The Adaptor Protein Nck Links Receptor Tyrosine Kinases with the Serine-Threonine Kinase Pak1*

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Nck is an adaptor protein composed of a single SH2 domain and three SH3 domains. Upon growth factor stimulation, Nck is recruited to receptor tyrosine kinases via its SH2 domain, probably initiating one or more signaling cascades. In this report, we show that Nck is found in living cells to the serine-threonine kinase Pak1. The association between Nck and Pak1 is mediated by the second SH3 domain of Nck and a proline-rich sequence in the amino terminus of Pak1. We also show that Pak1 is recruited by activated epidermal growth factor (EGF) and platelet-derived growth factor receptors. Moreover, Pak1 kinase activity is increased in response to EGF in HeLa cells transfected with human Pak1, and the kinase activity was enhanced when Nck was co-transfected. It is concluded that Nck links receptor tyrosine kinases with Pak1 and is probably involved in targeting and regulation of Pak1 activity.

The Nck protein (1) belongs to the class of signaling molecules termed adaptors which, like Grb2, are composed solely of SH2 and SH3 domains, with no intrinsic catalytic activity. These proteins exert their biological function by coupling upstream signals, usually those initiated by activation of receptor tyrosine kinases, to downstream elements in the cell (2). The role of the adaptor protein Grb2 in the Ras signaling pathway has been well established (3).

Nck associates directly with activated PDGF-β-receptor via its SH2 domain (4) and is recruited to EGF receptor upon EGF stimulation (5–7). Nck contains three SH3 domains and could potentially bind to at least three effector proteins. However, the proteins which form a complex with Nck in vivo to transmit downstream signals are largely unknown. It has been reported that upon overexpression Nck can bind to the guanine nucleotide exchange factor Sos (8), and that Nck can interact with p120cbl (9) and the Wiskott-Aldrich syndrome protein WASP (10). The physiological relevance of these interactions remains uncertain. Recently, the Drosophila homologue of Nck has been identified (termed dock) in a genetic screen for mutants that disrupt photoreceptor guidance and targeting (11), thus providing a potential clue for a biological role of Nck in vertebrates. It was proposed that the Drosophila Nck may play a role in linking receptor tyrosine kinases with changes in organization of the actin cytoskeleton.

Pak (p21-associated kinase) proteins are serine-threonine kinases (12) homologous to the yeast Ste20 kinase, an enzyme involved in linking pheromone-activated G protein-coupled receptors to a MAP kinase cascade (13). Several distinct members of the Pak family have been identified (12, 14–17). It has been shown that Pak proteins can be activated in vitro by the small GTP-binding proteins Cdc42 and Rac1. In addition, Pak proteins appear to participate in the activation of both the J NK and p38 MAP kinase signaling pathways (18–21). Physiological substrates for Pak proteins have not been identified.

In a recent report, Bagrodia et al. (16) noted the existence of proline-rich sequences similar to canonical SH3 domain binding regions at the amino terminus of Pak3; similar sequences are also present in other Pak proteins. It was demonstrated that GST fusion proteins containing the SH3 domains of Nck and PLCγ can bind to Pak3 in an in vitro binding assay. In addition, a serine-threonine kinase activity was shown to be associated with Nck in a variety of cell types (22). The second SH3 domain of Nck was sufficient for association with the kinase activity. This kinase activity did not appear to be modulated by a variety of mitogenic stimuli, and it appeared to be localized exclusively in the particulate fraction. The molecular mass of the Nck-associated kinase was estimated to be ~65 kDa. We note that this molecular mass is similar to that of Pak proteins.

In this report we show that Nck and Pak1 are constitutively complexed in living cells, and that their association is mediated through the second SH3 domain of Nck and the first proline-rich sequence of Pak1. In addition, we demonstrate that the Nck/Pak1 complex is recruited to EGF and PDGF receptors upon growth factor stimulation. We also show that Nck modulates Pak1 activity upon EGF stimulation in vivo.

EXPERIMENTAL PROCEDURES

Plasmids—For mammalian expression, cDNA encoding wild-type human Pak1 (21, 23) was subcloned into the epiplasmid expression vector pCMVβ (24), then subcloned into pMSCV. For GST fusion proteins, cDNA constructs encoding individual SH3 domains of Nck and the full-length protein were amplified by polymerase chain reaction (Perkin-Elmer) using oligonucleotides with BamHIEcoRI linkers to facilitate subcloning into pGEX-ZT (Pharmacia Biotech Inc.). The constructs were sequenced on both strands using the Sequenase Quick Denature kit (U. S. Biochemical Corp.).

Antibodies—Rabbit polyclonal antibodies raised in rabbits against a GST fusion protein comprising full-length Nck were used for Nck immunoprecipitation. To facilitate visualization of Nck by SDS-PAGE (since it runs very close to the IgG heavy chain), anti-Nck antibodies were covalently cross-linked to protein A-Sepharose beads (Zymed) using dimethyl pimelimidate (Pierce), following the protocol described in Ref. 25. For Nck immunoblotting, we used either the antibodies described above or affinity-purified polyclonal antibodies (SC-290, Santa Cruz Biotechnology). For Pak1 immunoprecipitation and immunoblotting, affinity purified polyclonal antibodies (SC-881, Santa Cruz Biotechnology) raised against the last 19 amino acids of human or rat Pak1 kinase were used.

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Pak1 (12) were used. myc-Pak1 was immunoprecipitated and immunoblotted using a monoclonal anti-myc antibody (9E10, Santa Cruz Biotechnology). For PDGF receptor blotting, we used polyclonal antibodies against the myc epitope previously described in Ref. 27.

Cell Lines and Stimulation with Growth Factors—L6 (rat myoblasts), 293T (human kidney), and HeLa (human cervix epitheloid carcinoma) cells were grown in DMEM (Cellgro) supplemented with 10% FBS (Life Technologies, Inc.), glutamine, and antibiotics. L6 cells were starved in DMEM supplemented with 0.2% FBS before stimulation with PDGF (Intergen) at 30 ng/ml for 5 min, or insulin (Sigma) at 0.1 nm for 5 min. Transiently transfected HeLa cells were starved in DMEM with 0.1% FBS and stimulated with EGF (100 ng/ml) for different times. Cell starvation was for 16–24 h, and all stimulations were performed at 37°C.

Cell Lysis, Immunoprecipitation, and Immunoblotting—Cells were washed twice in cold phosphate-buffered saline and lysed in lysis buffer containing phosphatase inhibitors as described (27). Cell extracts were preclarified by centrifugation. For Nck immunoprecipitation, preclarified cell extracts were incubated with anti-Nck antibodies cross-linked to Protein A-Sepharose beads in a nutator at 4°C for 3 h. For Pak1 or myc-Pak1 immunoprecipitation, cell extracts were incubated with 15 µg of anti-Pak1 or anti-myc affinity-purified antibodies for 90 min, then recovered on 30 µl of protein A- or protein G-Sepharose beads for 90 min at 4°C. Immunocomplexes were washed three times with lysis buffer and eluted in SDS sample buffer. Upon SDS-PAGE (Bio-Rad), gels were transferred to nitrocellulose (MSI), incubated with TBS–5% bovine serum albumin (Intergen) for 2 h at room temperature or overnight at 4°C, followed by incubation with primary antibodies for 1 h at room temperature. Following extensive washes in TBS–0.1% Triton X-100, filters were incubated for 1 h with secondary antibodies (protein A or G conjugated to horseradish peroxidase) in TBS supplemented with 5% non-fat dry milk. Proteins bound to filters were visualized by enhanced chemiluminescence (Renaissance, DuPont NEN).

Transfected—293T cells were transfected by the calcium precipitation method (28) using 10 µg of total DNA per 6-cm dish. HeLa cells were transfected with 6 µg of DNA per 6-cm dish using LipofectAMINE (Life Technologies, Inc.), following the instructions of the manufacturer. In the experiment shown in Fig. 3, each time point corresponds to an individually transfected dish.

Stable Transformation of L6 Cells—To overexpress human Nck in L6 cells, Nck cDNA was subcloned into the retroviral vector SRE. Helper-free infectious retrovirus was produced by transient transfection into the retroviral packaging cell line BOSC 23 using calcium phosphate. Nck-containing retroviruses were used to infect L6 cells, and G418-resistant pools were selected. Expression of Nck was confirmed by immunoblotting.

Preparation of GST-Fusion Proteins—GST-fusion proteins comprising amino acids 2–66 (SH3-I), 103–170 (SH3-II), and 188–257 (SH3-III) of human Nck were expressed in Escherichia coli and purified by affinity chromatography on glutathione-Sepharose beads as previously described (29).

Pak Activity Assay—Pak1 immunoprecipitates on beads were washed four times in lysis buffer, followed by two washes in 2× kinase buffer (23). Kinase reactions were carried out in 30 µl of a solution containing 2 µg of MBP, 20 µM ATP, and 5 µCi of [γ-32P]ATP (6000 Ci/mmol) in kinase buffer. After incubation for 20 min at 30°C, reactions were stopped by addition of 15 µl of 3× SDS sample buffer followed by boiling the samples for 4 min. Results were visualized by SDS-PAGE and autoradiography.

Interaction Trap Binding Assay—Point mutations in human Pak1 were introduced by a unique site elimination protocol (30). Wild-type and mutant forms of Pak1 were subcloned into the bait vector pEG202 (31). Human Nck and Saccharomyces cerevisiae RNA polymerase subunit RP48 (32) were each subcloned into the activation domain plasmid pJG4-5 (31). Bait vectors, activation domain vectors, and a lacZ reporter vector were co-transformed into EGY48, and transformants were selected on dextrose-containing medium lacking uracil, leucine, and histidine. Three independent colonies from each transformation were analyzed for reporter activation. The colonies were replica-plated to galactose-containing medium to induce production of the bait protein, and the colonies were assayed for β-galactosidase production (33).

RESULTS AND DISCUSSION

We first used parental and Nck-overexpressing L6 cells to determine whether Pak1 and Nck form a complex in vivo.

Lysates from quiescent, PDGF-, or insulin-stimulated cells were prepared and subjected to immunoprecipitation and immunoblotting with anti-Nck or anti-Pak1 antibodies. Pak1 was detected in Nck immunoprecipitates from cells which express endogenous Nck (Fig. 1A) and at a higher level in immunoprecipitates derived from cells that overexpress Nck (Fig. 1B). The amount of Pak1 complexed with Nck does not appear to vary upon cell stimulation with PDGF or insulin (also with EGF, see below). These results argue for the presence of a preformed Pak1/Nck complex in vivo, and that complex formation is limited by the amount of Nck in the cells.

Upon stimulation of cells with EGF or PDGF, Pak1 is recruited by activated EGF and PDGF receptors by means of its SH2 domain (4–7). This therefore raises the question of the involvement of Pak1 in the Nck-mediated signal transduction pathway(s) initiated by growth factor stimulation. Serum-starved L6 cells stably overexpressing Nck were PDGF- or mock-stimulated, and lysates were immunoprecipitated with anti-Pak1 or anti-Nck antibodies and probed with anti-PDGFR receptor antibodies. PDGFR receptor was detected in PDGF-stimulated Pak1 immunoprecipitates (Fig. 2A, lane 4), demonstrating that Pak1 is able to associate with activated PDGFR receptors. The same blot was probed with anti-phosphotyrosine antibodies (Fig. 2D). Pak1 was not detectably tyrosine-phosphorylated upon PDGF stimulation; Pak1 does not appear to be a substrate for PDGFR receptor. However, two constitutively tyrosine-phosphorylated proteins of ~90 kDa and ~150 kDa, were present in the Nck (pp90 and pp150) and Pak1 (pp90) immunoprecipitates. Tyrosine-phosphorylated proteins of the same apparent molecular mass were observed in Nck and Pak1 immunoprecipitates from A431 and HER14 (NIH 3T3 overexpressing human EGF receptors) cells (data not shown). The identity of pp90 and pp150 is currently unknown.

The results discussed above suggest a role for Pak1 in a PDGFR receptor signal transduction pathway. Since EGF also triggers association between activated EGF receptor and Nck (5–7), we repeated this experiment with A431 and HER14 cells to examine the possible involvement of Pak1 in EGF receptor signaling. The results were essentially the same as in Fig. 2, with Pak1 detected in anti-Nck immunoprecipitates (stimulated or nonstimulated) and activated EGF receptor detected in Nck and Pak1 immunoprecipitates.2

Whether or not the recruitment of Pak1 to activated growth factor receptors was associated with activation of Pak1 kinase activity was explored in the next set of experiments. We tested whether EGF activates Pak1 in vivo. HeLa cells were transiently transfected with a migrastatin-tagged human Pak1 expression vector.2

FIG. 1. Nck co-immunoprecipitates with the serine-threonine kinase Pak1. A, lysates from mock (lane 1), PDGF- or insulin-stimulated L6 cells were immunoprecipitated with anti-Nck or anti-Pak1 antibodies. The co-precipitating proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was divided in two parts. The lower part was probed with anti-Nck antibodies, and the upper part was probed with anti-Pak1 antibodies. B, as in A, but the lysates are from an L6 line which stably overexpresses human Nck.

2 M. L. Galisteo and J. Schlessinger, unpublished results.
Pak1, we performed mediated by the second SH3 domain of Nck. We demonstrate that the interaction between the two proteins is specific. Pak1 antibodies (Fig. 4) showed that full-length Nck associated with Pak1, as well as the second SH3 domain. These results fit the consensus for SH3 domain binding. The most amino-terminal of these (KPPAPPMMR) is highly conserved in all three human Pak proteins and is also present in the Drosophila homologue of Pak1. In order to determine which, if any, of these motifs mediate binding to Nck, we carried out an interaction-trap analysis in S. cerevisiae, using as bait wild-type Pak1 or point mutants in each of the three proline-rich regions, respectively (Fig. 5A). The interaction between Pak1 and Nck is easily detectable in this system, resulting in the strong activation of the reporter $\beta$-galactosidase (Fig. 5B). Replacement of proline 13 (located in the first proline-rich domain) with alanine completely abolishes binding to Nck, whereas similar mutations within the second and third proline-rich regions are without effect. Pak1 does not detectably interact with an irrelevant partner, RPB4 (32), indicating that the interaction with Nck is specific.

It was recently demonstrated that a GST-fusion protein containing the three SH3 domains of Nck was able to bind to Pak3 in an in vitro "pull-down" assay, as well as to a GST protein containing the SH3 domain of PLCv (16). The second SH3

**Fig. 2. Pak1 is recruited to activated PDGF receptor.** Lysates from mock (-) or PDGF-stimulated L6 cells were immunoprecipitated with anti-Nck or anti-Pak1 antibodies. Upon SDS-PAGE and transfer to nitrocellulose, the membrane was cut into three parts which were analyzed with the following antibodies. A, anti-PDGF receptor; B, anti-Pak1; C, anti-Nck; D, anti-Tyr(P) blot was performed after stripping the membrane. 40 $\mu$g of PDGF-stimulated L6 lysate were also analyzed in lane 5.

![Image](http://www.jbc.org/)

**Fig. 3. Pak1 activity is increased upon EGF stimulation of HeLa cells.** HeLa cells transfected with 6 $\mu$g of a myc-Pak1 construct (lanes 1-4) or with 3 $\mu$g of myc-Pak1 plus 3 $\mu$g of Nck (lanes 5-8) were starved and stimulated with 100 ng/ml EGF for the times shown in the figure and lysed. Four-thirds of each lysate were subjected to immunoprecipitation with anti-myck antibody. Resultant immunocomplexes were split in two and subjected to kinase assays in the presence of MBP (A) and immunoblotting using $\alpha$-myck antibody (a control for transfection and immunoprecipitation efficiency) (B). One-fourth of each lysate was subjected to immunoprecipitation with anti-EGF receptor antibodies and immunoblotting with anti-Tyr(P) antibodies (C) in order to follow the activation of the receptor upon EGF stimulation.

![Image](http://www.jbc.org/)

**Fig. 4. The second SH3 domain of Nck mediates association with Pak1.** Lysates from 293T cells transfected with a myc-tagged human Pak1 construct were incubated with 5 $\mu$g of GST-fusion proteins expressing the individual SH3 domains of Nck (SH3 I, II, III) or the full-length protein. After SDS-PAGE and transfer to nitrocellulose, the filter was probed with anti-Pak1 antibodies. GST-Nck ran closely to the myc-Pak1 protein in the gel, accounting for the deformation of the band in this lane.
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WASP has been shown to be a mediator between Cdc42 and the actin cytoskeleton (35, 36), and it has been reported recently to be complex with Nck in hematopoietic cells (10). These results are consistent with the proposal that the Drosophila homologue of Nck (dock), may link tyrosine kinase receptor activation with intracellular signaling pathways that regulate cytoskeletal changes in the growth cone (11). By analogy, the Nck adaptor protein may act as a link between tyrosine kinase receptors and changes in the actin cytoskeleton in mammalian cells, and its function may be modulated by the action of Rho-like GTPases that are present at the plasma membrane.

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