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

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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 (Amgen, 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 NaVO₄). 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...
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 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

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 fission 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 blot 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 interacts 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).

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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