adherent cells lysate was lesser than the amount bound by Nck1 from adherent cells (lane 2). This difference may be explained by the stability of the pre-existing complexes between Nck1 and RasGAP in adherent cells. No Nck or RasGAP were detected in the control sample without Nck antibodies (lane 1).

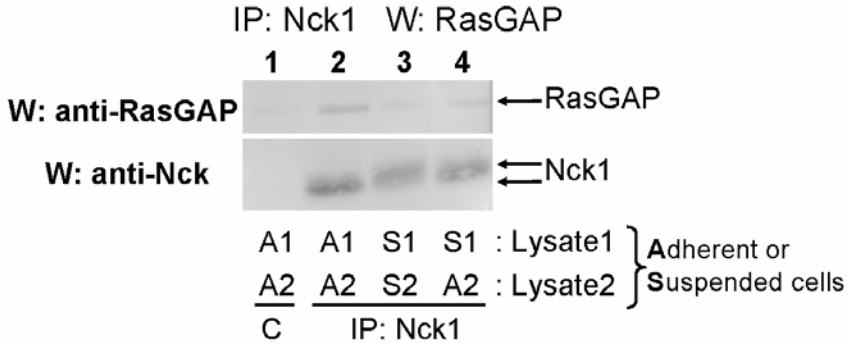


Fig. 12. **Nck1 after the cell detachment from the substrate binds RasGAP.** H-WT cells were left attached (A1) or detached (S1) with trypsin-EDTA solution and lysed. The lysates were immunoprecipitated with anti-Nck1 antibodies. The immunoprecipitates from the cells left attached (A1) were subjected to the second immunoprecipitation from the lysates of the attached (A2, lane 2) cells. The immunoprecipitates from the detached cells (S1) were subjected to the second immunoprecipitation from the lysates of the detached (S2, lane 3) or attached (A2, lane 4) cells. The immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was subjected to Western blot analysis with the antibodies indicated in the left-hand margin. No Nck or RasGAP were detected in control immunoprecipitates (C; lane 1).

Data suggest that RasGAP protein from the detached cells might be subjected to some kind of modification or structural changes, which diminishes dramatically its association with Nck1. To check this possibility we employed Far Western assay for RasGAP immunoprecipitated from adherent or detached cells to bind Nck1. In this assay RasGAP is denaturated by the SDS-PAGE step. Therefore, all structures or additional unknown RasGAP-binding proteins that might be involved in preventing RasGAP to bind Nck1 from detached cells should dissipate. On the contrary, if RasGAP undergoes chemical modification that displaces Nck1 binding from RasGAP in detached cells will be left intact in the Far Western and will be reflected as the diminution in RasGAP and Nck1 association.

Therefore, we immunoprecipitated RasGAP from adherent or detached H-WT cells and subjected immunoprecipitates to Far Western assay with wild-type GST-Nck1 (Fig. 13). GST-Nck1 was able to bind RasGAP from both adherent (A, lane 3) and detached (S, lane 4) cells. The amount of immunoprecipitates in both samples was similar (lanes 1-2). GST alone did not bind RasGAP (lane 5). So, we assume that RasGAP is not modified covalently on the Nck1-binding site. On the other hand, data suggest that upon cell detachment RasGAP might undergoes conformational changes that could lead to the complex dissociation.

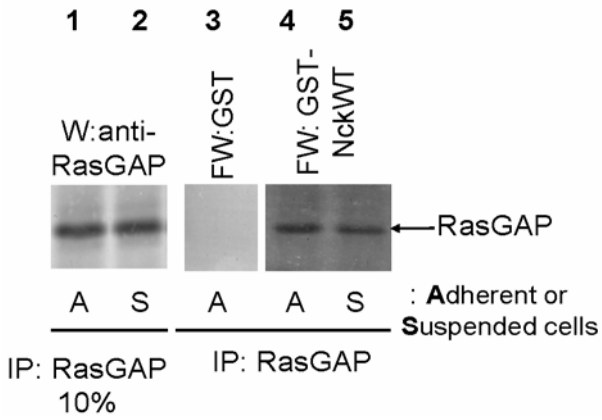


Fig. 13. **Denaturated RasGAP from detached cells binds Nck1.** The lysates of attached (A) or detached (S) H-WT cells were used for immunoprecipitation with anti-RasGAP antibody. The immunoprecipitates were subjected to Far Western blotting using recombinant GST-fused wild type Nck1 (GST-NckWT; lanes 3-4) or GST (lane 5). Ten percent of the immunoprecipitate were subjected to Western blot with anti-RasGAP (antiGAP; lanes 1-2) for a positional control.

**Nck1 association with RasGAP increases RasGAP catalytic activity towards H-Ras**. The main function of RasGAP is the activation Ras GTPase activity and the reduction of active GTP-bound Ras level. Therefore, we tested the possibility that Nck1 binding to RasGAP might affect the catalytic activity of RasGAP. To do this we immunoprecipitated RasGAP from H-WT cells and incubated immunoprecipitates with or without recombinant Nck1 *in vitro*. Recombinant H-Ras was preloaded with GTP and incubated with the RasGAP immunoprecipitates for 45 min. The amount of H-Ras-GTP complex left after the incubation with RasGAP was determined in the precipitation assay using GST and Ras-binding domain (RBD) of Raf-1 fusion protein which associates exclusively with GTP-bound H-Ras. The level of H-Ras in RBD precipitates have been evaluated by Western blot with anti-H-Ras antibodies (Fig. 14B, top panel). A similar amount of H-Ras was present in all the samples (middle panel). The analysis of active H-Ras level shows that the Nck1-preloaded RasGAP reduced the level of H-Ras-GTP (Fig. 14A, 3) more efficiently than RasGAP without Nck1 (Fig. 14A, 2). The RasGAP level in both samples was the same (Fig. 14B, bottom panel). Thus, data show that the association of Nck with RasGAP increases RasGAP catalytic activity *in vitro*.

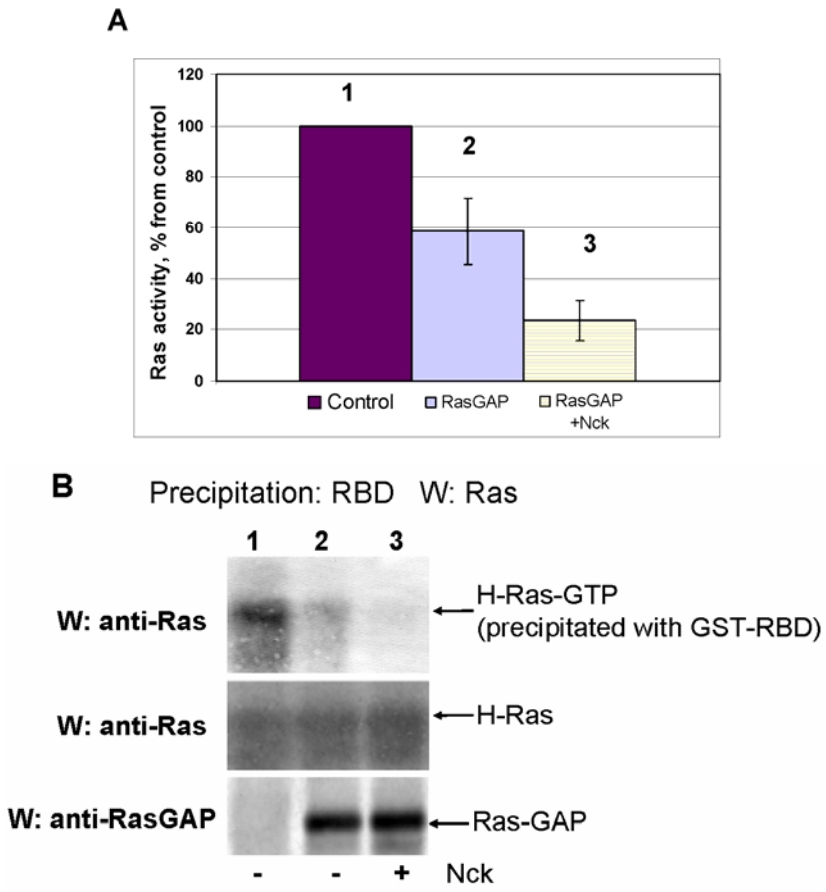


Fig. 14. **Nck1 association with RasGAP increases RasGAP catalytic activity towards H-Ras *in vitro***. The lysates of H-WT cells were used for immunoprecipitation with anti-RasGAP antibody. The immunoprecipitates were loaded or not with recombinant Nck1 and incubated with recombinant GTP-bound H-Ras. The catalytic activity of RasGAP was assayed by the amount of H-Ras-GTP left after the incubation without Nck1 (2), preloaded with Nck1 (3) or no RasGAP addition (1). The relative amount of GTP-bound H-Ras was measured by H-Ras ability to associate with GST-RBD and visualized by Western blot with anti-H-Ras antibodies. (A) The amount of active H-Ras was measured with Image J software and represented as percent from the amount of active Ras in sample incubated without RasGAP. (B) Corresponding Western blot. RBD-bound active H-Ras (top panel) and the total amount of H-Ras was visualized by Western blot with anti-H-Ras antibodies (middle panel). The amount of RasGAP without (-) or with (+) Nck-1 was visualized by Western blot with anti-RasGAP antibodies (bottom panel).

To show that Nck1 can play a role in Ras regulation in cell we tested how over-expression of Nck1 affected the level of Ras-GTP in H-WT cells. HA-H-Ras was over-expressed in H-WT cells with or without wild-type HA-Nck1. Quiescent cells were not treated or treated with PDGF-BB, lysed and the level of active HA-H-Ras was assayed by the precipitation with GST-RBD. The level of HA-H-Ras in RBD precipitates was analysed by Western blot with antibodies against HA epitope (Fig. 15). Data show that PDGF treatment increases Ras-GTP amount in cells with endogenous Nck1 level (Fig. 15 A and B). Unexpectedly, over-expression of Nck1 have resulted in

the increase of active Ras-GTP level. However, PDGF treatment of Nck1 over-expressing cells have not led to higher accumulation of Ras-GTP compared to cells with endogenous Nck1 level. Data show that although Nck1 over-expression alone increases the amount of active Ras, after the treatment with PDGF-BB the level of active Ras in both Nck over-expressing cells and cells with endogenous Nck1 level is similar. Therefore, Nck over-expression diminishes PDGF-BB-induced Ras activity.

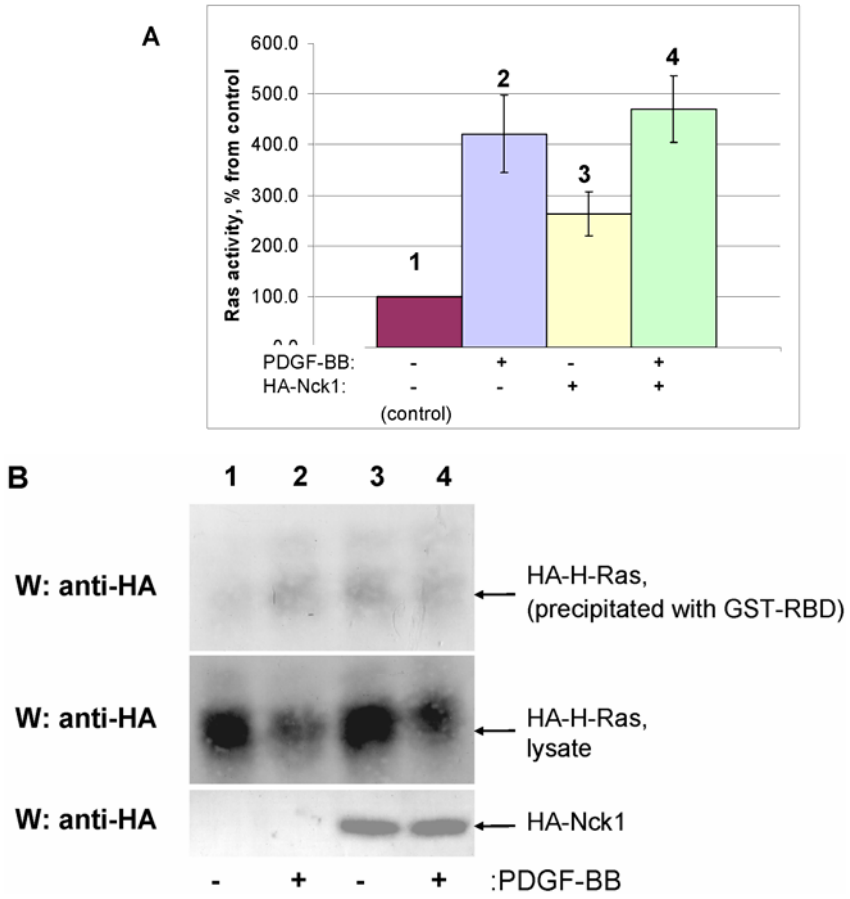


Fig. 15. **Nck1 over-expression affects Ras activity**. HA-H-Ras was co-transfected with or without HA-Nck1. The quiescent cells were treated or not with PDGF-BB and lysed. Active HA-H-Ras in the lysates was assayed by precipitation with GST-RBD and detected by Western blot with antibody against HA epitope. (A) The amount of active HA-H-Ras was measured with Image J software and represented as percent from the amount of active Ras in PDGF-untreated H-WT cells without Nck1. (B) Corresponding Western blot. Top panel – RBD-bound active HA-H-Ras, middle panel – the level of HA-H-Ras in lysates, lower panel – HA-Nck1 expression.

Since the complex between Nck1 and RasGAP dissociates upon cell detachment from the substrate we tested how Ras-GTP level changes in the detached cells. H-WT cells were transfected with pcDNA3 vector expressing HA-H-Ras for 48 hours, made quiescent and left adherent or suspended with trypsin/EDTA and lysed. Active HA-H-

Ras was precipitated with Sepharose-immobilized GST-RBD or GST as the blank control (Fig. 16B, lane 1) and visualized by Western blot with antibodies against HA epitope (Fig. 16B, upper panel). Data were evaluated with Image J image analysis software (Fig. 16A). As expected HA-H-Ras expression in all the samples was similar, as well as the amount of GST-RBD that was used for the precipitation (Fig. 16B). Data show that in the detached cells Ras activity is elevated compared to the adherent cells. Such increase correlates with the dissociation of the complex between Nck1 and RasGAP.

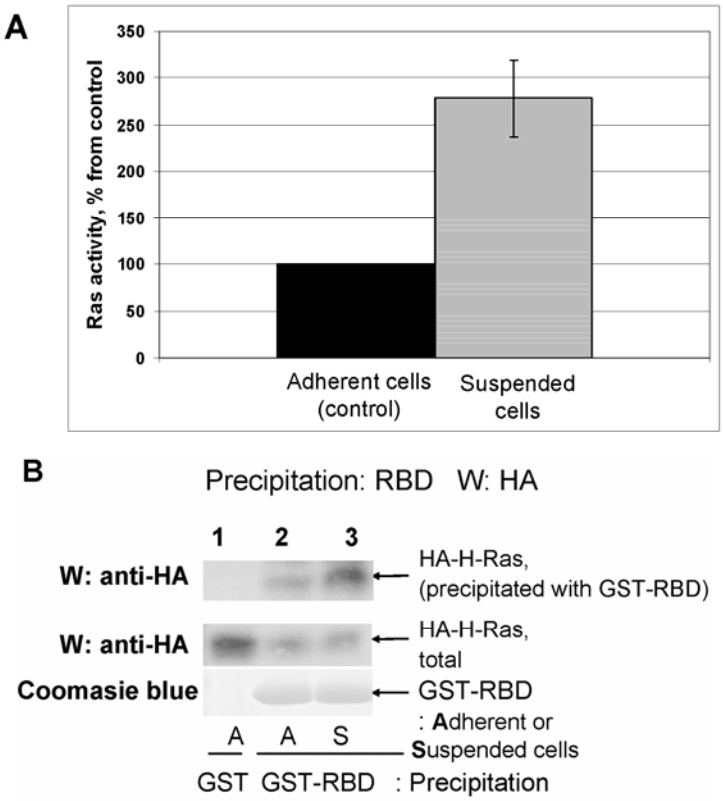


Fig 16. **Ras activity increases upon cell detachment**. H-WT cells were transfected with pcDNA3-HA-H-Ras vector. 48 h after the transfection cells were left adherent or suspended and lysed. Active HA-H-Ras from the lysates was precipitated with GST-RBD protein and detected by Western blot with antibody against HA tag. (A) The amount of active HA-H-Ras was measured with Image J software and represented as percent from the amount of active Ras in adherent cells. (B) Lane 1 – precipitation with GST, lane 2 – Ras activity in adherent cells, lane 3 – Ras activity in suspended cells. Upper panel – the amount of active HA-H-Ras, middle panel – level of total HA-H-Ras in lysates; detected by Western blot with antibody against HA tag. Lower panel – the amount of GST-RBD in samples stained with Coomasie blue R250 dye.

We next examined RasGAP activity in the suspended versus adherent cells. RasGAP was immunoprecipitated from adherent or detached H-WT cells and incubated

with GTP-bound recombinant H-Ras. As is shown in Fig. 17B, input H-Ras-GTP level (line 1) was dramatically reduced after pre-incubation with RasGAP immunoprecipitate from the adherent cells. However, the results of the pre-incubation of H-Ras-GTP with RasGAP from the detached cells was less severe (Fig. 17, A bars 2 and 3, and B lines 2 and 3). Therefore, data show that RasGAP from suspended cells inactivates H-Ras less efficiently than RasGAP from adherent cells. This fact is in line with our previous observation that Nck1 increases RasGAP activity toward H-Ras-GTP and that Nck1-RasGAP complex dissipates after cell detachment from the substrate.

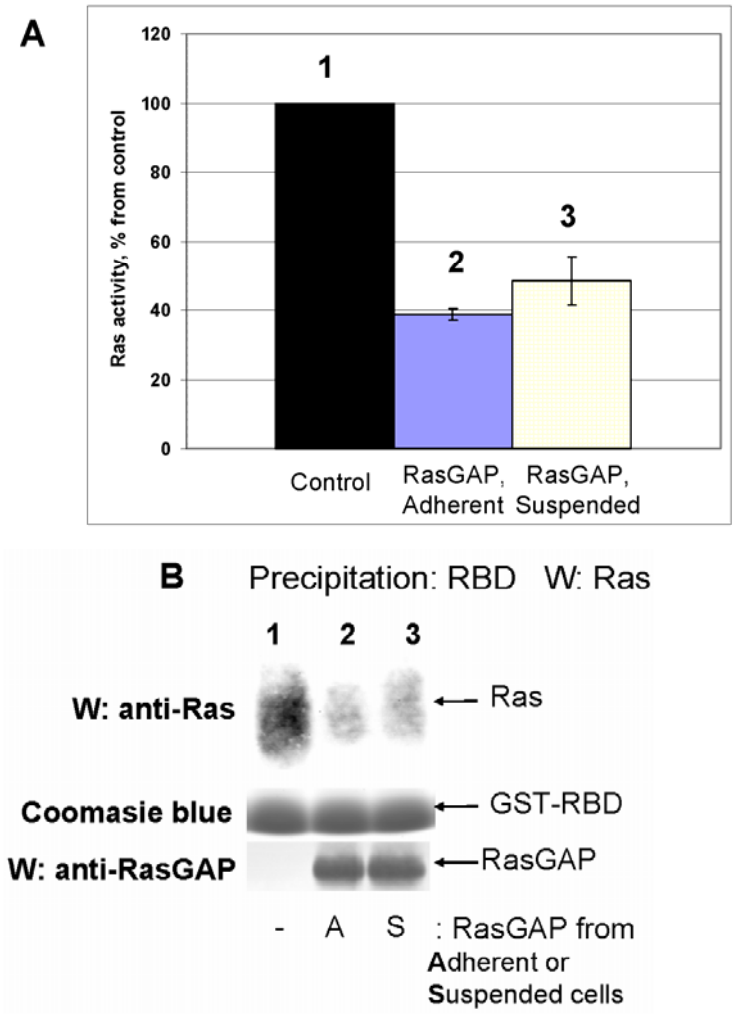


Fig. 17. **RasGAP activity depends on cell detachment**. RasGAP was immunoprecipitated from adherent or suspended cell lysates and incubated with GTP-bound H-Ras. H-Ras left active after the incubation without RasGAP (1), or with RasGAP from adherent (2) or suspended (3) cells was precipitated with GST-RBD and analysed by immunoblot with antibodies against Ras. (A) The amount of active H-Ras was measured with Image J software and represented as percent from the amount of active Ras in sample incubated without RasGAP. (B) Corresponding Western blot. Upper panel – the amount of active H-Ras. Middle panel – the amount of GST-RBD stained with Coomasie blue R250 dye. Lower panel – the amount of RasGAP in immunoprecipitates, visualizes by Western blot with antibodies against RasGAP.

In summary, the first and the third SH3 domains of Nck1 directly bind proline-rich sequence of RasGAP and lead to RasGAP activation. Over-expression of Nck1 decreases the activation of Ras by PDGF-BB in cells. The dissociation of complex between Nck1 and RasGAP after the cell detachment results in the decrease of RasGAP activity and the increase of Ras-GTP species.

## DISCUSSION

Ras family GTPases have key roles in regulating cell proliferation, differentiation and apoptosis, endo-exocytic cycle, actin cytoskeleton rearrangement. Mutations affecting the activity of Ras or Ras regulators contribute to the development of various disorders of cell functions and diseases. That is why the mechanisms of Ras regulation must be understood thoroughly. Ras GTPase activating protein (RasGAP) has major role in the negative regulation of Ras G-proteins. In this study we have investigated the interaction between RasGAP and adaptor protein Nck1. We have elucidated the mechanism of complex formation between RasGAP and Nck1 and have determined that the integrity of the complex depends on the cell adhesion to the substrate. We have also demonstrated the influence of Nck1 association with RasGAP on the activity of H-Ras protein in cells and *in vitro*.

The adaptor protein family Nck consists of two members: Nck1 and Nck2. Despite rather high homology the functions of Nck proteins in the cell may diverge (Chen, She et al. 2000; Guan, Chen et al. 2007). Nck proteins take part in the regulation of actin cytoskeleton, cell cycle, protein translation (Cardin, Latreille et al. 2007; Lettau, Pieper et al. 2009).

While investigating the role of Nck family proteins in the signal transduction by PDGF receptor-β we have discovered that Nck1 (Ger, Tunaitis et al. 2003) and Nck2 associate with RasGAP (Fig. 2). This is a novel previously unreported interaction. There is a number of proteins that form complex with both RasGAP and Nck and could potentially mediate the interaction between these two proteins. For example, PDGFR-β associates with Nck (Nishimura, Li et al. 1993) and with RasGAP (Kazlauskas, Kashishian et al. 1992). Nck1 adaptor protein have been shown to associate with RasGAP in mouse neuroblastoma and rat glioma hybrid cell line NG108 (Holland, Gale et al. 1997). However, this report shows that the complex in the cells is formed only

upon the stimulation with ephrin-B1, and that the SH2 domains of Nck1 and RasGAP are responsible for the complex formation, as well as the complex between Nck1 and RasGAP is mediated by the adaptor protein p62DOK1. Whereas our data clearly shows that complex between Nck proteins and RasGAP exist in the cell irrespectively of PDGF stimulation. Additionally, Nck1 and RasGAP complexes have been found in various cell lines of various species under the quiescent conditions (Fig. 4). However, the amount of RasGAP and Nck1 complex found might depend on various conditions including expression levels of these proteins. We have not assayed NG108 cells used by Holland et al., but there is possibility that the Nck1 and RasGAP do not associate in these cells or the amount of the complex is too low for the detection.

Both Nck1 and Nck2 bind the similar amount of RasGAP (Fig. 2, 3) so we did not analyze the potential distinctions between Nck proteins and focused our research on Nck1.

Nck1 and RasGAP are able to interact in the cell only if they are located in same compartments of the cell, that is why they have to colocalize. Nck1 and RasGAP colocalization in fixed cells was elucidated with immunostaining and subsequent laser scanning confocal microscopy. Data presented in Fig. 5 show that Nck1 and RasGAP colocalize within the cell, which is further substantiated by other type of experiments including immunoprecipitation and precipitation with recombinant proteins. However, beside obvious colocalization of RasGAP and Nck1 in the nucleus and lamella-like structures, cytoplasmic organelles or structures harbouring this protein complex remain to be determined.

Colocalization with the certain cell structure might give a clue to the biological role of the Nck-RasGAP protein complex. Nck1 and RasGAP together are detected in the lamellipodia-like structures of cells. Such colocalization is in the agreement with the other studies, for instance Nck (Buday, Wunderlich et al. 2002) and RasGAP (Ligeti, Dagher et al. 2004; Shang, Moon et al. 2007) are involved in the regulation of small Rac GTPases which are responsible for the lamellipodia formation.

Nck localization in the nucleus is a well-known fact (Kremer, Adang et al. 2007). However RasGAP has always been regarded as a specific cytoplasm marker (Zhang, Zhang et al. 1993). We detect RasGAP in the nucleus using various RasGAP antibodies and staining techniques. Currently, we cannot ascertain whether RasGAP localization in

the nucleus is some microscopy artefact or this localization has remained undetected previously due to some restrictions of the biochemical methods.

We have tested the possible colocalization of Nck1 and RasGAP with some defined cell structures like actin and tubulin network, Golgi, mitochondria and endocytotic vesicles. Unfortunately, no definitive Nck1-RasGAP colocalization have been found in these structures.

As the interaction between Nck1 and RasGAP has been confirmed, we sought to determine which of the domains of the adaptor protein Nck preferentially interacts with RasGAP. The adaptor protein Nck1 consists of three SH3 domains and one SH2 domain (Lehmann, Riethmuller et al. 1990). SH2 domains provide interactions with a phosphorylated tyrosine residue in the context of a longer peptide motif within a target protein most often in cells stimulated with growth factor that induce tyrosine phosphorylation. However, SH3 domains bind specific proline-rich motives and SH3 domain-mediated interactions usually are constant. Holland S. et al. describe the interaction between Nck1 and RasGAP which is growth-factor-dependent and mediated through Nck1 and RasGAP SH2 domains. On the contrary, our data clearly shows that the association between Nck1 and RasGAP does not depend on stimulation with growth factor (Fig. 2, 3). Using Nck1 constructs with inactivating point mutations in SH3 or SH2 domains we have shown that the first and the third SH3 domains of Nck1 are responsible most for the interaction with RasGAP. As expected, Nck1 with point mutations in the first or in the third SH3 domains bind less RasGAP than wild-type Nck1 (Fig. 6). The mutations in the second SH3 domain or in the SH2 domain have no effect on the association with RasGAP. Association with RasGAP of Nck1 mutant with only the first or the third intact SH3 domain is weaker than that of Nck1 mutant with all three SH3 domains left functional (Fig. 7). This shows that the thirst and the third SH3 domains of Nck1 contribute most to complex formation and this contribution is cumulative.

The ligands of SH3 domains are specific proline-rich motives. The amino acid sequence of the RasGAP was analyzed with the Scansite program (http://scansite.mit.edu/). This led to the identification of the region from 135 to 145 amino acid with several proline-rich motives potentially capable to interact with SH3 domains. This region with surrounding amino acids 68-168 was cloned by PCR and

expressed in H-WT cells as EGFP-fusion protein. By precipitation with the wild-type GST-Nck1 or GST-Nck1 with impaired SH2 domain we have shown that this region of RasGAP binds SH3 domains of Nck1 (Fig. 8). In summary, we have found that the association between Nck1 and RasGAP is provided by the two Nck1 SH3 domains and N-terminal proline-rich sequence of RasGAP.

Is this interaction direct or do other proteins contribute to Nck1-RasGAP complex formation? To elucidate this, we have employed Far Western blot technique where one of the proteins tested (RasGAP in our case) is concentrated by immunoprecipitation, separated by SDS-PAGE and transferred to membrane. Another protein partner to be analyzed (Nck1) is expressed as recombinant GST fusion protein. The membrane with the RasGAP is incubated in solution of the recombinant GST-Nck1 and the interaction is visualized with the antibody against the GST part (Fig. 9). Data show that GST-Nck1-WT and GST-Nck$(SH3)_3$ can bind RasGAP directly while GST-NckSH2 does not bind RasGAP. Interestingly, data with Far Western blot show that RasGAP immunoprecipitates contain 190 kDa protein that readily interacts with GST-Nck1-WT. We have hypothesized that this protein could be p190 RhoGAP which binds strongly to RasGAP (Ellis, Moran et al. 1990). The association of Nck1 to p190 RhoGAP was tested by precipitation with recombinant GST-Nck1-WT and GST-Nck$(SH3)_3$ proteins, however we have not detected any of p190 RhoGAP in the precipitates indicating that 190 kDa protein detected in the Far Western by Nck is not the p190 RhoGAP.

Nck1 and RasGAP proteins are involved in cell processes associated with cytoskeleton reorganization. Our data consistently showed that both proteins interacted constantly. Cell adhesion to the substrate coordinate cytoskeleton dynamics, therefore, we assumed that by forcing cells to detach from the substrate we will disorganize cytoskeleton and this might effect the Nck1-RasGAP complex formation. Therefore we have tested whether cell detachment from the substrate effects Nck1 and RasGAP association (Fig. 10). Data show that cell adhesion to the substrate is absolutely required for the complex formation because cell detachment from the substrate with trypsin or EDTA treatment instantly dissipated the complex. Moreover, Nck1-RasGAP complex has been partially restored only at the fifth hour after re-plating on the fibronectin.

Next, we have investigated which of the two proteins becomes incapable of the complex formation after the cell detachment. Our data show that Nck1 from detached

cells retains the ability to bind RasGAP from adherent cells (Fig. 12). On the contrary, RasGAP from detached cells or cells re-plated only for four hours did not bind recombinant GST-Nck1 (Fig. 11). As expected, RasGAP in the re-plated for 7 hours cells is already capable to recruit Nck1. Thus, data shows that it is RasGAP that becomes incapable of binding Nck1 in the detached cells.

Cell adhesion to the substrate is known to be able to affect protein-protein interactions. RasGAP associates with protein kinase FAK only in suspended but not in adherent U-251MG malignant astrocytoma cells. However, the suspendation of these cells also models cell migration and invasion that is why suspendation alone cannot be hold responsible for the complex formation (Hecker, Ding et al. 2004). The adaptor protein Nck1 after the cell detachment dissociates from protein kinase Pak but the complex reforms instantly after the cell re-plating (Howe 2001) while the complex between Nck1 and RasGAP is partially restored only after the five hours of re-plating. Thus, the interaction between Nck1 and RasGAP is regulated not only by mere cell adhesion, but also by some processes to be identified that take place after cell re-plating.

The following major causes might contribute to RasGAP inability to bind to Nck1: 1) posttranslational modification of Nck1-binding site that blocks binding; 2) posttranslational modification of RasGAP or/and its binding to unidentified protein that changes the conformation of RasGAP protein making the association with Nck1 compromised; (3) some protein that interacts with RasGAP after the cell detachment and competes with Nck1.

The analysis of the interaction between Nck1 and RasGAP by Far Western blot shows that denaturated RasGAP from detached cells can bind Nck1 with the same efficiency as RasGAP from adherent cells (Fig. 13). This shows that the most likely reason of complex dissociation is some change in RasGAP molecular conformation due to chemical modification or/and binding to unidentified protein. Further studies will be focused on search for allosteric modification of RasGAP or for the protein interacting with RasGAP after the cell detachment.

The activity of RasGAP proteins can be modulated by protein translocation, secondary messengers, posttranslational modifications or association with regulatory proteins (Bos, Rehmann et al. 2007). The regulation of p120 RasGAP is rather poorly elucidated. p120 RasGAP has domains usually responsible for the association with

secondary messengers: one of the functions of PH domain is the interaction with phosphatidylinositol lipids (Ferguson, Lemmon et al. 1995), C2 domain often provides regulation by calcium ions (Sutton and Sprang 1998). PH domain can interact with the catalytic domain of RasGAP and inhibit its activity. Inositol triphosphate and inositol (4,5)-biphosphate bind to PH domain and reduce its capability to interact with catalytic domain (Drugan, Rogers-Graham et al. 2000). As of posttranslational modifications, phosphorylation by Src kinase may diminish RasGAP catalytic activity (Giglione, Gonfloni et al. 2001). But the dominant paradigm of RasGAP regulation is thought to be the translocation to the cell membrane where targeted Ras GTPases are localized. This model is demonstrated to be true in the system where RasGAP is translocated to the cell membrane through the complex with annexin VI (Grewal, Evans et al. 2005). There are also precedents when RasGAP catalytic activity is inhibited by the association with other proteins, for example p190 RhoGAP (Moran, Polakis et al. 1991) or p62 DOK (Kashige, Carpino et al. 2000).

However, there have been no evidence of the interaction that would enhance the catalytic activity of RasGAP. We have demonstrated that when immunoprecipitated RasGAP is loaded with Nck1 RasGAP catalytic activity towards Ras increases and the amount of active Ras in the system accordingly decreases (Fig. 14). When we have over-expressed Nck1 we have detected the increase in the basic level and decrease of the PDGF-BB induced level of active H-Ras (Fig. 15). We have not determined the exact cause of the increase of basic level of H-Ras activity. However, it might be explained by the known fact that Nck1 interacts with several proteins that could effect H-Ras activity. It has been shown that Nck binds Ras guanine nucleotide exchange factor Sos (Hu, Milfay et al. 1995), also Nck interacts with p62 DOK adaptor protein (Tang, Feng et al. 1997) which negatively regulates Ras activity (Kashige, Carpino et al. 2000). Nck binds adaptor protein Abi1 (Yamamoto, Suzuki et al. 2001) which can act as a part of the complex modifying Sos1 specificity (Innocenti, Tenca et al. 2002). The basic level of H-Ras after Nck over-expression might be increased because of changes in equilibrium of any of these complexes due to the excess of Nck1. Our data shows that Nck1 over-expression in the cell decreases the level of PDGF-BB induced H-Ras activity. This complies with the data from the experiments *in vitro*: Nck1 association with RasGAP increases RasGAP catalytic activity and decreases the amount of active H-Ras. Also, our

data are in agreement with the well-established fact that RasGAP mostly affects PDGF-induced Ras-GTP level, but not the basic level of Ras activity (Kulkarni, Gish et al. 2000).

As was indicated above, the complex between Nck1 and RasGAP dissociates upon cell detachment from substrate. We have assayed RasGAP and Ras activity in detached cells. RasGAP from suspended cells has activated Ras GTPase weaker than RasGAP from adherent cells (Fig. 17). On the other hand, the Ras-GTP level in detached cells accordingly was higher than in adherent cells (Fig. 16). Similary, Ras-GTP level in suspended versus adherent cells corresponds with other studies (Hecker, Ding et al. 2004). Altogether, these experiments show that the dissociation of the complex between Nck1 and RasGAP correlates with the decrease of RasGAP catalytic activity and the increase of the amount of Ras-GTP. The goal of subsequent studies will be the analysis of causal relationship between these events.

## Conclusions

1. Nck1 and Nck2 proteins form complex with p120 Ras GTPase-activating protein (p120 RasGAP). This complex is present in different cell lines tested.
2. Adaptor protein Nck1 and RasGAP colocalize within the cell in the nucleus, lamellipodia-like structure and in the unidentified cytoplasmic structures.
3. The association between adaptor protein Nck1 and RasGAP is mediated most by the first and the third SH3 domains of Nck1 and RasGAP N-terminal proline-rich sequences. The adaptor protein Nck1 associate with RasGAP directly *in vitro*.
4. The complex between adaptor protein Nck and RasGAP dissociates after the adherent cell detachment.
5. RasGAP association with adaptor protein Nck1 enhances the catalytic activity of RasGAP toward H-Ras-GTP.

## List of publications

Articles:

**Ger M**, Tunaitis V, Valius M. 2003. *Elucidation of the Complex Formation between Nck-α, RasGTPase Activating Protein and Platelet-derived Growth Factor Receptor-β*. Biologija Nr.3: 7-9

**Ger M**, Tunaitis V, Stoskus M, Valius M. 2005. *Identification of Ras GTPase-activating protein-binding sites in adaptor protein Nck-α*. Biologija Nr.1: 35-7

**Ger M**, Magnusson KE, Valius M. 2006. *Proteomics Analysis of Proteins Associating with Nck-α*. Biologija Nr.4: 1-4.

Gurskiene V, **Ger M**, Valius M. 2006. *Differential association of Nck family proteins with PDGF receptor-β and Ras GTPase activating protein*. Biologija Nr.3: 106-8.

**M. Ger**, Z. Zitkus, M. Valius. 2009. *Adaptor protein Nck1 interacts with Ras GTPase-activating protein and regulates its activity*, FEBS Letters, submitted

Conference reports:

**Ger M**., Tunaitis V., Valius M. *Identification of Ras GTPase-activating protein-binding sites in adaptor protein Nck-α.* Conference of Lithuanian Biochemical Society. On 18-20 June 2004.

**Ger M**., Magnusson K.E., Valius M. *Proteomics Analysis of Proteins Associating with Nck-α*. Conference of Lithuanian Biochemical Society. On 16-18 June 2006.

**Reziumė**

Mažieji G-baltymai Ras yra svarbūs ląstelės dalijimosi, diferenciacijos ir išgyvenimo reguliatoriai. Ras šeimos baltymų mutacijos yra viena dažniausių vėžio atsiradimo priežasčių. p120 Ras GTPazę aktyvinantis baltymas (p120 RasGAP) yra pagrindinis Ras aktyvumo neigiamas reguliatorius. Šiame darbe buvo nustatyta, jog Nck šeimos adaptoriniai baltymai, Nck1 ir Nck2, sąveikauja su RasGAP. Ši sąveika nepriklauso nuo trumpalaikio poveikio trombocitų kilmės augimo faktoriumi (PDGF) bei vyksta skirtingo tipo ląstelėse. Baltymai Nck1 ir RasGAP kolokalizuojasi ląstelėje. Už sąveiką su RasGAP yra atsakingi pirmasis ir trečiasis adaptorinio baltymo Nck1 SH3 domenai bei RasGAP N-galinės prolino turtingosios sekos, sąveika gali vykti tiesiogiai. Adaptorinio baltymo Nck1 ir RasGAP kompleksas disocijuoja po adhezinių ląstelių sąveikos su substratu suardymo ir pradeda atsistatyti tik praėjus penkioms valandoms po sąveikos su substratu atstatymo. Už sąveikos suardymą yra atsakingas RasGAP baltymo konformacijos pokytis. Adaptorinio baltymo Nck1 sąveika su RasGAP didina RasGAP katalizinį aktyvumą, o ląstelėje Nck1 perteklinė ekspresija mažina RasGAP reguliuojamą PDGF indukuotą Ras aktyvumą. Nck1 ir RasGAP komplekso disociacija po ląstelių suspendavimo koreliuoja su RasGAP aktyvumo sumažėjimu bei Ras aktyvumo ląstelėje padidėjimu.

Šiame darbe buvo ištirtas komplekso tarp p120 RasGAP ir adaptorinio baltymo Nck1 susidarymo mechanizmas ir vaidmuo ląstelėje. Gauti duomenys leidžia daryti prielaidą, jog adaptorinis baltymas Nck1 dalyvauja GTPazės Ras aktyvumo reguliavime.

## CURRICULUM VITAE


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| 1998-2000 | Senior research assistant, Instute of Botany, Lithuania |
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**Scientific visits**

1. 2003 m. – Linköping Universitety, Sweden. Visit period – 3 months. Purpose: analysis of the complex between adaptor protein Nck and RasGTPase-activating protein with the methods of confocal microscopy and MALDI mass spectrometry.
2. 2003 m. – Institute of Bioorganic chemistry, Russia. Visit period– 1 week. Purpose: course on yeast two hybrid system.
3. 2008 m. – Applied Biosystems, Germany. Visit period – 1 week. Purpose: 4800 TOF/TOF mass spectrometer training course.
4. 2008 m. – Applied Biosystems, Germany. Visit period – 1 week. Purpose: 4000 QTrap mass spectrometer training course.

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