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Identification of Ras GTPase-activating protein-binding sites in adaptor protein Nck-α

M. Ger,
V. Tunaitis,
M. Stoškus,
M. Valius

Department of Developmental Biology, Institute of Biochemistry, Vilnius, Lithuania

E-mail: marija.ger@bchi.lt

Adaptor proteins consisting of Src homology (SH) 2 and 3 domains mediate various cellular signaling events initiated by receptor protein tyrosine kinases. Nck-α is one of the adaptor proteins implicated in the coordination of multiple intracellular signal transduction pathways emanating from the ligand-activated PDGF receptor-b. In our previous studies we have shown that Nck-α constantly associates with RasGTPase-activating protein (RasGAP). Here we show that SH3 domains of Nck-α are responsible for constitutive association with RasGAP. Moreover, Nck-α and RasGAP interact directly *in vitro*. These data provide a new insight into the molecular mechanism of RasGAP and Nck-α interaction.

Key words: PDGF receptor, Nck, RasGAP

INTRODUCTION

Nck-α belongs to a family of Src homology (SH) 2/SH3 domains-containing adaptor proteins, a group of proteins consisting of SH2 and SH3 domains and lacking any intrinsic enzymatic activity. SH2 domains associate with specific phosphotyrosine-containing sites. SH3 domains bind proline-rich motives, and generally these interactions are phosphorylation-independent [1, 2]. Nck-α has three consecutive SH3 and one SH2 domains. Adaptor proteins through their SH3 domains can associate with a number of signaling proteins and upon cell stimulation with a growth factor recruit them to tyrosine-phosphorylated cytoplasmic or membrane-attached proteins [3]. Nck-α is involved in the signaling pathways controlling actin cytoskeleton dynamics, DNA synthesis initiation, gene expression and protein translation [4–6].

RasGTPase-activating protein (RasGAP) is known mainly to regulate the steady-state level of activated ras. It is also involved in the regulation of actin cytoskeleton, however, the exact molecular mechanism remains to be determined [7].

In this study, we have elucidated mechanism by which Nck-α adaptor protein forms molecular complex with RasGAP.

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 cell line, devoid of endogenous PDGFR-β, was used for a stable expression of PDGFR-β (H-PR) using the retrovirus expression vector as described previously [8].

HepG2 cells were grown to a 70–80% confluence and made quiescent by culturing in serum-free DMEM overnight. Cells were stimulated or unstimulated with 30 ng/ml PDGF-BB (Amgene, USA) for 10 min at 37 °C, washed 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, 2 mM $NaVO_4$). The lysates were cleared by centrifugation at 20.000 × g for 15 min (0 °C).

Construction of SH2 domain-lacking Nck-α and generation of GST fusion proteins. A DNA fragment encoding three consecutive SH3 domains of Nck-α but lacking SH2 domain (called 3SH3, aminoacids 5-251) was generated by PCR and subcloned into the pGex2T bacterial expression vector (Amersham

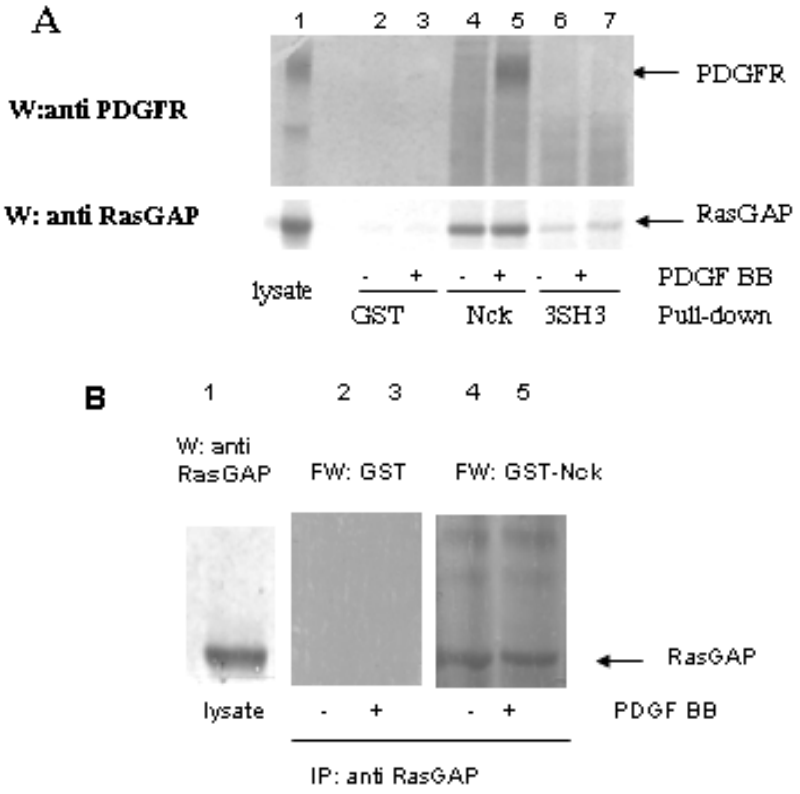

Figure. The mechanism of interaction between Nck-α and RasGAP.
A. Quiescent cultures of H^4-WT cells were left resting (–) or stimulated for 10 min with 30 ng/ml of PDGF-BB (+), lysed and used for pull-down assay with GST (lanes 2 and 3), GST-Nck-α (lanes 4 and 5) or GST-3SH3 (lanes 6 and 7) recombinant proteins. Precipitates were subjected to PDGFR (top portion of panel A) or RasGAP (bottom portion of panel A) Western blot analysis. Nck – GST-Nck-α, 3SH3 – GST-3SH3
B. Quiescent cultures of H^4-WT cells were left resting (–) or stumulated for 10 min with 30 ng/ml of PDGF-BB (+), lysed and immunoprecipitated with a RasGAP antibody. Immunoprecipitates were subjected to Far Western blot analysis with GST (lanes 2 and 3) or GST-Nck-α (lanes 4 and 5). Simultaneously a RasGAP Western blot analysis was performed against H^4-WT cell lysate to determine the exact position of RasGAP (lane 1)

Biosciences, USA). GST proteins were generated and purified as described earlier [9].

Far Western blot and pull-down assay. Lysates from PDGF-treated or untreated H-PR cells were immunoprecipitated with RasGAP antibodies for 2 h (0 °C). Immunoprecipitates were collected with Protein-A Sepharose (Amersham Biosciensces, USA) for 1 h at 4 °C. Then sepharose beads were washed with EB^+ buffer and prepared for SDS-PAG electrophoresis. Supernatants were sepatated by SDS-PAGE electrophoresis, transferred to PVDF membrane and blotted for 3 h with 10 μg/ml of GST or GST-Nck-α protein 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 and probed with alkaline phosphatase-conjugated secondary antibody (Sigma, USA). Blots were developed in a solution NBT/BCIP (Roth, Germany). Pull-down experiments were performed as described earlier [9].

RESULTS AND DISCUSSION

SH3 domains of Nck-α are responsible for constant association with RasGAP. We have previously reported that Nck-α constantly associates with RasGAP [9]. Constant interactions of adaptor proteins usually are mediated by SH3 domains [4]. To test this possibility, we have constructed an Nck-α mutant GST fusion protein lacking SH2 domain (GST-3SH3) and used it along with GST-Nck-α protein in a pull-down assay from PDGF-stimulated or unstimulated H-PR cells. Data show that both GST-Nck-α (Fig. 1A, lanes 4 and 5) and GST-3SH3 (Fig. 1A, lanes 6 and 7) associate with RasGAP in either PDGF-treated or untreated cells. GST-Nck-α fussion protein associates with PDGF receptor only in PDGF-treated cells; GST-Nck3SH3 does not associate with PDGF receptor-β, because this interaction requires SH2 domain [10]. GST alone binds neither PDGF receptor nor RasGAP (Fig. 1A, lanes 2 and 3).

Nck-α associates with RasGAP directly. To determine whether Nck-α and RasGAP interact directly, we have immunoprecipitated RasGAP from PDGF-treated and untreated H-PR cells and performed a Far Western assay with GST-Nck-α or GST. Data show that GST alone does not interact with RasGAP (Fig 1B, lanes 2 and 3), however, GST-Nck-α associates with RasGAP directly in PDGF-independent maner (Fig. 1B, lanes 4 and 5). The only interaction between Nck-α and RasGAP reported earlier was indirect and occurred only in growth factor-stimulated cells [11].

Taken together, the data show that Nck-α and RasGAP proteins interact directly. This interaction is mediated by Nck-α SH3 domains and does not depend on cell stimulation with PDGF. Further studies will be needed to map the exact sites responsible for the complex formation between Nck-α and RasGAP and to determine the intracellular role of such interaction.

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M. Ger, V. Tunaitis, M. Stoškus, M. Valius

SU RAS GTPAZÆ AKTYVUOJANÈIU BALTYMU SÀVEIKAUJANÈIØ DOMENØ NUSTATYMAS ADAPTORINIAME BALTYME NCK-α

Santrauka

Ið Src homologijos (SH) 2 ir 3 domenø sudaryti adaptoriniai baltymai perduoda ávairius signalus ið receptoriniø tirozino proteinkinaziø. Nck-α yra vienas iš adaptoriniø baltymø, dalyvaujantis daugybës nuo PDGF receptoriaus-b priklausomø vidulàsteliniø signalø reguliavime. Anksèiau iðsiaiðkinome, kad Nck-α nuolat sàveikauja su Ras GTPazæ aktyvuojanèiu baltymu (RasGAP). Ðiame darbe nustatëme, kad nuolatinæ Nck-α ir RasGAP sàveikà nulemia Nck-α SH3 domenai. Be to, *in vitro* Nck-α ir RasGAP sàveikauja tiesiogiai. Gauti duomenys leidþia geriau suprasti Nck-α ir RasGAP sàveikos molekuliná mechanizmà.