Interaction of the Nck Adapter Protein with p21-activated Kinase (PAK1)*

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The p21-activated kinases (PAKs) link G protein-coupled receptors and growth factor receptors (S. Drharmawardhane, R. H. Daniels, and G. M. Bokoch, submitted for publication) to activation of MAP kinase cascades and to cytoskeletal reorganization (M. A. Sells, U. G. Knaus, D. Ambrose, S. Bagrodia, G. M. Bokoch, and J. Chernoff, submitted for publication). The proteins that interact with PAK to mediate its cellular effects and to couple it to upstream receptors are unknown. We describe here a specific interaction of the Nck adapter molecule with PAK1 both in vitro and in vivo. PAK1 and Nck associate in COS-7 and Swiss 3T3 cells constitutively, but this interaction is strengthened upon platelet-derived growth factor receptor stimulation. We show that Nck binds to PAK1 through its second Src homology 3 (SH3) domain, while PAK1 interacts with Nck via the first proline-rich SH3 binding motif at its amino terminus. The interaction of active PAK1 with Nck leads to the phosphorylation of Nck at multiple sites. Association of Nck with PAK1 may serve to link this important regulatory kinase to cell activation by growth factor receptors.

Nck has been identified as one of the so-called adapter proteins, consisting of one Src homology 2 (SH2) and three Src homology 3 (SH3) domains (1). Adapter proteins are believed to play important roles in coupling activated receptors, particularly protein-tyrosine kinases, to various signaling pathways (2). Nck has been shown to be recruited to both the activated EGF and PDGF receptors (3, 4). However, there is limited knowledge about the effector proteins that interact with Nck. Overexpression of Nck has been shown to transform mammalian cells, suggesting that it interacts with protein components important for regulation of normal cell growth (3, 5). Perhaps related to this phenotype, the binding of Nck through its second SH3 domain to the mammalian Ras exchange factor Son of Sevenless (SOS) has been reported (6). Additional proteins able to bind with Nck are the IRS-1 protein in insulin-stimulated cells via the Nck SH2 domain (7), WASP, the protein defective in Wiskott–Aldrich syndrome via the third Nck SH3 domain (8), and a poorly characterized protein termed Nap1 via the first Nck SH3 domain (9). Unidentified serine/threonine kinase(s) of 65–69 kDa have also been reported to associate with the second SH3 domain of Nck both in vitro and in vivo (10). Nck is rich in potential phosphorylation sites (1), and it has been observed that Nck becomes phosphorylated on serine, threonine, and tyrosine residues in response to activation of a number of growth factor receptors, including those for epidermal growth factor, nerve growth factor, and platelet-derived growth factor (3, 11, 12). Nck is also phosphorylated in response to forskolin and phorbol ester treatment (11), suggesting it serves as a substrate for cAMP-dependent protein kinase (PKA) and protein kinase C.

The p21-activated kinases (PAKs) were identified as serine/threonine kinases whose activity is regulated by the small GTPases Rac and Cdc42 (13–15). PAKs appear to initiate the protein kinase cascades leading to activation of the p38 and JNK (16–19) kinases. Additionally, there is evidence that PAKs mediate some of the cytoskeletal effects of Rac and Cdc42.3 PAKs have been shown to be stimulated by both G protein-coupled receptors (15) and growth factor receptors.3 However, the mechanisms involved in this activation have not yet been elucidated.

In this study, we describe the specific interaction of Nck with PAK1 both in vitro and in vivo. Stimulation of the PDGF receptor in Swiss 3T3 cells enhances the level of associated Nck and PAK1. We map the site of this interaction to the second SH3 domain of Nck and to the first proline-rich SH3 binding domain of PAK1. PAK1 phosphorylates Nck in vivo on serine/threonine residues, at least some of which are distinct from those phosphorylated by cAMP-dependent protein kinase. Nck may thus serve as a means to link PAK1 to activation of tyrosine kinase receptors and additional signaling components.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—Full-length and isolated SH domains of Nck were generated by polymerase chain reaction using the human nck cDNA as template (1) and cloned into pGEX-2T (Pharmacia Biotech Inc.) for expression as glutathione S-transferase (GST) fusion proteins. The Nck SH3 first domain construct encoded residues 1–68 of Nck; the Nck second, residues 101–166; SH3 third, residues 191–257; the second plus third SH3 domains, residues 101–257; and the SH2 domain, residues 275–377. These proteins were isolated using glutathione-Sepharose beads according to the manufacturer's instructions (Pharmacia) and gave essentially single bands on Coomassie Blue-stained SDS-polyacrylamide gels, with the exception of GST-full-length Nck (designated GST-Nck), which degraded to yield several smaller fragments.

Full-length Nck was cloned in-frame with the hemagglutinin tag into the mammalian expression vector pCGN (22). PAK1 was prepared in pCMV6 with an amino-terminal Myc epitope tag as described in Footnote 2. Point mutations in Pak1 were introduced using a unique site-elimination protocol (23). Transient expressions in COS-7 cells were

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† The abbreviations used are: SH2, Src homology 2; SH3, Src homology 3; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PKA, cAMP-dependent protein kinase; PAK, p21-activated kinase; GST, glutathione S-transferase; GTPγS, guanosine 5′-3-O-(thio)triphosphate; aa, amino acids.

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FIG. 1. PAK1 binds to the second SH3 domain of Nck. The binding of PAK1 to GST fusion proteins containing various portions of Nck was determined as described under “Experimental Procedures.” PAK1 was detected using the 9E10 Myc epitope antibody at 1:3000 dilution.

performed essentially as described in Ref. 16.

Cell Culture—Swiss 3T3 and COS-7 cell lines were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 10 mM HEPES, 2 mM t-glutamine at 37 °C in an atmosphere of 10% CO2.

Preparation of Cell Lysates and Immunoprecipitation—Cells were plated in 100-mm tissue culture dishes and serum-starved for ~18–18 h prior to the start of the experiment. The cells were then treated with or without stimuli as indicated and then rapidly scraped from the dish into ice-cold lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl2, 1 mM dithiothreitol, 150 mM NaCl, 1% glycerial, 1% Nonidet P-40, 2 mM sodium vanadate, 50 IU/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin). In some experiments, cells were lysed in hypotonic buffer (5 mM Tris-HCl, no NaCl) in the absence of Nonidet P-40. After 15 min on ice, the lysates were pelleted for 5 min at 1500 rpm at 4 °C and the clarified supernatants removed.

Aliquots of cell lysates were incubated with the indicated antibody overnight at 4 °C and then with 80 μl of a 1:1 slurry of Protein A or Protein G-Sepharose beads for 45–60 min. Beads were pelleted and washed twice with 1 ml of lysis buffer containing Nonidet P-40 and once with 1 ml of lysis buffer without Nonidet P-40 and then used for immunoblots. For kinase assays, the beads were washed an additional time with lysis buffer without Nonidet P-40 and then twice with 1 ml of kinase buffer (see below).

In Vitro Kinase Assays—Kinase assays were performed essentially as described in Ref. 15. Washed immunoprecipitates were incubated in a 60-μl volume containing kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 0.2 mM dithiothreitol), 5–10 μM ATP, 5 μCi of [γ-32P]ATP (6000 Ci/mmol, DuPont NEN) and in the presence or absence of 1 μg of Cdc42-GTPyS or Rac1-GTPyS for 20 min at 30 °C. Incubations were stopped in Laemmli sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis on 10% gels.

Solid Phase Binding Experiments—To assess interactions of PAK1 with GST-Nck fusion proteins, COS-7 cell Nonidet P-40 lysates were incubated for 2 h at 4 °C with equivalent amounts (~5 μg) of pure GST fusion protein and then washed as described above prior to SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose. Gel electrophoresis, transfer of proteins to nitrocellulose membranes, and immunoblotting were performed as described in Ref. 24.

RESULTS AND DISCUSSION

Nck Interacts with PAK1 in Vitro through Its Second SH3 Domain—PAKs have a carboxyl-terminal Ser/Thr kinase catalytic domain that is highly conserved in the Ste20-related kinases (12, 13). In contrast, the amino-terminal regulatory domains of PAKs containing the GTPase binding site are relatively divergent. Studies in our laboratory on the ability of PAK1 to regulate the actin cytoskeleton have indicated the importance of the amino terminus of PAK for interactions with the actin cytoskeleton.7 We therefore initiated experiments to identify proteins that interact with the amino terminus of PAKs. As shown in Fig. 1, we incubated lysates from COS-7 cells that were transiently transfected to overexpress PAK1 with various purified GST fusion protein constructs, washed them, and evaluated binding by immunoblotting. PAK1 did not bind to control GST-coated beads (lane 1) nor to several other GST fusion proteins including the SH3 domains from p120 Ras GAP and the SH2 domain of Nck (data not shown). In contrast, PAK1 bound effectively to full-length Nck (lane 2). Using the same methods, we examined binding to each of the individual SH3 domains of Nck, as well as to a construct encompassing both the second and third SH3 domains. PAK bound to the second SH3 domain but only weakly interacted with the first or third SH3 domains. Binding to the combined second and third SH3 domains was always greater than to the second SH3 domain itself, suggesting that the weak interaction with the SH3 third domain might synergize with the binding to the SH3 second domain. In contrast, the combined second and third Nck SH3 domain constructs bound PAK1 with comparable or slightly greater efficiency than did full-length Nck, indicating there was no additional synergy when the first SH3 domain was also present.

Nck Binding to the First Proline-rich Motif of PAK1—PAK1 has two proline-rich motifs in the amino terminus that have the characteristic PXXP (where X indicates a variable amino acid) structure of SH3 binding domains (20). These consist of the sequences PPAPP (aa 12–16) and PLPPNP (aa 40–45). We prepared peptides encompassing these domains and determined their ability to compete with PAK1 for Nck binding. As shown in Fig. 2, only the peptide (QDKPPAPPMRN) including the first putative SH3 binding site, but not the second (SKLPPNPPEEK), effectively blocked PAK binding to Nck. Both a control peptide from another site in PAK1 rich in proline residues (DATPPPVIAPRPE, aa 182–194) and a peptide derived from an SH3 binding domain on the p85 subunit of phosphotidiole 3-kinase (KISPPTPKRPRPTVPAFG) were unable to block binding. Similar results were obtained using the peptides in a dot blot assay. The peptide results were confirmed by mutagenesis of proline 13 to alanine in order to disrupt the first SH3 binding motif. This mutation in PAK1 caused at least a 10-fold decrease in Nck binding affinity, as determined by semiquantitative dot blot assays (data not shown). Nck therefore binds, at least partially, through the first amino-terminal SH3 binding motif of PAK1. Interestingly, the amino acid sequence of this site is nearly identical in human PAK2 and mouse PAK3, and mouse PAK3 was recently reported to bind to full-length Nck in vitro (21). Therefore, we predict that interaction of Nck with all three PAK family kinases may be important for their regulation.

PAK1 Interacts with Nck in Cells—In order to determine whether Nck binds to PAK1 in intact cells, we transiently overexpressed Nck in COS-7 cells and then immunoprecipitated with specific antibodies to evaluate the association with endogenous PAK1 (Fig. 3A). We observed that we precipitated a 68-kDa kinase, which autophosphorylated only in the presence of recombinant GTPyS-loaded RacI and Cdc42 (Fig. 3A, first panel). This kinase co-migrated exactly with PAK1 precipitated from the same cells with a specific PAK1 antibody (15). Identification of this kinase as PAK1 was supported by the fact that we could also directly detect PAK1 in the Nck precipitate by immunoblotting (Fig. 3A, second panel). Conversely, immunoprecipitation with the PAK1 antibody brought down Nck as well (Fig. 3A, third panel). These data indicated that Nck

FIG. 2. PAK1 binds to Nck via an amino-terminal SH3 binding motif. Binding of PAK1 to GST-Nck second/third SH3 domain protein was performed as described under “Experimental Procedures” but in the presence of the indicated concentrations of peptides. Pro #1, QDK-PPAPPMRN; Pro #2, SKLPPNPPEEK, Pro-R #3, DATPPPVIAPRPE, p85 ProA, KISPPTPKRPRPTVPAFG. C, control (blank) with GST beads alone; Lys, COS cell lysate containing PAK1.
expressed in COS cells interacts with PAK1.

We have shown that PAK1 activity is stimulated by PDGF in Swiss 3T3 cells. We therefore examined the interactions of endogenous Nck and PAK1 in Swiss 3T3 cells in the presence or absence of PDGF (Fig. 3B). In non-stimulated cells, Nck co-precipitated with a Ser/Thr kinase that co-migrated with endogenous PAK1 and whose activity was stimulated by addition of recombinant Rac- or Cdc42-GTP5S (Fig. 3B, first panel). Again, we also observed that this associated kinase was PAK1 as determined by immunoblotting (Fig. 3B, second panel). We examined lysates from Swiss 3T3 cells after stimulation with PDGF for various times. At all times examined from 0 to 10 min, we observed an association of PAK1 with Nck. This is consistent with the report by Chou and Hanafusa (10) that the association of a 68-kDa Ser/Thr kinase with Nck in cells was consistent with the report by Chou and Hanafusa (10) that the association of a 68-kDa Ser/Thr kinase with Nck in cells was constitutive. In Fig. 3B, second panel, we show that there is a ~2.5-fold increase (determined by densitometry) in the amount of PAK1 found in the Nck precipitate after treatment with PDGF for 2 min, suggesting that receptor activation increases or stabilizes the binding of these two proteins to each other. In contrast, treatment of the cells with phorbol 12-myristate 13-acetate, which has been reported to stimulate the phosphorylation of Nck (11), did not increase the level of Nck-associated PAK1.

**PAK1 Phosphorylates Nck at Sites Distinct from PKA**—We examined whether the interaction of Nck with PAK1 had any functional consequences on PAK1 activity in vitro. Binding of Nck to PAK1 had no direct stimulatory effect on PAK1 catalytic activity nor did it alter the ability of PAK1 to interact with and be stimulated by Rac- or Cdc42-GTP5S (data not shown). However, we found that Nck served as an effective substrate for phosphorylation by recombinant constitutively active GST-PAK1 in vitro (Fig. 4). Full-length Nck was phosphorylated as efficiently by PAK1 as it was by PKA, for which Nck is a known substrate (11). Immuno-precipitated and non-activated wild-type PAK1 itself catalyzed very little phosphorylation of Nck but did phosphorylate when stimulated by Cdc42-GTP5S, suggesting that activation of a PAK1-Nck complex by GTPase results in Nck phosphorylation. We used various GST-Nck fusion proteins to evaluate the regions on Nck that became phosphorylated and observed that PAK1 phosphorylated fragments containing the first SH3 domain (aa 1–68) and the SH2 domain (aa 275–377) of Nck. PAK1 also phosphorylated additional sites in the second and third SH3 domain constructs that were not phosphorylated by PKA. These constructs encompass aa residues 101–166 and 191–257, respectively, of Nck, a region that contains multiple serine and threonine residues. The fusion protein containing both the second and third SH3 domains also includes residues 167–190, and this construct serves as a substrate for both PAK1 and PKA. Thus, the association of an activated PAK1 with Nck catalyzes the phosphorylation of Nck on multiple sites. The significance of these phosphorylations on Nck activity is unknown.

In conclusion, the data we have presented here establish the specific interaction of PAK1 with the adapter protein Nck. In intact Swiss 3T3 cells, this interaction is increased by stimulation through the PDGF receptor, suggesting that Nck serves as a means to couple PAK1 activity to PDGF receptor activation. Since Nck has been reported to co-precipitate with the activated PDGF and EGF receptors, it will be of interest to examine whether Nck physically links PAK to such growth factor receptors. We have detected PAK1 in EGF receptor precipitates from A431 cells but have not yet determined if this association is mediated via Nck.

**Fig. 3. Interaction of Nck with PAK1 in intact cells.** The binding of Nck to PAK1 was assessed in COS-7 (Panel A) or Swiss 3T3 (Panel B) cells as described under "Experimental Procedures." The kinase assays shown in Panel A were in the presence of 1 µg of Cdc42-GTP5S; Rac5S in Panel B indicates the presence or absence of 1 µg of Rac preloaded with GTP5S in the kinase assay. The antibodies (Ab) used in each immunoprecipitate (IP) shown are indicated: C, for nonimmune control; P, PAK1 antibody (15); N, Nck polyclonal antibody (Upstate Biotechnology); PMA, phorbol 12-myristate 13-acetate.

**Fig. 4. Phosphorylation of Nck by activated PAK1 compared with PKA.** ~2 µg of GST fusion protein was incubated with either 1 µg of constitutively active GST-PAK1 (15) or 40 units/ml activated catalytic subunit of PKA (Sigma) for 15 min at 30°C under kinase assay conditions (see "Experimental Procedures"). Samples were analyzed on 10% polyacrylamide gels by autoradiography. The numbers at the right of each panel refer to the relevant GST-Nck SH3 constructs. Lane 1, kinase with GST alone (blank); lane 2, full-length (FL) GST-Nck, which is the uppermost band below PAK1 itself (the lower bands are breakdown products of GST-Nck); lane 3, GST-NckSH2; lane 4, GST-NckSH3#1; lane 5, GST-NckSH3#2; lane 6, GST-NckSH3#3; lane 7, GST-NckSH3#2 + SH3#3.

[4] R. H. Daniels and G. M. Bokoch, unpublished observations.
binding domain is critical to the activity of PAK1 to stimulate assembly of the actin cytoskeleton. Mutations in the PAK1 amino terminus that increase its ability to assemble actin also enhance the affinity of PAK1 for binding Nck. These data suggest that the interaction of proteins with SH3 binding domains at the PAK1 amino terminus is a dynamic process that contributes to the regulatory effects of PAK1 on cell function. The role which the Nck-PAK interaction plays in this process remains to be determined in future studies.

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