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 adapter 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 incapable 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 (AAACTTAATCCTCCTGATGAATCTGGTCCTGGTTGTATG 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...
using the CaCl₂ 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 NaVO₄) 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 Blotblock, 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 PDGFR-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.

Data show that immunoprecipitated Nck-β interacts with PDGFR-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 PDGFR 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 PDGFR 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 PDGFR 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 PDGFR 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 binds 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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References


Nck ŠEIMOS BALTYMŲ SĄVEIKAI SU PDGF RECEPTORIUMI Î IR RAS GTPazę AKTYVUOJANČIŲ BALTYMU

Santrauka
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 su jungdami tirozinosforilintus baltymus su kitomis įtakos signalinėmis molekulėmis. Šiame darbe nustatėme, kad Nck-β sąveikauja su ligandu aktyvuotu PDGF receptoriumi β ir Ras GTPazę aktyvuojačiu baltymu (RasGAP) in vivo. Nuolatine Nck-β sąveika su RasGAP padidėja aktyvavus receptorius. Tuo tarpu Nck-α nuolat sąveikauja su RasGAP, bet negali sąveikauti su PDGF receptoriumi β. Mūsų duomenimis, Nck šeimos nariai skirtingai sąveikauja su signalų perduodančiais baltymais.