Identification of Nck Family Genes, Chromosomal Localization, Expression, and Signaling Specificity*

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Already a dozen molecules share binding to the Src homology (SH) 3 domains of human Nck, an SH3-SH3-SH2 adapter protein. We reason that there may be multiple gene members of Nck to accommodate the large binding repertoires. Here we report identification of novel human and mouse Nck genes and rename them as the Nckα and Nckβ genes (including the human Nckα, human Nckβ, mouse Nckα, and mouse Nckβ genes). Nckα and Nckβ share 68% amino acid identity, whereas the two Nckα and two Nckβ across the species show 96% identity to each other. The human Nckβ gene is mapped to 2q12, whereas the human Nckα gene has previously been mapped at 3q21. Antibodies specifically against Nckα and Nckβ detect Nckα and Nckβ with an identical molecular mass in the same cells of various origins. Ec topically expressed Nckβ, but not its SH2 domain mutant, strongly inhibits epidermal growth factor- and platelet-derived growth factor-stimulated DNA synthesis. Consistently, epidermal growth factor receptor and platelet-derived growth factor receptor preferentially interact with Nckβ over Nckα in vitro. This study indicates that Nck is a multiple gene family and that each gene may have its own signaling specificity. Because previous anti-Nck (human Nckα) antibodies cross-react with Nckβ, reassessment of those studies with specific Nck genes would be necessary.

Src homology domain- (SH2 and SH3 domain)-containing adapter proteins, including Crk, Grb2 (Ash/Sam5), and Nck, are composed exclusively of SH2 and SH3 domains and lack any other enzymatic or functional motifs (1–4). They act by coupling tyrosine phosphorylation via SH2 domains to downstream effectors through SH3 domains (2, 4–6). For example, Grb2 links EGFR receptor (EGFR) to Ras by binding to the pYXVN motif (where pY represents phosphotyrosine) in EGFR via its SH2 domain while associating through PPPVPRP motifs with Sos, a guanine nucleotide exchange factor for Ras, through the SH3 domains. As a result, Sos is translocated to the plasma membrane, where it activates Ras (5, 6).

The Nck gene was first isolated from a cDNA library of a human melanoma cell line (7). The deduced amino acid sequence reveals an SH3-SH3-SH2 protein with variable lengths of interval sequences. A partial cDNA fragment of a mouse Nck homologue, which showed an overall 64% amino acid identity with the human Nck, was initially reported as one of the EGFR-binding proteins (8). We have recently isolated the full-length cDNA of this mouse Nck clone. Nck-like genes have also been identified in Xenopus and in Drosophila (dreadlocks gene, or dork), which share overall 87 and 44% amino acid identity, respectively, with the human Nck gene (9, 10).2 While the specific function of Nck in mammals remains unclear, mutations in the Drosophila gene, dork, disrupted the photoreceptor cell (R cell) axon guidance and targeting, implying that the dork links yet unidentified upstream tyrosine kinase(s) to the actin cytoskeleton (9). Tanaka et al. (13) showed that SH3 domain mutants of the human Nck interfered with dorso-ventral patterning in Xenopus laevis embryos, confirming a role of Nck in cell differentiation.

In mammalian cells, Nck has been shown to be a common target of phosphorylation for a variety of growth factor receptors, cell surface antigens, and adhesion molecules (11, 12, 14–23). Anti-Nck antibodies co-immunoprecipitate a number of phosphotyrosine proteins, including EGFR, PDGFR, Eph, insulin receptor substrate-1, focal adhesion kinase, integrins and p62(DOK) (12, 16–18, 21–23). In vitro binding experiments indicate that these interactions are mediated through the SH2 domain of Nck. Close to a dozen either previously known or unknown proteins have been reported to interact with SH3 domains of the human Nck. They include Ab1 protein-tyrosine kinase (24), Sos (25), Nck-associated kinase (26), the p21^Rac1-activated kinase (27–30), the Rho effector PKN-related kinase, PRK2 (31), the proto-oncogene c-cal (32), the human Wiskott-Aldrich syndrome protein (33, 34), a novel serine-threonine kinase (Nck-interacting kinase) (35), casein kinase-1 γ-2 (36); Sam-68 (37), Nck-associated protein 1 (38), and Nck-, Ash-, and PLCγ-binding protein 4 (39). While the function of Nck in these complexes remains elusive, it has been

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AFO43119, AFO43259, and AFO43260 (for human Nckα, mouse Nckα, and mouse Nckβ, respectively).

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1 The abbreviations used are: SH2 and SH3, Src homology domain 2 and 3, respectively; DOK, downstream of kinases; EGFR, epidermal growth factor; EGFR, EGFr receptor; PDGFR, platelet-derived growth factor; PDGFR, PDGFr receptor; HA, influenza virus hemagglutinin; GST, glutathione S-transferase; DAPI, 4,6-diamidine-2-phenylindole.

2 A. J. Wong and B. J. Mayer, GenBank™ accession number U85781.
shown that Nck translocates p21^Cdc42/Rac-activated kinase to the plasma membrane, and the membrane attachment appears to be sufficient for activating the kinase activity (40). Nck was reported as a proto-oncogene, since elevated expression weakly transformed cultured mouse and chicken fibroblast cells (11, 12).

While the number of Nck-binding proteins continues to rise, we speculated that Nck may be encoded by a family of genes, in order to accommodate the binding capacity or specificity. In this study, we have undertaken approaches to identify Nck gene homologues and orthologues in humans and mice. We found that both humans and mice have at least two Nck genes, which are even co-expressed in the same cells. More importantly, different Nck play distinct roles in receptor tyrosine kinase signaling.

**MATERIALS AND METHODS**

A partial mouse Nck cDNA (Grb4) was originally obtained from Ben Margolis (Howard Hughes Medical Institute, University of Michigan, Ann Arbor) (8). A human heart cDNA library in ZAP II vector and random primer labeling kit were from Stratagene (La Jolla, CA). The 3.5 kb cDNA library, constructed from total mRNA of human 293 cells, was used as described previously (41). Mouse pEXLox (+) cDNA expression library was obtained from Novagen (Madison, WI). Mouse embryo (17.5 days) 5’-stretch cDNA library ML1029b and multiple-tissue Northern blots (2 μg of poly(A)^+ RNA/lane) were purchased from CLONTECH (Palo Alto, CA). The Oligotex mRNA isolation kit was from Qiagen (Chatsworth, CA). Human recombinant EGF and PDGF-bb were purchased from Intergen (Purchase, NY). Anti-Nck antiserum (number 66) and anti-phosphotyrosine antibody (number 72) were used as described previously (12). Anti-PDGF-Receptor (number 2879.2) was a gift from John Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA). Anti-p25^Nck (number 276) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal rabbit anti-HA antibodies (a gift from Junlin Guan (Cornell University, Ithaca, NY) and purchased from Babco (Richmond, CA), respectively. The DNA sequencing kit, [α-35S]dATP, and [γ-32P]dCTP were purchased from Amersham Pharmacia Biotech. 125I-Protein A was from ICN Biomedicals, Inc. (Costa Mesa, CA).

**Mouse Nck Cloning and DNA Sequencing**—DNA templates were labeled by using the Prime II random primer labeling kit (Stratagene) in the presence of [α-32P]dCTP. The average specific activity of labeled probes was 1 × 10^8 cpm/μg of DNA. Approximately 1 × 10^9 cpm/ml was used in hybridization reactions. A cDNA library made from human 293 cells was first screened by the partial mouse Nck cDNA (8). Hybridization with the 32P-labeled human Nck probe was carried out overnight at 42 °C in the presence of 50% formamide, 5 × SSC, 0.1% SDS, and 1 × Denhardt’s solution. Filters were washed in 0.1× SSC and 0.1% SDS four times at 65 °C and subjected to autoradiography at −70 °C. Eight independent plaque clones were isolated from a total of five million phages after titer screening. cDNA inserts were subcloned into pBluescript and subjected to nucleotide sequencing analysis. A 5’-stretch human heart cDNA library (Stratagene) was screened for a full-length cDNA clone under the same conditions by using a 32P-labeled cDNA fragment of the newly identified Nck gene. Similarly, to identify novel mouse Nck genes, the mouse embryonic cDNA library (pEXLox (+)) and the mouse embryo 5’-stretch cDNA library (ML1029b, CLONTECH) were screened under high stringency using 32P-labeled human Nck cDNA (7) as the probe. Eight independent positive plaques were isolated after three rounds of screening a total of five million phages. Following nucleotide sequencing analysis, a full-length novel mouse Nck cDNA (now termed mouse Nckα) was identified from five of the eight plaques. A DNA sequence encoding three repeats of the nine-amino acid epitope (YPDVPDYA) from the influenza virus HA was linked in frame to N-termini of the open reading frames of the newly cloned human Nck and Nckβ cDNAs. These HA-Nck fusion proteins were subcloned by PCR into pR5 expression vector under a cytomegalovirus promoter and a 3’ SV40 enhancer, and the entire Nck sequences were confirmed by nucleotide sequencing analysis. To confirm expression of the HA-tagged Nck genes, these constructs were transiently expressed in COS cells, and Triton X-100 extracts of the cells were analyzed by Western blot analysis using either anti-HA tag monoclonal antibody or antiserum (numbers 66 and 68) previously raised against the human Nckα (12).

**Generation of Anti-Nckα and Anti-Nckβ-specific Antibodies**—Rabbit anti-Nckα antibodies were raised against the peptide SLRKGSL-SNGGQSR, which shares only three amino acids (20%) with the corresponding peptide in the Nckα (this has been established with the laboratory of Zymed Laboratories, Inc., P.O. Box 6060, San Francisco, CA). Antisera were tested for recognizing HA-tagged human Nckα. Anti-Nckβ-specific antibodies were further purified by peptide affinity chromatography, and tested for Western immunoblotting assay and immunoprecipitation. We found that the optimal condition for Western immunoblot was 1:500 dilution. This antibody did not appear to work in immunoprecipitation experiments. Following previously raised rabbit anti-GST-Nck (human Nck0) fusion protein antiserum (number 66; see Ref. 12). This antiserum recognizes both HA-Nckα and HA-Nckβ (data not shown). To obtain an anti-Nck-specific antisem, antiserum 66 was subjected to a GST-Nckβ affinity column to remove the portion of anti-Nckα antibodies. The flow-through was tested for its specificity of recognizing HA-Nckα. It proved to only recognize HA-Nckα and not HA-Nckβ.

**Northern Blot Analysis**—Human and mouse multitranscript blotting were purchased from CLONTECH (Palo Alto, CA) and subjected to prehybridization and hybridization according to the manufacturer’s instructions. Briefly, the RNA blots were incubated in ExpressHyb solution for 3 h at 42 °C without radioactive probes. 32P-λ-CTP-labeled human and mouse Nckα and Nckβ cDNA probes (1 × 10^6 cpm/ml, 3 × 10^5 cpm/ml for GAPDH) were heated at 95 °C for 5 min, added to the reactions, and incubated at 42 °C for 12 h. The membranes were washed in 0.1× SSC and 0.1% SDS three times at 60 °C and exposed to Kodak XAR film at −70 °C.

**Site-directed Mutagenesis, Cell Transfection, and [3H]Thymidine Incorporation**—Mutagenesis was carried out by using QuickChange™ site-directed mutagenesis kit (Stratagene). The oligonucleotide primers that were used to generate human Nck-R308K and human Nckβ-R312K mutants were as follows: 5’-GGGGATTTCTACTTAAAGGATTATCATCCGGCC and 5’-GGCGAGAATCTACTTAAATAGGAAATCCCG for the human Nckα and 5’-GGGCACTTCCATTTAGGAGAACGAGTTCTCG and 5’-CGAGGACTGTGTCTGCTTTTAATAGGGAGT for the human Nckβ. PCR reactions were carried out in an initial denaturation step, followed by 35 cycles under the same conditions, except for an overlap extension, by using F6/4 DNA polymerase to generate SH2 domain mutants in which the conserved arginine residue in the FLVQES motif was changed to lysine. After DpnI digestion of parental cDNA templates, the mutant clones were transformed into XL1-Blue competent bacteria, and the plasmids were isolated. Mutations were confirmed by nucleotide sequencing analysis.

**Generation of GST Fusion Proteins**—Oligonucleotides containing engineered restriction sites (BamHI/EcoRI) were used to amplify the entire coding sequence of Nckα and Nckβ genes by polymerase chain reaction. The DNA fragments were gel-purified, cleaved with BamHI and EcoRI, and repurified prior to being ligated into pGEX-3X vector. Competent XL-1 or BL-21 bacteria were transformed with the constructs, and the recombinant clones were screened for production of the corresponding GST fusion proteins following isopropyl-1-thio-β-D-galac-
toppersin. GST fusion proteins were purified by affinity chromatography using glutathione-agarose beads as described previously (12).

**GST Fusion Protein Pull-down Assay**—Subconfluent HER14 cultures (80%) were starved in low serum (0.2%) medium for 16 h and treated with or without EGF (500 ng/ml) for 2 min or PDGF-bb (50 ng/ml) for 5 min at 37 °C. Cells were washed three times with ice-cold PBS and solubilized in the lysis buffer as described previously (12). The postnuclear extracta (total lysates (TL), 10,000 × g for 10 min at 4 °C) were incubated with various amounts of the full-length GST-Nck fusion proteins immobilized on glutathione beads for 2 h at 4 °C. The beads were washed three times with the corresponding lysis buffer and heated at 95 °C for 5 min in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Supernatants were analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with corresponding antibodies (see figure legends). Results were visualized by 125I-protein A and autoradiography.

**RESULTS**

**Identification of Novel Nck Family Genes and Comparison with Nck Homologues in Xenopus, Drosophila, and Caenorhabditis elegans**—Since an overall 64% amino acid identity between the human Nck (7) and the partial mouse Nck (Grb4; see Ref. 8) was too low to be considered as a pair of orthologue genes. We compared the two human Nck genes to the previously isolated Nck-like genes from Xenopus laevis, from Drosophila (dreadlocks or dork) (9), and C. elegans (Cosmid ZK470, GenBankTM accession number U39651). As indicated in Table I, human Nck shows 87, 44, and 33% amino acid identity to Xenopus Nck, Drosophila dork, and C. elegans Nck-like, respectively, and human Nck shares 68, 43, and 34% amino acid identity, respectively. While the Xenopus Nck gene is clearly more related to the human Ncka gene, it is not obvious which of the two human Nck genes is evolutionarily closer to the Drosophila dork and the C. elegans Nck genes. Comparison of changes in the F strand, the BG loop, and the “BC loop” suggested that dork gene is also more closely related to the human Ncka gene. It is possible that Xenopus and Drosophila may also have as yet unidentified Nckβ-related genes.

To examine expression of Nck genes and to compare the relative abundance of Ncka and Nckβ messages, human and mouse tissue mRNA (2 μg/lane) blots were hybridized with radioactively labeled human Ncka and mouse Nck DNA probes, respectively. Following detection of Ncka genes, the same blots were stripped off for Nckβ probes and hybridized to human and mouse Nckβ DNA probes. Expression of the β-actin gene was used as control. As shown in Fig. 1, both human Ncka (A) and human Nckβ (B) genes were expressed in almost all of the tissues tested, although variations in the relative abundance of Ncka and Nckβ expression among different tissues were observed. Equal amounts of human β-actin were detected (C). Similar results were obtained for the mouse Ncka (D) and mouse Nckβ (E) genes, which were expressed in most of the tissues studied. The mouse β-actin gene expression was included as the control (F). These data indicate that Ncka and Nckβ are generally expressed in most tissues.

**Nckβ and Ncka Genes Reside on Different Chromosomes**—We have previously mapped the human Ncka gene to 3q21, a region in which mutations correlate with occurrence of a number of human inherited disorders and neoplasias (49). To identify the chromosomal location of the human Nckβ gene, we performed fluorescence in situ hybridization of a biotin-labeled Nckβ probe to normal human metaphase chromosomes. Hybridization of this probe resulted in specific labeling only of chromosome 2 (Fig. 2). Specific labeling of 2q11–14 was observed on four (four cells), three (seven cells), two (nine cells), or one (six cells) chromatid(s) of the chromosome 2 homologues in 26 cells examined. Of 103 signals observed, 61 (60%) were located at 2q11–14. Of these, two signals (3%) were located at 2q11, 50 signals (82%) were located at 2q12, 3 signals (5%) were located at 2q13, 4 signals (4%) were located at 2q14, and 5 signals (5%) were located at 2q15.
mouse multiple-tissue Northern blots (MTN; 2 µg of mRNA/lane; CLONTECH) were hybridized sequentially with 32P-labeled human or mouse Nckα and Nckβ cDNA probes (1 × 10^6 cpm/ml) or β-actin cDNA probes (5 × 10^5 cpm/ml), according to the manufacturer’s instructions. Panel A, human MTN blotted with human Nckα probe (hNckα); panel B, human MTN blotted with human Nckβ probe (hNckβ); panel C, human MTN blotted with human β-actin probe; panel D, mouse MTN blotted with mouse Nckα probe (mNckα); panel E, mouse MTN blotted with mouse Nckβ probe (mNckβ); panel F, mouse MTN blotted with mouse β-actin probe. H, heart; B, brain; Pl, placenta; S, spleen; L, lung; Li, liver; Sk, skeletal muscle; K, kidney; T, testis; P, pancreas. The blots were washed and subjected to autoradiography. Exposure time for panels A, B, D, and E was 48 h; for panels C and F, exposure time was 6 h.

located at 2q13, and 6 signals (10%) were located at 2q14. Forty-two single background signals were observed at other chromosomal sites. None of these chromosomal bands were labeled more than once, and none were doublet signals. We observed a specific signal at 2q12 in two additional hybridization experiments using this probe. These results suggest that the human Nckβ gene is localized to chromosome 2, band q12. No recurring chromosomal abnormalities involving 2q12 have been identified in human malignancies.

![Image](image1.png)

**Fig. 1. Tissue expression of Nck family genes.** Human and mouse multiple-tissue Northern blots (MTN; 2 µg of mRNA/lane; CLONTECH) were hybridized sequentially with 32P-labeled human or mouse Nckα and Nckβ cDNA probes (1 × 10^6 cpm/ml) or β-actin cDNA probes (5 × 10^5 cpm/ml), according to the manufacturer’s instructions. Panel A, human MTN blotted with human Nckα probe (hNckα); panel B, human MTN blotted with human Nckβ probe (hNckβ); panel C, human MTN blotted with human β-actin probe; panel D, mouse MTN blotted with mouse Nckα probe (mNckα); panel E, mouse MTN blotted with mouse Nckβ probe (mNckβ); panel F, mouse MTN blotted with mouse β-actin probe. H, heart; B, brain; Pl, placenta; S, spleen; L, lung; Li, liver; Sk, skeletal muscle; K, kidney; T, testis; P, pancreas. The blots were washed and subjected to autoradiography. Exposure time for panels A, B, D, and E was 48 h; for panels C and F, exposure time was 6 h.

![Image](image2.png)

**Fig. 2. Chromosomal localization of the human Nckβ gene.** In situ hybridization of a biotin-labeled Nckβ cDNA probe to human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes is shown. The chromosome 2 homologues are identified with arrows; specific labeling was observed at 2q12. The inset shows partial karyotypes of two chromosome 2 homologues illustrating specific labeling at 2q12 (arrowheads). Images were obtained using a Zeiss Axiophot microscope coupled to a cooled charge coupled device camera. Separate images of DAPI-stained chromosomes and the hybridization signal were merged using image analysis software (NU200 and Image 1.57).

![Image](image3.png)

**Fig. 3. Co-expression of Nckα and Nckβ proteins in the same cells.** To test newly generated anti-Nckα- and anti-Nckβ-specific antibodies, COS cells were transfected with the HA-Nckα or HA-Nckβ construct. Lysates of the cells were directly subjected to Western immunoblotting analysis (15 µg/lane) using anti-HA tag antibody, preimmune serum, anti-Nckα, or anti-Nckβ-specific antibodies (A–D). Total lysates (30 µg/lane) of seven human, monkey, and mouse cell lines indicated were analyzed by Western blot analyses for expression of Nckα (E) and Nckβ (F) using the anti-Nckα- and anti-Nckβ-specific antibodies. Anti-Cdk4 antibody blot (G) was used as a control for the relative amount of proteins in each lane. The results on blots were visualized by incubating with 125I-protein A, followed by autoradiography. Exposure times were 8 h (A), 15 h (B–D), and 48 h (E–G).

**Generation of Specific Antibodies and Detection of Nckα and Nckβ Co-expression in a Wide Variety of Cell Types—** In order to study whether or not Nckα and Nckβ are expressed simultaneously in the same cells and, if they are, whether they have distinct functional assignments, rabbit anti-Nckβ-specific antibodies were raised against a unique peptide, SLRKGASL-SNGQGSR, derived from Nckβ cDNA. Anti-Nckα-specific antibody serum was generated by removing the anti-Nckβ antibody portion from the previously raised anti-GST-human Nckα antiseraum (numbers 66–68; see Ref. 12) with a GST-Nckβ affinity column (see “Materials and Methods”). We tested the specificity of these antibodies by Western immunoblotting and immunoprecipitation assays. The total cell extracts of HA-Nckα- or HA-Nckβ-transfected COS cells were resolved in an SDS gel, transferred to nitrocellulose membrane, and immunoblotted by preimmune serum, anti-HA, anti-Nckβ, or anti-Nckα antibodies. As shown in Fig. 3, anti-HA antibody detected both HA-
Nckα (Fig. 3A, lane 1) and HA-Nckβ (Fig. 3A, lane 2). Preimmune serum of the anti-Nckβ antibody recognizes neither the 48-kDa HA-tagged Nck proteins nor the 45-kDa endogenous Nck proteins (3B, lanes 1 and 2). The negative results of the preimmune serum for the anti-Nckα antiserum (number 66) were as previously shown (12). Anti-Nckβ antibody recognized the 48-kDa HA-Nckβ (Fig. 3C, lane 2) but not HA-Nckα (Fig. 3C, lane 1) and recognized the endogenous Nckβ in both of the cell extracts. On the contrary, anti-Nckα antiserum recognized the HA-Nckα (Fig. 3D, lane 1) but not HA-Nckβ (Fig. 3D, lane 2) and recognized the endogenous Nckα in both of the cell extracts. Unfortunately, these antibodies did not work well for immunoprecipitation tests (data not shown).

To detect endogenous expression of Nckα and Nckβ proteins in various cell types, cell extracts were prepared from the various cell lines indicated and subjected to Western immunoblot analysis as described previously. As shown in Fig. 3E, the expression of a 45-kDa Nckα was detected by anti-Nckα antiserum in all of the cell lines included. When a duplicate membrane with identical lines of samples was blotted with the anti-Nckβ antibody, a 45-kDa Nckβ was observed in every cell line tested (Fig. 3F). Moreover, an additional 47-kDa species, which was only recognized by the anti-Nckβ antibodies, was revealed from the three mouse cell lines (lanes 4, 6, and 7). The slowly migrating species represents a differentially phosphorylated form of Nckβ, since it was no longer detectable following alkaline phosphatase treatment of the same cell lysates (data not shown). Levels of Cdk4 kinase was shown by anti-Cdk4 antibody blotting and included as a control for relative protein loading (Fig. 3G). While we cannot be sure about the ratios of Nckα and Nckβ in these cells, these results clearly indicate that Nckα and Nckβ are simultaneously expressed in the same cells of various origins.

Nckβ Inhibits EGF- and PDGF-stimulated DNA Synthesis in an SH2-dependent Manner—We have previously shown that Nck co-immunoprecipitates with activated EGFR and PDGFR (12). Overexpressed human Nckα in fibroblasts was reported to have a weak transforming activity in fibroblasts, suggesting that Nck is involved in growth control (11, 12). To gain insights into functional differences between Nckα and Nckβ genes, we studied effects of overexpression of either wild type or SH2 domain mutants of human Nckα and human Nckβ genes on EGF- and PDGF-stimulated DNA synthesis. HER14 cells were transiently transfected with either vector alone or various Nck constructs using SuperFect reagent, which has been demonstrated to obtain more than 70% transfection efficiency in NIH-3T3 cells (Qiagen). Our anti-HA antibody staining showed 85% of transfection efficiency in HER14 cells (data not shown). The HA-tagged wild type and SH2 mutants of human Nckα and human Nckβ gene constructs were schematically shown in Fig. 4A. Expression of the corresponding Nck constructs was detected by Western blot analyses using anti-HA antibody. As shown in Fig. 4B, similar levels of the four HA-tagged Nck proteins (48 kDa) were detected, which were 7–9-fold higher than the endogenous Nckα and Nckβ protein levels (Fig. 3 and data not shown).

A DNA synthesis study was conducted by measuring [3H]thymidine incorporation in these cells in response to EGF and PDGF (Fig. 4C). A 7–8-fold increase was detected in the cells transfected with the vector alone (open bars). Neither the wild type (dark solid bars) nor the SH2 mutant (Nckβ-R308K) (light solid bars) of Nckβ had any inhibitory effects on EGF-stimulated DNA synthesis. Instead, wild type Nckα enhanced the EGF stimulation. However, we detected a moderate inhibition of the PDGF-stimulated DNA synthesis only by the wild type and not by the Nckα-R308K mutant under these conditions. Interestingly, we found that the wild type Nckβ strongly inhibited (more than 50%) both the EGF- and PDGF-stimulated DNA synthesis (dark hatched bars). This inhibition by Nckβ was SH2 domain-dependent, since SH2 mutation of Nckβ (Nckβ-R312K) abolished the inhibitory effects (light hatched bars). These results suggest that Nckα and Nckβ have distinct signaling specificities in intact cells, and the newly identified Nckβ gene has a stronger regulatory function in growth factor signaling.

EGFR and PDGFR Preferentially Interact with Nckβ—To better understand the differential effects of Nckα and Nckβ on EGFR and PDGFR signaling, we compared interaction of Nckα and Nckβ with these receptor tyrosine kinases. In vitro pull down experiments using GST-Nckα and GST-Nckβ fusion proteins were carried out. Postnuclear extracts of HER14 cells, either untreated or treated with EGF or PDGF-bb, were incu-
bated with different amounts of GST-Nckα and Nckβ fusion proteins immobilized on glutathione-agarose beads. Following washing, the bead-bound EGFRs were eluted in sample buffer by 95°C heating for 5 min. Supernatants were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-phosphotyrosine (anti-PY) antibody. Results were visualized by 125I-protein A autoradiography. B, the Coomassie Blue staining of a duplicate SDS gel of A, similar to A, except 20 µg of the same group of GST fusion proteins plus GST alone were incubated with the lysates of the PDGF-stimulated HER14 cells. D, the Coomassie Blue staining of a duplicate SDS gel for C. E, the cell lysates were immunoprecipitated with anti-p62DOK antibody, followed by anti-phosphotyrosine immunoblotting. F, a duplicate membrane of A was blotted with anti-p62DOK antibody (M276; Santa Cruz Biotechnology). G, the cell lysates were subjected to heat and the SDS denaturation process prior to incubation with the GST fusion protein beads (20 µg). Exposure time in A, C, and F was 30 h; exposure time in E was 96 h; and exposure time in G was 37 h.

Fig. 5. EGFR and PDGFR preferentially interact with Nckβ. HER14 cells were either untreated or treated with EGF (500 ng/ml, 2 min at 37°C) or PDGF-bb (50 ng/ml, 5 min at 37°C), and the postnuclear extracts were subjected to either in vitro binding assays or immunoprecipitations (IP). A, the cell lysates were incubated with indicated amounts of GST-Grb2-SH2, GST-Nckα, or GST-Nckβ fusion proteins immobilized on glutathione-agarose beads. Following washing, the bead-bound EGFRs were eluted in sample buffer by 95°C heating for 5 min. Supernatants were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-phosphotyrosine (anti-PY) antibody. Results were visualized by 125I-protein A autoradiography. B, the Coomassie Blue staining of a duplicate SDS gel of A, similar to A, except 20 µg of the same group of GST fusion proteins plus GST alone were incubated with the lysates of the PDGF-stimulated HER14 cells. D, the Coomassie Blue staining of a duplicate SDS gel for C. E, the cell lysates were immunoprecipitated with anti-p62DOK antibody, followed by anti-phosphotyrosine immunoblotting.

The amount of Nckβ-bound EGFR is 10-fold higher than that of Nckα-bound EGFR at any of the concentrations used (lanes 5 and 6 versus lanes 2 and 3). The amount of Nckβ-bound EGFR is actually comparable with that of Grb2-SH2-bound EGFR (lanes 7 and 8) (Grb2 has previously been observed as one of the strongest binding proteins to EGFR) (34). GST alone bound neither inactive nor tyrosine-phosphorylated EGFR (Ref. 12 and data not shown). Coomassie Blue staining of the GST fusion proteins used in the experiment is shown in Fig. 5B. The bands below the GST-Nck proteins are probably degraded GST-Nck products. As shown in Fig. 5C, GST-Nckα bound the activated PDGFR (lane 6), consistent with previous reports (12, 52). However, GST-Nckβ bound PDGFR 10 times more strongly than the GST-Nckα (lane 8 versus lane 6). The intensity of GST-Nckβ-bound PDGFR is even significantly greater than that of GST-Grb2-SH2-bound PDGFR (lane 8 versus lane 4). GST alone was included as a negative control (lanes 1 and 2). The Coomassie Blue-stained SDS gel of the GST fusion portions indicates the amounts of GST fusion proteins used (Fig. 5D). Since our newly generated anti-Nck bodies did not immunoprecipitate the native antigens, we were unable to confirm these results in intact cells. Moreover, since Nck proteins migrate too close to the immunoglobulin heavy chains in SDS-polyacrylamide gel elec-
trophoeresis, we could not be certain about the receptor-bound Nck bands when anti-EGFR and anti-PDGFR antibodies were used for immunoprecipitations followed by anti-Nck antibody immunoblotting.

We have previously shown that Nck binds directly to PDGFR but indirectly to EGFR in HER14 cells (21, 52). Instead, Nck directly binds to tyrosine phosphorylated p62DOK, known as the GAP-associated p62 phosphotyrosine protein (54, 55), in HER14 cells in response to EGF (21). We therefore proposed that Nck associates with EGFR via p62DOK. To test whether or not p62DOK is indeed associated with the EGFR in vivo, we carried out co-immunoprecipitations of the extracts of EGF- or PDGF-stimulated HER14 cells. It is clearly shown in Fig. 5E that anti-p62DOK antibody co-immunoprecipitated the tyrosine-phosphorylated (lane 2), but not the unphosphorylated (lane 1), 170-kDa EGFR. Tyrosine phosphorylation of p62DOK was also clearly detected in the EGF-stimulated cells (lane 2). The identities of EGFR and p62DOK were further confirmed by anti-EGFR and anti-p62DOK antibodies (data not shown). PDGF stimulation causes much less tyrosine phosphorylation of the p62DOK and its association with the PDGFR in these cells. These data are the first evidence that p62DOK physically associates with activated EGFR and PDGFR.

To test if binding of Nckβ to p62DOK is also stronger than that of Ncka, a duplicate membrane of that shown in Fig. 5A was immunoblotted with anti-p62DOK antibody. As shown in Fig. 5F, Nckβ bound 2–3-fold more strongly to the p62DOK than Ncka from EGF-stimulated (lanes 2 and 3 versus lanes 5 and 6) but not unstimulated (lanes 1 and 4) cells. We then tested if Nckβ, unlike Ncka, is able to bind directly to the EGFR. GST-Nck fusion proteins were incubated with prenatened cell lysates (see “Materials and Methods”). Under these conditions, in which the preexisting protein complexes are destroyed, only the phosphotyrosine proteins, which are able to directly bind the SH2 domain of Nck, were detected. It is shown in Fig. 5G that neither GST-Ncka nor GST-Nckβ was able to bring down the EGFR (lanes 4 and 6). As expected, GST-Grb2 was able to bind the denatured tyrosine-phosphorylated EGFR (lane 2). GST alone did not show any binding (lanes 8 and 9). Thus, the stronger binding of Nckβ to EGFR could be at least partially due to its increased binding to p62DOK. In contrast, we found that both GST-Ncka and GST-Nckβ were able to bind denatured tyrosine-phosphorylated PDGFR (data not shown). Interestingly, no apparent differences between Ncka and Nckβ in their SH3 binding were detected, including binding to Wiskott-Aldrich syndrome protein, p21<sup>cdc42</sup>/rac-activated kinase, PRK2, and Sos in vitro (data not shown).<sup>3</sup>

**DISCUSSION**

To study whether Nck is encoded by a single gene or by a family of genes is important for ultimate understanding of the specific function of Nck. Previous studies have all been based upon an assumption that Nck is a single gene-encoded protein and have yielded inconsistent results. In this paper, we have shown that Nck is encoded by a multiple-gene family, by identifying a novel human Nck gene (the human Nckβ gene) and the two mouse Nck counterparts (the mouse Ncka and Nckβ genes). We suggest renaming the previously cloned human Nck gene (7) as the human Nckα gene. We have established, based on amino acid sequence homology, relationships of Nck genes in humans and mice that show that the human Ncka/mouse Ncka and likewise human Nckβ/mouse Nckβ genes are two pairs of orthologue genes across the species. We have identified chromosomal localization of the human Nckβ gene to 2q12, different from the previously reported location of the human Nckα gene at 3q21 (49). Immunoblotting analyses using specific anti-Nckα and anti-Nckβ antibodies demonstrate that Nckα and Nckβ are expressed in the same cells of a wide range of tissue origins, suggesting that they have different functional assignments. More importantly, Nckα and Nckβ exhibit dramatically different effects on EGFR- and PDGF-stimulated DNA synthesis in NIH-3T3 (HER14) cells. Exogenous expression of Nckβ, but not its SH2 mutant, inhibits both EGFR- and PDGF-induced <sup>3</sup>H]thymidine incorporation. Under similar conditions, wild type Nckα has a much reduced inhibitory effect. Both EGFR and PDGF bind Nckβ significantly more strongly than Nckα in vitro. These results indicate that Nckα and Nckβ have distinct signaling specificities.

Multiple homologues of Nck in the same cells would enhance the capacity of accommodating various upstream signals and interacting with common or distinct downstream effectors. There are at least three possibilities. First, different Ncks may link, via SH2 domains, tyrosine phosphorylation of various surface receptors to the same downstream effectors, such as Sos, p21<sup>cdc42</sup>/rac-activated kinase, and Wiskott-Aldrich syndrome protein. In this case, having multiple Nck members in a cell would only increase the upstream binding repertoire. Second, SH2 domains of different Nck members bind to the same upstream activators, but the SH3 domains interact with distinct downstream effectors. In this case, specific Nck proteins would carry their specific cellular functions. Third, none of the SH2 and the SH3 domains in different Nck molecules share any common binding motifs. In this case, except for a common mechanism of action, they do not have any functional overlaps. While certain variations in the EF and the BG loops of the SH2 domains and the RT loops of the SH3 domains between Nckα and Nckβ have been noticed, it is not clear whether or not these amino acid differences indeed cause changes in their binding specificity. Other yet unidentified differences, such as subcellular localization and phosphorylation, between Nckα and Nckβ may also play roles in determining their signaling specificity. Park (50) recently reported cloning of a mouse Nck cDNA that is identical to the mouse Nck reported here.

The two SH2 domains of Nckα and Nckβ show a significant, at least quantitatively, difference in binding to two previously known Nck-interacting receptor tyrosine kinases. The newly identified Nckβ is able to bind similar amounts of tyrosine-phosphorylated EGFR and PDGFR in a concentration 10-fold less than Nckα. We have previously shown that Nckα did not directly bind EGFR, and proposed that the interaction with EGFR is mediated through tyrosine-phosphorylated p62DOK (21). In this paper, we have confirmed for the first time that p62DOK indeed associates with activated EGFR and PDGFR in intact cells (Fig. 6E). Consistently, Nckβ appears to bind 2–3-fold better than Nckα to p62DOK. But this may not explain the 10 times better binding of Nckβ to these receptors. Similarly to Nckα, Nckβ does not directly bind to EGFR. Thus, these results suggest that either the Nckβ-p62DOK complex binds, via an unknown mechanism (such as involving a third molecule), to EGFR much more strongly than the Nckα-p62DOK complex, or alternatively differences in the SH3 domains may also contribute to the interaction between Nckβ and the EGFR. It remains to be studied whether or not p62DOK itself is able to directly bind to EGFR and, if it does, what the mechanism of the interaction would be. Moreover, it would be important to understand if Nckα and Nckβ bind at the same site(s) or different site(s) in p62DOK and if only the SH2 domains or both SH2 and SH3 domains of Nck are involved in the interaction.

Results from testing the effects of Nckα, Nckβ, and the SH2 mutants in EGFR- and PDGF-stimulated DNA synthesis have several important implications. First, these data clearly indi-
cate a signaling difference between Nckα and Nckβ, irrespective of whether or not Nck are indeed involved in control of DNA synthesis. Previous studies have reported opposite findings concerning whether Nck signals through the Ras/mitogen-activated protein kinase pathway (25, 51, 52). Second, a stronger inhibitory effect of Nck on PDGF-stimulated, but less on EGF-stimulated, DNA synthesis was somewhat expected. We have previously shown that Nck binds directly to PDGFR (45), and, furthermore, the SH2 domain of Nckα shares a binding site, Tyr751, in the human PDGFR-β with one of the SH2 domains of phosphatidylinositol 3-kinase (45). Since binding to phosphatidylinositol 3-kinase has been demonstrated to be required for PDGF-induced mitogenesis (53), one mechanism, by which overexpressed Nckα inhibits PDGF-induced DNA synthesis, would be to compete with phosphatidylinositol 3-kinase for binding to the same pool of the PDGFR. Third, the observation that Nckβ potently inhibits, in an SH2-dependent manner, both EGF- and PDGF-stimulated DNA synthesis raises an intriguing question of whether Nckβ shares the same binding site with phosphatidylinositol 3-kinase or different tyrosine residue(s) in the PDGFR. Nckβ indeed also binds directly to the PDGFR. It is possible that Nckβ binds at different tyrosine residues in PDGFR, which also play essential roles in mitogenesis, such as sites for SH2 domains of phospholipase Cγ and Src kinases. Experiments are under way to address these issues.

Finally, the majority of the currently available anti-Nck (human Nckα) antibodies cross-react with Nckβ. This observation indicates that most of the previous experiments, especially the experiments involving co-immunoprecipitations and peptide mapping, were actually dealing with two distinct gene products, which have differences both in amino acid sequences and even in signaling specificities. The current study, therefore, urges a reassessment of those experiments by using specific anti-Nck antibodies and genes.

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