Elucidation of the complex formation between Nck-α, RasGTPase activating protein and platelet-derived growth factor receptor-β

M. Ger,
V. Tunaitis,
M. Valius

Institute of Biochemistry,
Mokslininkų 12,
LT-2600, Vilnius, Lithuania

Many of the protein–protein interactions that are essential for eukaryotic cell intracellular signal transduction are mediated by protein-binding modules, including SH2 and SH3 domains. Nck-a is a SH2- and SH3-domain-containing adaptor protein implicated in coordination of variuos intracellular signal transduction pathways emanating from the ligand-activated PDGF receptor-β. Here we show that Y740, Y751, Y771 and Y1009 phosphotyrosines in the ligand-activated PDGF receptor-β are responsible for complex formation with Nck-α. In addition, we have found that in PDGF-unstimulated cells Nck-α constantly associates with the RasGTPase activating protein (RasGAP). Data show that RasGAP associates with Y771 phosphotyrosine in the ligand-activated PDGF receptor and might mediate Nck-α and the receptor complex formation *in vivo*.

Key words: intracellular signal transduction, protein–protein interaction, protein binding

INTRODUCTION

The group of proteins composed almost entirely of protein–protein interaction mediating domains but lacking any intrinsic enzymatic abilities is referred to as adaptor proteins. SH2/SH3 domain-containing adaptor proteins, such as the Nck family, play a major role in regulating tyrosine kinase signalling pathways. SH2 domains associate with specific phosphotyrosine-containing sites. SH3 domains bind proline-rich motives; generally these interactions are phosphorylation-independent, unless phosphorylation changes protein conformation. Adaptor proteins through their SH3 domains can associate with a number of signaling proteins and upon cell stimulation can recruit them to tyrosine-phosphorylated cytoplasmic or membrane-attached partners [1].

The Nck family contains the proteins Nck-α and Nck-β. They consist of three SH3 and one SH2 domains that share 68% amino acid sequence homology between isoforms, although they appear to have distinct functional assignments in the same cells [2, 3]. Nck is involved in the signaling pathways controlling cell mitogenesis, differentiation and apoptosis [4]. Recent studies suggest that Nck play an important role in mediating receptor tyrosine kinase signalling to the actin cytoskeleton [1, 2, 5].

In this study, we identify the RasGTPase activating protein (RasGAP) as a binding partner of Nck-α in the PDGF-activated and non-activated cells. Moreover, data suggest that the rasGAP-Nck-α complex might associate with the phosphotyrosine 771 in the ligand-activated PDGF receptor-β.

MATERIALS AND METHODS

Cell culture and preparation of extracts. HepG2 cells were maintained on Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% foetal bovine serum (Biochrome KG). HepG2 cell line, devoid of endogenous PDGFR-β, was used to create cell lines that express different PDGFR-β mutants by means of the retroviral infection system described previously [6].

The nature of the different PDGFR-β mutants has been extensively described and characterised by Montmayer et al. [7]. Briefly, WT is a HepG2 cell line expressing the wild-type PDGF-β receptor. pLXSN has been infected with the empty retroviral vector pLXSN. F5 expresses a PDGFR-β mutant in which all the tyrosines that bind the SH2 domains of GAP, SHP-2, PLCγ and PI3K have been mutated into phenylalanine. Starting with the F5 mutant, se-

veral binding sites were selectively restored (add-back mutants): GAP$^+$ for binding of GAP (Y771), PLCγ^+ for binding of PLCγ (Y1021), PI3K$^+$ for binding of PI3K (Y740 and Y751), SHP-2$^+$ for binding of SHP-2 (Y1009) and Y740$^+$ and Y751$^+$, single PI3K-binding sites.

HepG2 cells were grown to 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 thrice 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, 20 mg/ml aprotinin, 1 mM PMSF, 2 mM $NaVO_4$). Lysates were cleared of nuclei by centrifugation at 20,000 × g for 15 min.

Generation of GST-Nck-α fusion protein. Full-length Nck-α cDNA was fused with the *S. japonicum* GST gene in the pGex2T vector and expressed in the bacterial strain BL21. The GST-Nck-α fusion protein was purified by affinity chromatography using Glutathione Sepharose 4B (Amersham, UK).

GST-Nck-α pull-down assay. Pull-down experiments were carried out using 10 μg of GST-Nck-α fusion protein or GST alone immobilised on Glutathione Sepharose 4B beads per sample. Beads with immobilised GST-Nck-α or GST were incubated with postnuclear lysates for 1 h at 4 °C and mixed continuously. Then the beads were washed four times with EB^{++} buffer and heated for 3 minutes at 100 °C with 1 × electrophoresis sample buffer. Supernatants were analysed by 8% SDS-PAGE electrophoresis, transferred to PVDF membrane and blotted with antibodies against PDGF receptor, RasGAP or PI3K. The antibodies used in this study were crude polyclonal rabbit antiserum raised against respective GST-fusion proteins. Then the membranes were probed with alkaline phosphatase-conjugated secondary antibody (Sigma, USA) and blots were developed in a solution of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate tolidium salt (Roth, Germany).

RESULTS AND DISCUSSION

Y740, Y751, Y771 and Y1009 phosphotyrosines of PDGF receptor-β are responsible for complex formation with Nck-α. In order to find out which of the PDGF receptor-β (PDGFR) phosphotyrosines are responsible for direct or indirect binding of Nck-α SH2 domain, we have performed a pull-down assay with bacterially synthesised GST-Nck-α fusion protein using lysates from PDGF-treated and untreated HepG2 cells ectopically expressing the PDGF receptor and its add-back mutants. Data show that PDGFR activation by the ligand is essential for Nck-α binding to the receptor. GST-Nck-α, but not the GST portion of the protein, associates with the ligand-activated wild type (WT) PDGFR but binds very little of F5 mutant PDGFR (Fig. 1, A, line 8 and 6). Thus, the mutated tyrosines of F5 PDGFR might be involved in the Nck-α binding to the receptor. Nck-α associates with GAP$^+$ (Figure, A, line 12), PI3K$^+$ (Figure, A, line 14), SHP-2$^+$ (Fig. 1, A, line 16) and also with Y740$^+$ (Figure, B, line 10) and Y751$^+$ (Figure, B, line 12) add-back receptor mutants. However, association with the PLCγ^+ (Figure, A, 10) add-back mutant is similar to F5, indicating that phosphorylation of Y740, Y751, Y771 and Y1009 but not Y1021 in the receptor is responsible for complex formation with Nck-α. The role of Y740, Y751 and Y1009 in association with Nck has been shown previously [7, 8], although it was not specified for binding of which of the Nck isoforms they are responsible. Phosphotyrosine Y771 has never been identified as Nck-α binding site.

Nck-α associates with RasGTPase activating protein (GAP) in PDGF-unstimulated cells. To elucidate the mechanism by which Nck-α associates with GAP+ add-back PDGFR mutant, we investigated how Nck-α associates with GAP. Data show that Nck-α associates with GAP even in PDGF-unstimulated cells. As expected, after stimulation of PDGFR WT or GAP$^+$ receptor mutant expressing cells the

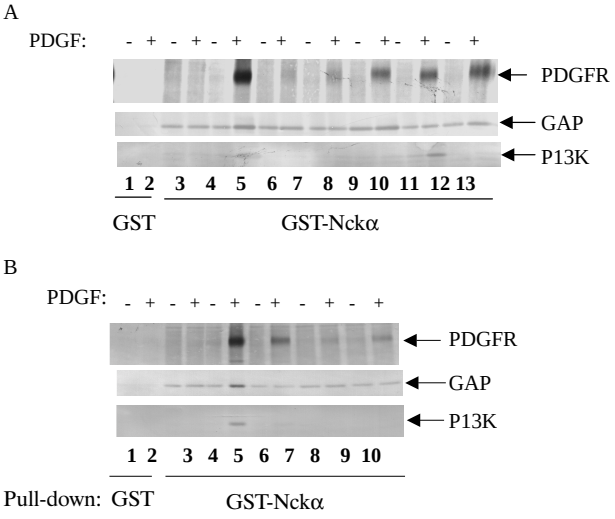

Figure. Nckα association with PDGFR, RasGAP and PI3K. HepG2 mutant cells were either treated or not with PDGF and the postnuclear lysates were subjected to pull-down assay with GST (A 1–2, B 1–2) or GST-Nckα. Cell lines: A: 3–4 – $pLXS_N$, 1–2, 5–6 – WT, 7–8 – F5, 9–10 – PLCγ^+, 11–12 – GAP$^+$, 13–14 – PI3K$^+$, 15–16 – SHP-2$^+$; B: 3–4 – $pLXS_N$, 1–2, 5–6 – WT, 7–8 – PI3K$^+$, 9–10 – Y740$^+$, 11–12 – Y751$^+$. Bead-bound proteins were eluted in electroforesis sample buffer and analysed by SDS-PAGE electroforesis and subsequent Western imunoblotting using antibodies against PDGFR, RasGAP or PI3K, respectively

amount of GAP in the complex increased considerably (Figure 1, A, lines 6 and 12). However, the presence of PI3K in the complex could be observed only in the ligand-activated PDGFR WT or PI3K$^+$ receptor expressing cells (Figure, A, lines 6 and 14). In a pull-down experiment with GST-Nck-α we could not detect the presence of PI3K in the PDGF-activated Y740$^+$ and Y751$^+$ receptor mutants; perhaps Nck-α and PI3K compete for the same binding site in the PDGF receptor.

Data suggest that Nck-α interacts with PI3K through the ligand-activated PDGF receptor. On the contrary, Nck-α and GAP associate constantly, directly or through some other protein. After stimulation with PDGF, Nck-α binds some additional GAP through activated PDGFR or other tyrosine-phosphorylated proteins, for instance p62DOK [9]. While indirect ligand activation-depended Nck-α and GAP association was reported earlier [9], no constant complex formation has been described before. We suggest that this PDGF-independent interaction might be mediated through one or several SH3 domains of Nck-α and that it might be responsible for Nck-α association with phosphorylated Y771 of the PDGF receptor via the GAP.

ACKNOWLEDGEMENTS

This work was supported by the Lithuanian Science and Studies Foundation (grants: K-005 and G-046).

References

1. Buday L. Biochimica et Biophysica Acta 1999; 1422: 187–204.
2. Chen M, She H, Davis EM et al. J Biol Chem 1998; 273(39): 25171–8.
3. Chen M, She H, Kim A et al. Mol Cell Biol 2000; 20(21): 7867–80.
4. McCartey JH. Bioessays 1996; 20.11; 20913–21.
5. Buday L, Wunderlich L, Tamas P. Cellular Signalling 2002; 14(9): 723–31.
6. Lint JV, Ni Y, Valius M et al. J Biol Chem 1998; 273(12): 7038–43.
7. Montmayer JP, Valius M, Vandenheede J et al. J Biol Chem 1997; 272(51): 32670–8.
8. Nishimura R, Kashishian A, Mondino A et al. Mol Cell Biol 1993; 13(11): 6889–96.
9. Holland SJ, Gale NW, Gish GD et al. EMBO J 1997; 16(130): 3877–88.

M. Ger, V. Tunaitis, M. Valius

KOMPLEKSO TARP NCKA, RASGTPAZĘ AKTYVUOJANČIO BALTYMO IR TROMBOCITŲ KILMĖS AUGIMO VEIKSNIO RECEPTORIAUS TYRIMAS

S a n t r a u k a

Daugybė eukariotų viduląstelinių signalų perdavimui būtinų sąveikų tarp baltymų vyksta per specialiai šiam tikslui skirtas struktūras, tarp jų ir SH2 ir SH3 domenus. Nck-α, adaptorinis baltymas, sudarytas iš SH2 ir SH3 domenų, dalyvauja perduodant į ląstelės vidų signalą nuo ligandu aktyvinto trombocitų kilmės augimo veiksnio (PDGF) receptoriaus-β. Šiame darbe atskleidėme, kad ligandu aktyvintame PDGF receptoriuje-β už komplekso su Nck-α sudarymą yra atsakingi fosfotirozinai Y740, Y751, Y771 ir Y1009. Be to, nustatėme, kad PDGF nestimuliuotose ląstelėse Nck-α nuolat asocijuoja su RasGTPazę aktyvinančiu baltymu (RasGAP). Gauti duomenys rodo, kad RasGAP, sąveikaudamas su ligandu aktyvinto PDGF receptoriaus-β fosfotirozinu Y771, gali užtikrinti Nck-α ir receptoriaus komplekso susiformavimą *in vivo*.