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Differential association of Nck family proteins with PDGF receptor-β and Ras GTPase activating protein

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The Src homology (SH) domains are protein modules found in many different proteins. The Nck family proteins consist of Nck-α and Nck-β proteins composed entirely of SH2 and SH3 domains and have no any putative catalytic domain. These proteins act as adaptors by linking tyrosine-phosphorylated proteins to other signaling molecules. Here we show that Nck-β associates with the ligand-stimulated platelet-derived growth factor (PDGF) receptor-β and Ras GTPase-activating protein (RasGAP) *in vivo*. Nck-β interaction with RasGAP is constant, but also increases after PDGF receptor-β stimulation. In contrast, Nck-α does not interact with ligand-activated PDGF receptor-β, but constantly associates with RasGAP.

Key words: PDGF receptor-β, RasGAP, Nck

INRODUCTION

Intracellular signaling pathways usually start from receptor activation by a ligand that reflects changes in the environment. After ligand-induced activation of a growth factor receptor it becomes tyrosine-phosphorylated and associates with the number of adaptor proteins or other cytoplasmic molecules [3]. One of such tyrosine kinase receptors is the platelet-derived growth factor (PDGF) receptor-β (PDGFR-β). After stimulation with PDGF the receptor becomes phosphorylated on tyrosine residues in the kinase domain. These phosphotyrosines serve as docking sites for cytoplasmic molecules. A large number of SH2 domain-containing proteins bind to PDGFR-β. Some of these molecules are enzymes, such as a phosphatidylinositol 3'-kinase (PI3K), phospholipase C-γ (PLC), the Src family of tyrosine kinases, or regulators of enzymes, like GTPase activating protein for Ras (RasGAP). Other PDGF receptor-binding molecules, such as Grb2, Crk, and Grb7, lack enzymatic activity and play an adaptor function [7].

Two members of the Nck family of adaptor proteins, Nck-α and Nck-β, are composed of three SH3 and one SH2 domains and share 68% of amino acid identity. The SH2 domain of Nck has been shown to bind to the epidermal growth factor receptor, insulin receptor substrate-1, and the ephrin receptor. On the other hand, Nck via it SH3 domains interacts with Ras GTP exchange factor (GEF) and p21-activated kinase. It has been shown that Nck-β binds significantly better than Nck-α to the receptor and nonreceptor tyrosine kinases and has distinct functional assignments in the same cell [1–5]. Nck proteins are implicated in cellular signal transduction, the regulation of cytoskeleton re-organization, gene expression, cell growth and differentiation regulation [6].

In this study, we identify differential binding of Nck-α and Nck-β proteins to the PDGF receptor-β and RasGAP. Data show that Nck-β but not the Nck-α association with RasGAP partially depends on PDGF receptor-β activation.

MATERIALS AND METHODS

Cell lines. The hepatocellular carcinoma-derived cell line HepG2 was cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% foetal bovine serum (HyClone, USA). HepG2 cell line, devoid of endogenous PDGFR-β, was used for a stable expression of PDGF receptor-β [8]. In a few experiments, the PDGF receptor-β F5 receptor mutant uncapable to bind the RasGAP, SHP-2, PI3K and PLC-γ [8] was used.

Expression constructs, antibodies. pRK5-HA-Nck-α and pRK5-HA-Nck-β expression vectors containing wild-type Nck cDNA were provided by W. Li. The Nck-β gene was linked in frame with the Ras farnesylation sequence (AAACTTAATCCTCCTGATGAATCTGGTCCT GGTTGTATG TCTTGTAAATGTGTTCTTTCT) which provides the membrane attachment [1].

The anti-PDGF receptor-β and anti-RasGAP antisera were raised in our laboratory. Anti-HA antibodies (sc-7392) were purchased from Santa Cruz Biotechnology (USA).

Transient transfection. Cells were transfected with 20 μg of pRK5-HA-Nck-α or pRK5-HA-Nck-β constructs

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using the $CaCl_2$ method. After 48 h the cells were starved in serum-free DME medium overnight.

Preparation of cell lysates. Resting cells were stimulated or unstimulated with PDGF-BB (Amgene, USA) 50 ng/ml for 10 min at 37 ˚C, washed three times with ice-cold PBS, 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 $NaVo_4$) and centrifuged 15 min at 20,000 *g* at 4 ˚C.

Immunoprecipitation. Postnuclear lysates were used for immunoprecipitation with anti-HA antibody for 2 h at 4 ˚C. The precipitates were collected with protein-A/G Sepharose (Santa Cruz Biotechnology, USA) for 1 h at 4 ˚C. The immune complexes 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% NaN_3), blotted for 3 h with anti-PDGFR, anti-RasGAP and anti-HA antibody as indicated, and visualized with alkaline phosphatase conjugated secondary antibody stained with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate tolidium salt.

RESULTS AND DISCUSSION

Nck-β associates with activated PDGF receptor-β *in vivo*. To test whether Nck-α and Nck-β interacts with PDGFR-β *in vivo*, we transfected HepG2-WT and HepG2-F5 cells with a wild type HA-Nck-α or HA-Nck-β construct. Cells were stimulated or not with 50 ng/ml PDGF-BB and lysed. Lysates were used for immunoprecipitation with anti-HA antibody. Immunoprecipitates were analyzed by Western blot using PDGFR, RasGAP or HA antibody, as indicated in Figure.

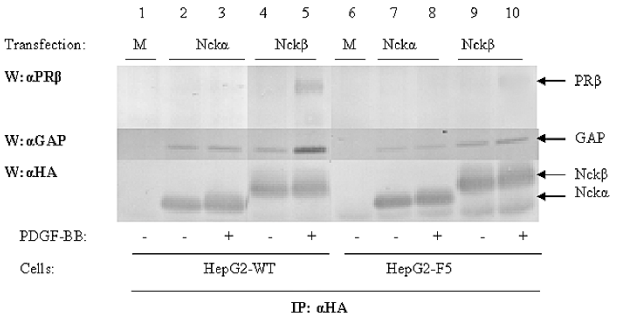

Figure. Nck-α and Nck-β differential association with PDGF receptor-β and RasGAP.
HepG2-WT (1-5) or F5 (6-10) cells were transfected with pRK5-HA-Nck-α (2–3, 7–8) or Nck-β (4–5, 9–10) constructs by $CaCl_2$ for 48h. Mock-transfected without any vector HepG2-WT and HepG2-F5 cells were used as negative control (1, 6). The cells were treated (+) or untreated (-) with 50 ng/ml PDGF-BB for 10 min, lysed, and the postnuclear lysates were used for immunoprecipitation with anti-HA antibody. Immunoprecipitates were separated by SDS-PAGE electrophoresis and subjected to PDGFR, RasGAP and HA Western blot analysis

Data show that immunoprecipitated Nck-β interacts with PDGF receptor-β, and this interaction occurs only in PDGF-BB stimulated HepG2-WT cells (Figure, lanes 4, 5). However, in stimulated HepG2-F5 cells Nck-β binds very little of mutant PDGF receptor F5 (Figure, lanes 9, 10). Data show that Nck-β binding to stimulated PDGFR-β *in vivo* depends on one or more phosphotyrosines that are responsible for the recruiting of PI3K, RasGAP, PLC-γ and Syp. On the contrary, no PDGF receptor-β was detected in immunoprecipitates from Nck-α overexpressed in HepG2-WT (Figure, lanes 2, 3) or HepG2-F5 (Figure, lanes 7, 8) cells, or in mock-transfected cells (Figure, lanes 1, 6).

Nck family proteins interact with RasGAP *in vivo*. To examine the capability of Nck proteins to associate with RasGAP and the dependence of this association on PDGF receptor-β activation, we performed immunoprecipitation of transiently transfected HA-Nck-α or Nck-β from HepG2-WT and HepG2-F5 cells stimulated or unstimulated with PDGF-BB. Immunoprecipitates were used for Western blot analysis with anti-RasGAP antibody. Data show that Nck-α (Figure, lanes 2 and 7) and Nck-β (Figure, lanes 4 and 9) interact with RasGAP in unstimulated WT and F5 HepG2 cells. After the activation of WT PDGF receptor-β with the ligand, the amount of RasGAP in the Nck-β immunoprecipitate, but not in Nck-α immunoprecipitate increased markedly (Figure, lane 5). However, the amount of RasGAP coimmunoprecipitated with HA-Nck-β from stimulated F5 cells was the same as from unstimulated cells (Figure, lanes 9, 10). The quantity of RasGAP coimmunoprecipitated with anti-HA-Nck-α did not change upon stimulation with PDGF-BB (Figure, lanes 2, 3, 7, 8).

Data show differences in Nck proteins binding to PDGFR-β and RasGAP. Nck-β interacts only with activated PDGFR-β and its interaction with RasGAP is constant, but after PDGFR-β stimulation with PDGF-BB Nck-β binds some additional RasGAP. As distinct from Nck-β, Nck-α does not associate with the receptor, and its interaction with RasGAP is constant in PDGF stimulated and unstimulated cells. So, it is possible that Nck-β in certain environmental conditions interacts with RasGAP directly and in other conditions serves as a link between PDGFR-β and RasGAP or RasGAP links Nck-β to PDGFR. The membrane attachment of Nck-β shows the localization of the Nck-β and RasGAP complex.

Further studies are needed to determine the mechanism and biological role of these interactions.

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Nck ŠEIMOS BALTYMŲ SĄVEIKA SU PDGF RECEPTORIUMI β IR RAS GTPazę AKTYVUOJANČIU BALTYMU

S a n t r a u k a

Src homologijos (SH) domenai yra baltymų moduliai, aptinkami skirtinguose baltymuose. Nck šeimai priklauso Nck-α ir Nck-β baltymai, sudaryti tik iš SH2 ir SH3 domenų ir neturintys jokių katalitinių domenų. Šie baltymai veikia kaip adaptoriai sujungdami tirozinfosforilintus baltymus su kitomis ląstelės signalinėmis molekulėmis. Šiame darbe nustatėme, kad Nck-β sąveikauja su ligandu aktyvuotu PDGF receptoriumi β ir Ras GTPazę aktyvuojančiu baltymu (RasGAP) *in vivo*. Nuolatinė Nck-β sąveika su RasGAP padidėja aktyvavus receptorių. Tuo tarpu Nck-α nuolat sąveikauja su RasGAP, bet negali sąveikauti su PDGF receptoriumi β. Mūsų duomenimis, Nck šeimos nariai skirtingai sąveikauja su signalą perduodančiais baltymais.