Nckβ Adapter Regulates Actin Polymerization in NIH 3T3 Fibroblasts in Response to Platelet-Derived Growth Factor bb

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The SH3-SH3-SH2 adapter Nck represents a two-gene family that includes Nckα (Nck) and Nckβ (Grb4/Nck), and it links receptor tyrosine kinases to intracellular signaling networks. The function of these mammalian Nck genes has not been established. We report here a specific role for Nckβ in platelet-derived growth factor (PDGF)-induced actin polymerization in NIH 3T3 cells. Overexpression of Nckβ but not Nckα blocks PDGF-stimulated membrane ruffling and formation of lamellipodia. Mutation in either the SH2 or the middle SH3 domain of Nckβ abolishes its interfering effect. Nckβ binds at Tyr-1009 in human PDGFR receptor β (PDGFRβ) which is different from Nckα's binding site, Tyr-751, and does not compete with phosphatidylinositol-3 kinase for binding to PDGFR. Microinjection of an anti-Nckβ but not an anti-Nckα antibody inhibits PDGF-stimulated actin polymerization. Constitutively membrane-bound Nckβ but not Nckα blocks Rac1-L62-induced membrane ruffling and formation of lamellipodia, suggesting that Nckβ acts in parallel to or downstream of Rac1. This is the first report of Nckβ's role in receptor tyrosine kinase signaling to the actin cytoskeleton.

Src homology (SH) domains, including SH2 and SH3 domains, are protein modules found in many otherwise functionally distinct molecules (25). The ligands for SH2 and SH3 domains are phosphotyrosine-containing peptides (pY-X-X-X) and proline-rich peptides (P-X-X-P), respectively. The C-terminal amino acid residues of the phosphotyrosines and the flanking amino acid residues of proline-rich segments determine binding affinity and specificity (26, 35). A family of SH2 and SH3 domain-containing proteins, including Crk, Grb2, and Nck, contain only SH2 and SH3 domains and have no other functional motifs (2, 19). They are therefore regarded as adapters. They act by binding to tyrosine-phosphorylated proteins via SH2 domains and associating with P-X-X-P motif-containing proteins through SH3 domains. SH3-associated proteins often get translocated to the proximity of phosphotyrosine proteins (32). Thus, SH2 and SH3 domains act as a second messenger connecting protein tyrosine phosphorylation to a variety of intracellular signaling networks. The best-characterized adapter is the SH3-SH2-SH3 protein Grb2. Grb2 binds to two separate pY-X-X-V motifs in the epidermal growth factor receptor (EGFR) via its SH2 domain and associates through its two SH3 domains to the PPIX/PPRRR motifs in Sos, a guanine nucleotide exchange factor for Ras. As a result, Sos is translocated to the plasma membrane and activates Ras (32).

Nck contains three consecutive SH3 domains and one SH2 domain, which together occupy more than 70% of Nck's 377 amino acids (16). Similar to Grb2, Nck is widely expressed in various types of cells and acts as an adapter by linking receptor tyrosine kinases to downstream signaling networks. It has also been reported that there is a fraction of Nck that is associated with Sam68 in the nucleus (14), although its function remains unknown. The SH2 domain of Nck has been shown to bind either directly or indirectly to EGFR, platelet-derived growth factor receptor (PDGFR). Eph receptor, insulin receptor substate 1, p130cas, and p62Dok (16, 20). For example, tyrosine 751 (Y751) in human PDGFR-β was identified as the binding site for Nckα. Since Y751 is also one of the two binding sites in the PDGFR for the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) (the p85 subunit has two SH2 domains), Nckα and PI3-K may either compete with each other for binding to the PDGFR and thereby antagonize each other's function or bind to different pools of the cell surface PDGFR (22). There has been no evidence so far for or against either of these hypotheses. Stein et al. identified a binding site for Nck in the Eph family receptor, Eph1 (ELK). They showed that Y-594 in the juxtamembrane region of Eph1 recruits Nck to the plasma membrane. The membrane-bound Nck in turn causes, apparently via Nck-interacting kinase (NIK), activation of the JNK/SAPK pathway (1, 36, 37). The current list of Nck (α, β, or both) SH3-binding molecules includes the Abl protein tyrosine kinase, Sos, Nck-associated kinase (NAK), p21cdc42/60-inactivated kinases (PAK), Rho effecter PKN-related kinase PK2, protooncogene c-cbl, human Wiskott-Aldrich syndrome protein (WASP), the novel serine threonine kinase NIK, casein kinase 1 gamma-2, Sam68, Nap1 (Nck-associated protein 1), and NAP4 (Nck-, Ash- and PLCγ-binding protein 4) (16). We and others recently reported that Nck represents a family of genes including two human (Nck/hNckα and hNckβ/Nck2) and two mouse (mNckα and mNckβ/Grb4) Nck genes (3, 4, 25, 40). hNckα and hNckβ reside in different chromosomes (4, 10, 41) and are coexpressed in most but not all cells (3). The newly identified Nckβ binds significantly better than Nck (Nckα) to both receptor and nonreceptor tyrosine kinases (3, 4, 40). Moreover, Nckα and Nckβ appear to have distinct functional assignments in the same cells (4).

Recently, a growing number of studies have suggested that Nck plays an important role in mediating receptor tyrosine kinase
kinase signaling to the actin cytoskeleton. Rockow et al. showed that overexpression of Nck blocks nerve growth factor- and basic fibroblast growth factor-induced neurite outgrowth, a Rac1/Cdc42 GT-Pase-dependent actin cytoskeletal change, in rat adrenal pheochromocytoma cells PC12, through an extracellular signal-regulated kinase-independent mechanism (29). Two Nck-SH3-binding proteins, WASp and Pak1, have clear roles in regulation of the actin cytoskeleton through either Cdc42- and Rac-dependent or -independent mechanisms, Symons et al. showed that WASp binds to GTP-bound Cdc42 and clusters in polymerized actin (38). N-WASP (richest in neural tissues) is also involved in Cdc42 signaling to the actin cytoskeleton (21). Sells et al. reported that Pak1-induced actin organization depends upon binding to Nck but not upon Paxil kinase activity or binding to Rac1 and Cdc42 (33). They showed that a kinase-dead Paxil could mimic the effect of Rac and induce lamellipodia formation (33). Consistent with their observations, Obermeier et al. showed that brain-specific Pax (ypPak/Pak3) induces cell spreading, membrane ruffling, and increased lamellipodia formation (24). The strongest support of the notion that Nck links tyrosine kinases to the actin cytoskeleton comes from a genetic study of Drosophila melanogaster. Each of the eight R cells (R1 to R8) of the Drosophila compound eye is a distinct neuron and acts as a photoreceptor. Guidance and target recognition of these R cells toward axons are believed to be regulated by receptors at the surface of the growth cone, which resides at the leading edge of the axon. The growth cones receive extracellular cues and in turn control the intracellular actin cytoskeletal rearrangement. Zipursky and his colleagues found that the gene called Dreddocks, or Dock, was concentrated in the R-cell, or Dock, was confirmed by DNA sequencing analyses. The constructs were transfected into NIH 3T3 cells, and membrane attachment was confirmed by a cell fractionation study (see below).

Cell fractionation, immunoprecipitation, and immunoblotting. Transfected NIH 3T3 cells in 15-cm tissue culture dishes were scraped off in 2 ml of ice-cold phosphate-buffered saline (PBS) containing 1 mM sodium docosahexaenoic acid (DHA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM orthovanadate. Cell pellets, obtained by sequentially repeating the above procedure. The final PCR were purified and subcloned into pPRK at the Xhol site, and sequences were confirmed by DNA sequencing analyses. The constructs were transfected into NIH 3T3 cells, and membrane attachment was confirmed by a cell fractionation study (see below).

Site-directed mutagenesis. Human Nckα and Nckβ genes have been described previously (4). Mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene). PCRs were carried out using an overlap exten-
FIG. 1. Overexpression of Nckβ but not Nckα blocks PDGF-stimulated membrane ruffling. NIH 3T3 cells, cultured in fibronectin-coated (10 μg/ml, 2 h) eight-chamber culture slides, were either untransfected (A and B), transfected with vector alone mixed with a GFP-containing vector (C and D), or transfected with the wild-type Nckα (E to F') or Nckβ (G to H') construct at 0.5 μg/well. After 48 h, cells were starved in low-serum medium for an additional 18 h and treated (B, D, F, and H) or not (A, C, E, and G) with PDGF-bb (100 ng/ml) at 37°C for 15 min. Expression of the Nck proteins was monitored by anti-HA antibody blotting, followed by a secondary antibody conjugated with FITC. The actin cytoskeleton was revealed by rhodamine-labeled phalloidin staining. Eighty to 100 cells which showed positive FITC staining were selected and analyzed in each experiment. Vector-transfected cells were identified as GFP positive. Images were recorded with a Zeiss confocal microscope. Magnifications, × 150. This experiment was repeated four times.
FIG. 2. Nckβ binds at Y1009 in the PDGFR and its overexpression did not affect PI3-K binding to the PDGFR. (A) TRMP cells expressing wild-type (wt) or mutant PDGFR were serum starved and either untreated or treated with PDGF-βb (400 ng/ml) for 5 min. Total lysates (50 μg of protein per lane) of the cells were resolved in an SDS gel, transferred to a nitrocellulose membrane, and blotted with either antiphosphotyrosine (anti-PY) (a) or purified GST-Nckβ (3 μg/ml), followed by anti-GST antibody blotting (b) at 4°C for 2 h. Membranes were washed, and the results were visualized with ECL. (B) Lysates of cells (2 × 10⁶ cells/dish), untransfected (lanes 1 and 2) or transfected with 1.5 μg (lanes 3 and 4) or 5 μg (lanes 5 and 6) of Nckβ cDNA, and either untreated or treated with PDGF (5 min, 37°C), were immunoprecipitated (IP) with an anti-p85 antibody (Z-8; Santa Cruz). The immunoprecipitates were resolved in an SDS gel, transferred to a nitrocellulose membrane, and immunoblotted with either monoclonal anti-PDGFR antibody (61520.11; R&D Systems) (a) or the anti-p85 antibody (b), or the same set of total lysates (30 μg of protein/lane) were directly analyzed by Western blot using anti-HA (12CA5) antibody (c). Results were visualized by ECL. (C) Schematic representation of binding of the two Ncks to human PDGFR-β together with their shared binding partners.
PDGF was visualized by further immunoblotting with anti-GST antibody, followed by ECL.

**Generation of anti-Nck-specific antibodies.** Full-length Nckα and Nckβ cDNAs were fused with the bacterial GST gene in the pGEX vector and expressed in the bacterial strain XL-1. Fusion proteins were purified and used to immunize three rabbits per antigen. All the initial antisera cross-reacted to the portion of Nckα-interacting antibodies. Anti-Nckβ antibodies were further purified from the leftover supernatant with a GST-Nckβ fusion protein affinity column, in accordance with a previously published procedure (7). Likewise, to isolate anti-Nckβ-specific antibodies, each of the anti-Nck antisera was passed through a GST-Nck fusion protein column to remove the portion of Nckα-binding immunoglobulin G (IgG) molecules. The leftover supernatant was subjected to purification by a GST-Nck fusion protein affinity column, in accordance with a previously published procedure (7). Likewise, to isolate anti-Nckβ-specific antibodies, each of the anti-Nck antisera was passed through a GST-Nckα fusion protein column to remove the portion of Nckβ-binding immunoglobulin G (IgG) molecules. The leftover supernatant was subjected to purification by a GST-Nckα fusion protein affinity column, in accordance with a previously published procedure (7).

**Immunofluorescence microscopy.** Serum-starved parental cells, transfected cells, and microinjected cells on coverslips were either untreated or treated with PDGF-bb (100 ng/ml) for 15 min at 37°C. Cells were rinsed with PBS and fixed in freshly prepared 4% (wt/vol) paraformaldehyde in PBS for 10 min. Cells were rinsed twice with PBS and permeabilized in PBS containing 0.2% Triton X-100 for 5 min. Following a PBS rinse, cells were incubated with primary antibodies anti-HA (12CA5, 2 µg/ml) or rabbit anti-Myc antibody (N-262; Santa Cruz; 4 µg/ml) or both in PBS containing 1% BSA for 2 h. Cells were rinsed and incubated with secondary antibody mixtures containing FITC-conjugated rabbit anti-mouse IgG (Jackson Laboratory; 10 µg/ml) and/or AMCA (coumarin)-conjugated goat anti-rabbit IgG (Sigma, 10 µg/ml) together with TRITC (rhodamine)-conjugated phalloidin (0.1 µg/ml; Molecular Probes) for 45 min. Therefore, in cells simultaneously transfected with Myc-tagged Rac/Cdc42 and HA-tagged Nck, expression of these genes and changes in actin polymerization in a single cell could be visualized by triple (green [FITC], blue [AMCA], and red [TRITC]) staining. Cells were rinsed three times with PBS (10 min of incubation each time) and air dried. The coverslips were mounted with anti-fade reagent (Molecular Probes). Expression of transfected genes and actin polymerization in the cells was examined by confocal microscopy (at the University of Southern California Confocal Core Facility), using ZEISS 100X 1.0 oil immersion objectives. Thirty to 120 randomly selected cells from either vector-transfected population or gene-transfected double (FITC and TRITC)- or triple-stained (FITC, AMCA, and TRITC) populations were analyzed for peripheral filamentous actin in membrane ruffles. Images shown are representative of significantly responding cells under each condition. The percentage of cells that had undergone membrane ruffling was calculated as responding cells over total positively stained cells.

### RESULTS

**Overexpression of Nckβ but not Nckα blocked PDGF-induced actin polymerization.** Prompted by the finding that *Drosophila* Nck-like gene, *Dock*, plays a critical role in mediating extracellular cues to intracellular actin cytoskeleton at the growth cone during axon guidance and targeting (5, 27), we were interested in understanding whether or not Nck has a similar function in mammalian cells. We chose PDGF signaling in fibroblasts as the biological system, because it has been well established that in these cells PDGF stimulates, via Rac, actin polymerization, which leads to formation of membrane ruffles and lamellipodia (23, 28), and Nck is a direct target for the PDGF (15, 22). We started out by confirming the PDGF effect in NIH 3T3 cells and by testing whether or not Nck regulates PDGF signaling to the actin cytoskeleton. It is shown in Fig. 1 that in quiescent (serum-starved) cells, a...
A fine ring of polymerized actin at the periphery of the cell was seen by staining with rhodamine-labeled phalloidin (Fig. 1A). Following PDGF treatment for 15 min, a dramatic alteration in the actin cytoskeleton of the cell, including accumulation of polymerized actin in the peripheral plasma membrane and formation of lamellipodia and membrane edge ruffles, could be visualized (Fig. 1B) under the fluorescent microscope. Of the 212 parental cells examined, 208 showed this phenotype (see statistical analyses in Fig. 5). Similar results were observed in cells transfected with an empty pRK5 expression vector (Fig. 1D versus C). These results have established NIH 3T3 cells as an adequate cell culture system for studying PDGFR signaling to the actin cytoskeleton.

We have previously shown that Nckα binds directly to human PDGFR at Y751 (22), and Nckβ binds 10 times better than Nckα to the PDGFR, via an unknown site (4). We tested if overexpression of HA-tagged wild-type Nckα and Nckβ would interfere with (enhance and suppress) PDGF-stimulated actin polymerization. To examine expression of transfected HA-Nck and changes of the actin cytoskeleton in the same cells, HA-Nck-positive cells were identified by anti-HA antibody blotting, followed by FITC-conjugated secondary antibody staining, whereas the actin cytoskeleton was visualized by staining with rhodamine-labeled phalloidin, as previously used. It is also shown in Fig. 1E to H that enforced overexpression of wild-type Nckα or wild-type Nckβ had no significant effect on the actin structure in serum-starved, unstimulated cells (Fig. 1E and G). Expression of HA-Nckα or HA-Nckβ protein in the same cells was indicated by FITC staining (Fig. 1E’ and G’). In the PDGF-stimulated cells, cells transfected with wild-type Nckα exhibited a pattern of actin assembly similar to that in the cells transfected with the vector alone (Fig. 1F versus D), i.e., polymerized actin assembly at the leading edge of the plasma membrane and formation of membrane ruffles.

FIG. 4. SH2 and middle SH3 domains of Nckβ are required for the regulatory effect of Nckβ on PDGFR signaling. Cells were transfected with either the SH2 mutant Nckβ-R312K (A, A’, B, and B’), with the triple SH3 mutant Nckβ-W39/149/235K (C, C’, D, and D’), or with the individual SH3 mutants indicated (E to J’). The rest of the experimental procedures were identical to those described for Fig. 1. Three independent experiments were carried out, and they showed similar results.
expression of HA-Nckα protein in the same cell was indicated by FITC staining (Fig. 1F'). Surprisingly, in cells transfected with the wild-type Nckβ, the PDGF-stimulated accumulation of actin in membrane ruffles was dramatically inhibited (Fig. 1H) in more than 80% of HA-Nckβ-positive cells (71 of 87) examined (see statistical analysis in Fig. 5). Expression of transfected HA-Nckβ in the same cell was indicated by FITC staining (Fig. 1H'). These observations suggest that Nckβ but not Nckα participates in PDGF signaling to the actin cytoskeleton.

Nckβ binds to a distinct site in the PDGFR and does not compete with PI3-K binding. One could argue that the inhibitory effect of Nckβ was due to nonspecific binding competition
for PDGFR, occupying the binding sites of other signaling proteins such as PI3-K and Nckα, which have a common binding site, Y751 (22). To address this problem, we set out to identify the Nckβ binding site in human PDGFR-β. TRMP cells expressing all the possible PDGFR phosphorytrosine mutants, previously described (12), were used for the experiment. Lysates of these cells either untreated or treated with PDGF were resolved in duplicate in SDS gels, transferred to a nitrocellulose membrane, and blotted either with an antiphosphotyrosine antibody or with purified GST-Nckβ proteins. The GST-Nckβ-bound PDGFR was further visualized by anti-GST antibody immunoblotting, followed by ECL. The advantage of this technique is that it allows determination of direct interaction between Nckβ and PDGFR. It is shown in Fig. 2Aa that comparable amounts of PDGFR in various cell lines were subjected to the binding study. While GST-Nckβ was able to bind the wild type and most of the PDGFR mutants (Fig. 2Ab, lanes 2 to 8 lane 11), its binding to the PDGFR with a single mutation at Y1009 or double mutations at Y1009 and Y1021 was dramatically reduced (lanes 9 and 10). The slightly reduced binding to the Y740/751F mutant was not always reproducible.

Similar results were observed in co-immunoprecipitation experiments using our newly developed anti-Nckβ-specific antibodies (data not shown). These results demonstrate that Nckβ binds to Y1009 on human PDGFR-β. Since this site has previously been shown to be the binding site for the SH2 domain of SHP2 (13), Nckβ, similar to Nckβ, shares a binding site with another SH2-containing protein. We then tested whether or not overexpressed Nckβ would cause nonspecific competition for other binding sites on PDGFR. We compared the binding of PI3-K’s p85 subunit to PDGFR in control and Nckβ-overexpressing cells, particularly because PI3-K has been shown to play an important role in PDGF-stimulated actin polymerization (8, 42, 43) and shares the binding site Y-751 with the SH2 domain of Nckα. Figure 2B clearly shows that increasing concentrations of Nckβ expression in cells (c, lanes 3 to 6 versus lanes 1 and 3) did not affect the amount of p85-coimmunoprecipitated PDGFR (Fig. 2Ba, lanes 4 and 6 versus lane 2). Similar amounts of p85 were recovered by anti-p85 antibody immunoprecipitation (Fig. 2Bb, lanes 1 to 6). These results suggest that the observed inhibitory effect of Nckβ on PDGFR signaling to the actin cytoskeleton was probably not due to nonspecific binding competition, although we did not test this for each of the dozen previously shown PDGFR-binding proteins.

Unfortunately, because Y1009 is also shared by the SH2 domain of SHP2, the PDGFR-Y1009F mutant cannot be used to evaluate the specificity of Nckβ’s effect. Instead, another approach has been used; see below. A schematic representation of the binding of the two Ncks to human PDGFR is shown in Fig. 2C, in which both Nckα and Nckβ share a binding site with another PDGFR-interacting protein(s).

**Mutations in the SH2 and SH3 domains of Nckβ abolish its interfering effect.** To study the possible mechanism of Nckβ’s action, we generated HA-tagged SH2 and SH3 mutants of these two genes, as schematically shown in Fig. 3. The highly conserved arginine (R) of the FVLRES motif in the SH2 domains and the first tryptophan (W) of the characteristic double tryptophans in the SH3 domains were replaced with lysine residues (K). Figure 3A and B show the list of HA-tagged wild-type and SH2 mutants and SH3 mutants of Nckα and Nckβ, respectively. To confirm the expression of these transgenes, pRK5-cDNA constructs were transfected into NIH 3T3 cells, and lysates of the transfected cells were immunoblotted with anti-HA-tagged antibody (the transfection efficiency by Superfect reagent was around 35% for NIH 3T3 cells). It is shown in Fig. 3C and D that a similar level of protein expression of the various forms of Nckα (C) and Nckβ (D) genes was achieved. When the same samples were immunoblotted with an anti-Nckβ or anti-Nckβ antibody (71-2800; Zymed), which recognize both HA-tagged and endogenous Nck, five- to sevenfold-higher expression of HA-Nck over endogenous Nck was observed (data not shown).

We first investigated whether or not the SH2 domain of Nckβ was required for its dominant interfering effect. Cells were transfected with the Nckβ SH2 mutant, Nckβ-R312K, and either untreated or treated with PDGF. It is shown in Fig. 4 that expression of HA-Nckβ-R312K was indicated by anti-HA antibody blotting followed by FITC antibody staining (A’ and B’). Rhodamine-labeled phalloidin staining of the same cells revealed that Nckβ-R312K had little effect on actin polymerization in the absence of PDGF (Fig. 4A). However, in contrast to the effect of wild-type Nckβ, Nckβ-R312K was no longer able to block PDGF-induced membrane ruffling (Fig. 4B). These results suggest that binding to PDGF is essential for the function of Nckβ. We then tested the role of the three SH3 domains by using an SH3 triple mutant of Nckβ, Nckβ-W39/149/235K, Nckβ-W39/149/235K also failed to block PDGF-stimulated membrane ruffling (Fig. 4D versus C). Expression of HA-Nck-W39/149/235K was indicated by anti-PA antibody blotting followed by FITC antibody staining (Fig. 4C’ and D’). In 89 cells examined, all of which positively expressed Nckβ-W39/149/235K, we did not detect any significant inhibition of PDGF-induced membrane ruffling and lamellipodium formation (see statistical analysis in Fig. 5).

The effect of Nckβ-W39/149/235K on PDGFR signaling was unexpected. We initially had predicted that this mutant should have a strong dominant negative effect because its SH2 domain was still intact and could compete with endogenous Nckβ for binding to PDGFR. A possible explanation is that mutations in SH3 domains might have weakened SH2 binding to phosphotyrosine. Interestingly, while there is currently no evidence either for or against this hypothesis, similar results were previously reported for the *Drosophila* Nck-like gene Dock, for which it was shown that a similar mutant had no dominant negative effect (27). To identify the specific SH3 domain(s) which is required for the interfering action of Nckβ, we tested the effects of each of the individual SH3 domain mutations of Nckβ, W39K, W149K, and W235K. It is also shown in Fig. 4 that Nckβ-W39K (E and F) and Nckβ-W235K (I and J) were still able to block PDGF-stimulated membrane ruffling (F versus E and J versus I). Interestingly, the Nckβ-W149K mutant failed to inhibit PDGF-induced actin polymerization in the cell (Fig. 4G and H), resulting in clearly detectable PDGF-induced membrane ruffling (Fig. 4H versus G). These results indicated that the middle SH3 domain of Nckβ plays a critical role. The statistical analysis of these data is summarized in Fig. 5.

**Microinjection of anti-Nckβ-specific antibody inhibits PDGF-stimulated actin polymerization.** As mentioned previously, since Y1009 is also the binding site for SHP2, the PDGFR-Y1009F mutant became less useful for determining the specific effect of Nckβ on PDGFR-mediated actin polymerization. Therefore, we undertook a microinjection approach. We first generated anti-Nckα and anti-Nckβ antibodies that recognize the native forms of Nckα and Nckβ, respectively. It is shown in Fig. 6A that a commercial anti-Nck antibody (71-2800; Zymed) recognized both HA-tagged Nckα and Nckβ proteins (lanes 1 and 2). In contrast, our anti-Nckα and anti-Nckβ antibodies only recognized HA-tagged Nckα (lanes 3 versus 4) and HA-tagged Nckβ (lanes 5 versus 6), respectively. To confirm that
both Nckα and Nckβ are expressed in NIH 3T3 cells, total lysates of NIH 3T3 and seven other cell lines were immuno-
blotted with either anti-Nckα (Fig. 6B) or anti-Nckβ (Fig. 6C) antibody. It is clearly shown that Nckα is expressed in all the
cells tested (Fig. 6B), whereas Nckβ is expressed in most but
not all of the eight cell lines tested (Fig. 6C). Nonetheless,
Nckα and Nckβ are coexpressed in NIH 3T3 cells (indicated by
arrows). The anti-Nckα and anti-Nckβ antibodies showed neu-
tralizing effects in vitro, since they blocked GST-Nckα and
GST-Nckβ binding to PDGFR in a concentration-dependent
fashion (data not shown).

These antibodies were further purified and used for micro-
injection. Figures 6D to F’ show that microinjection of either
an irrelevant rabbit immunoglobulin (D and D’) or anti-Nckα
(E and E’) antibody did not affect PDGF-induced membrane
ruffling (D’ versus D and E’ versus E). In contrast, microin-
jection of the anti-Nckβ antibody significantly, albeit not com-
pletely, inhibited the effect of PDGF (Fig. 6F’ versus F). These
results were reproducible in three independent microinjection
experiments. We conclude that Nckβ regulates PDGFR sign-
aling to the actin cytoskeleton.

Membrane-bound Nckβ inhibits Rac signaling. To gain fur-
ther insights into the mechanisms of Nckβ action, we tested
whether or not Nckβ interferes with Rac1 signaling, which is
known to mediate PDGF-induced formation of lamellipodia
and membrane ruffles (28). A Myc-tagged, constitutively active
Rac1 (Rac1-L61) was introduced into NIH 3T3 cells with
and without cotransfection with Nckα or Nckβ. Constitutively ac-
tive Cdc42 (Cdc42-L61) and Rho (Rho-L63) were included as
controls. Previous studies indicate that membrane localization
is the key step for Nck to activate PAK (17, 34). Therefore, we
speculated that if the binding of Nckβ to PDGFR, i.e., relo-
cation from the cytoplasm to the plasma membrane, is an
essential step for Nckβ to execute its interfering effect on
PDGFR signaling, one would need to construct a constitutively
membrane-bound Nckβ to mimic the “active stage” (PDGFR
bound) of Nckβ.

Figure 7 shows that the farnesylation signal sequence of Ras,
KLNPFPDESGPGCMSGKCVLS, was fused to the carboxyl ter-
minal of Nckβ and Nckα to create Nckβ-mem and Nckα-mem,
respectively (Fig. 7A). To verify the effectiveness of the farnes-
ylation signal sequence, transfected cells were fractionated
into membrane, cytosol, and nucleus fractions. Equal portions
of the cellular fractions were resolved by an SDS gel, trans-
ferred to a nitrocellulose membrane, and immunoblotted with
anti-HA antibody. It can be seen that the majority of wild-type
Nckβ was detected in the cytosol fraction (Fig. 7B, lane 2
versus lanes 1 and 3), and a small amount was detected in the
nuclear fraction (lane 3). However, over 50% of the HA-Nckβ-
mem was found in the membrane fraction (lane 4 versus lanes
5 and 6). The small amount of Nckβ-mem that still remained
in the cytosol fraction (lane 5) is most likely the unfarnesylated
portion of Nckβ-mem. Similar results were observed for Nckα-
mem (Fig. 7C). The majority of the Nckα-mem was found in
the membrane fraction (lane 4 versus lane 1). The amounts of
membrane-associated Nckβ (Nckβ-mem) and Nckα (Nckα-
mem) should be regarded as highly significant, because even in
PDGF-stimulated cells, only a small percentage (~5 to 7%) of
Nck binds to the activated PDGFR (15, 22). These membrane-
bound Nck gene constructs were cotransfected with the Rho
GTPases, and their effects on the GTPases’ signaling were
investigated. In these experiments, coexpression of HA-Nck and
Myc-Rac1 in the same cells was differentiated by double stain-
ing with FITC-conjugated (green) rabbit anti-mouse IgG (against
anti-HA monoclonal antibody) and AMCA-conjugated (blue)
goat anti-rabbit IgG (against rabbit anti-Myc antibody), while

FIG. 5. Statistical analysis of data shown in Fig. 1 and 4. FITC-staining cells
(80 to 100 cells for each of the conditions) were randomly selected and analyzed
for membrane ruffling and lamellipodium formation in response to PDGF stimu-
luation. Values are [(number of actin-polymerized cells)/(total number of cells)] ×
100. Due to variations inogenous expression levels of any given HA-tagged
Nck construct in different cells, degrees of PDGF-stimulated actin polymeriza-

the changes in actin polymerization was again visualized by
TRITC-conjugated phalloidin.

Consistent with previously published studies (6), expression of
Rac1-L61 induced dramatic lamellipodia and membrane
ruffles as well as filopodia in NIH 3T3 cells (Fig. 8A). The
filopodium formation was likely due to activation of Cdc42 by
Rac1-L61 in these cells. Expression of Cdc42-L61 strongly in-
duced filopodium formation (Fig. 8F). For unknown reasons,
Rho-L63 did not cause clear actin stress fiber formation in
NIH 3T3 cells (data not shown). Cells cotransfected with wild-
type Nckα showed little inhibition of Rac1-L61-induced lamel-
lipodium formation and membrane ruffling, although filopodia
no longer appeared (Fig. 8B). Even the membrane-bound
Nckα produced no effect (Fig. 8C). Cells cotransfected with
wild-type Nckβ exhibited a moderate inhibition of lamellipo-
dium formation and membrane ruffling, although thickness of
the ruffled membrane was still evident (Fig. 8D versus 8A).
Moreover, this moderate inhibition occurred in only 15% of
the positively stained cells examined.

Interestingly, cotransfection with Nckβ-mem resulted in dra-
matic inhibition of Rac1-L61-induced lamellipodium forma-
tion (Fig. 8E) in more than 50% of the Nckβ-mem-positive
cells, in which membrane ruffling was almost completely gone
and a rather thin and smooth membrane appeared. The statistical
analysis of Nckβ-mem’s effect on Rac1-L61 is summarized in
Fig. 8K.

In contrast, neither Nckβ nor Nckβ-mem showed any inhibi-
tory effect on Cdc42-L61-induced filopodium formation (Fig.
8I and J), suggesting that the effect of Nckβ-mem on Rac1-L61
was specific. Similarly, neither Nckα nor Nckα-mem had any
effect on Cdc42-L61-induced filopodium formation (Fig. 8G
and H). As previously mentioned, since constitutively active
Rho1, Rho1-L63, did not cause significant stress fiber forma-
FIG. 6. Microinjection of anti-Nckβ but not anti-Nckα antibodies blocks PDGF-stimulated actin polymerization. (A) Lysates of HA-Nckα-transfected (lanes 1, 3, and 5) or HA-Nckβ-transfected (lanes 2, 4, and 6) cells were resolved in an SDS gel, transferred to a nitrocellulose membrane, and blotted with either 71-2800 (Zymed; cross-reacting with α and β) (lanes 1 and 2), anti-Nckα (lanes 3 and 4), or anti-Nckβ (lanes 5 and 6) antibody. Results were visualized by ECL. (B and C) Total lysates of the eight indicated cell lines were resolved in duplicate SDS gels and subjected to Western blotting using either anti-Nckα (B) or anti-Nckβ (C) antibody, followed by ECL. (D to F') Serum-starved NIH 3T3 cells, cultured in eight-chamber coverslips, were microinjected with either control IgG or antibodies (500 ng/ml), together with FITC-dextran as a marker protein to identify injected cells. Cells were then stimulated with PDGF-bb (100 ng/ml) for 15 min at 37°C. The actin cytoskeleton was revealed by rhodamine-labeled phalloidin staining as described in the text. Images were recorded with a Zeiss confocal microscope (magnification, ×150). For one experiment, 25 to 50 cells were injected with each antibody, and the experiment was repeated three times.
stimulated actin polymerization and subsequent membrane
fering effect of Nck

we have investigated the roles of Nck
have their own specific functions in cells. In the current study,

portions of each of the fractions were analyzed by Western blot analysis using

text) to obtain the membrane (m), cytosol (c), and nuclear (n) factions. Equal

cells. After 48 h, cells were subjected to a cellular fractionation procedure (see

tical analysis of Nck
's effect on Cdc42-L61 is summarized in

DISCUSSION

Nck has been implicated to play a role in cell mitogenesis and morphogenesis. Recent genetic studies in Drosophila sug-

t that Nck links cell surface tyrosine phosphorylation to the

in NIH 3T3 cells, we were not able to assess the effect of

Nckα-mem or Nckβ-mem on Rhoh1-L63 signaling. The statistical

analysis of Nckβ's effect on Cdc42-L61 is summarized in

the third SH3 domains could mediate the signaling events (27).

either by itself or in combination (27). We made sim-

ulations. We initially predicted that the middle SH3

mutation and the SH3 triple mutations should act in a strong
dominant negative fashion, but they did not. It is possible that

the middle SH3-binding protein plays a critical role, the over-

expressed Nckβ would have its middle SH3 domain unoc-

creregulator such as a protein tyrosine phosphatase, would

expression of Nckβ, similar to overexpression of a negative

regulator such as a protein tyrosine phosphatase, would

enhance its endogenous inhibitory effect. In fact, the results of

our mutagenesis studies favor this hypothesis, in which both

the SH2 and the triple and middle SH3 mutants are no longer

able to block PDGFR signaling, or the negative signal can no

longer be propagated through these mutants. While future

studies will be required to further distinguish between these

possibilities, the results of our microinjection experiments strongly

argue that Nckβ plays a direct role in PDGFR signaling to the actin cytoskeleton.

During the course of this study, a critical issue was the specific-

ity of Nckβ action. We initially argued that overexpressed

Nckβ may have had nonspecific competition for binding to

other phosphotyrosine sites in addition to binding to its own

site in the activated PDGFR. In this case, overexpressed Nckβ

could prevent other PDGFR-binding molecules from getting

into their sites, by which PDGFR signaling to the actin cyto-

skeleton was indirectly blocked. This argument has since been

challenged by three lines of evidence that strongly suggest that

the interfering effect of the overexpressed Nckβ was specific

for Nckβ. First, overexpression of the other Nck family mem-

ber Nckα, which has previously been shown to share a phos-
phototyrosine binding site with one of the two SH2 domains of the p85 subunit of PI3-K (22), did not show any interfering effect on either PDGFR or Rac-L61 signaling to the actin cytoskeleton, even though the SH2 domains of Nckβ and Nckα have a high degree (85%) of homology. In particular, since PI3-K has been reported to play a role in PDGF signaling to the actin cytoskeleton (6, 8), Nckα, not Nckβ, would be considered more likely to block PDGF-stimulated actin polymerization. The fact that Nckα did not inhibit PDGFR/PI3-K signaling to the actin cytoskeleton can be explained by the fact that the p85 subunit has two SH2 domains and its binding to Y740 has a much higher affinity than the binding to Y751 (11). p85 could even bind PDGFR with a mutation at Y751, where Nckα binds. It has recently been shown that tyrosine-778 (its binding protein remains unknown) in PDGFR-β plays an important role in PDGFR signaling to the actin cytoskeleton (31). Thus, multiple PDGFR-binding proteins may be involved in regulation of the actin cytoskeleton. Second, overexpression of membrane-bound Nckβ inhibited the constitutively active Rac-L61-induced membrane ruffling and lamellipodium formation, in which SH2 domain binding was apparently not involved because of a lack of PDGFR activation. This observa-
FIG. 8. Nckβ-mem but not Nckα-mem inhibits Rac1-induced membrane ruffling and lamellipodium formation. Cells were transfected with Rac1-L61 (A) or Cdc42-L61 (F)(0.3 mg/well) alone or cotransfected with Rac1-L61 plus wild-type Nckα (B), Rac1-L61 plus Nckα-mem (C), Rac1-L61 plus wild-type Nckβ (D), Rac1-L61 plus Nckβ-mem (E), Cdc42-L61 plus wild-type Nckα (G), Cdc42-L61 plus Nckα-mem (H), Cdc42-L61 plus wild-type Nckβ (I) or Cdc42-L61 plus wild-type Nckβ-mem (J) (Rac/Cdc42: Nck ratio, 0.3:2.5). To identify the double-transfected Rac1/Cdc42 plus Nck cells, staining with a combination of mouse anti-HA antibody 12CA5 followed by FITC-conjugated rabbit anti-mouse IgG and rabbit anti-Myc antibody followed by AMCA-conjugated goat anti-rabbit IgG was used. Changes in actin polymerization were detected by TRITC-conjugated phalloidin. Statistical analysis of Rac1 (K) and Cdc42 (L) was made from four independent experiments and they showed similar results.

The first two authors contributed equally to this work.

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