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Nckβ Adapter Controls Neuritogenesis by Maintaining the Cellular Paxillin Level▽

Shengxi Guan,[1] Mei Chen,[1] David Woodley,[1,2] and Wei Li[1,2]*

Department of Dermatology and USC/Norris Cancer Center, University of Southern California Keck School of Medicine, Los Angeles, California 90033,[1] and VA Greater Los Angeles Healthcare System, Sepulveda, California 91343[2]

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The SH2/SH3 adapter Nck has an evolutionarily conserved role in neurons, linking the cell surface signals to actin cytoskeleton-mediated responses. The mechanism, however, remains poorly understood. We have investigated the role of Nck/Nckα/Nck1 versus Grb4/Nckβ/Nck2 side-by-side in the process of mammalian neuritogenesis. Here we show that permanent genetic silencing of Nckβ, but not Nckα, completely blocked nerve growth factor-induced neurite outgrowth in PC12 cells and dramatically disrupted the axon and dendrite tree in primary rat cortical neurons. By screening for changes among the components reportedly present in complex with Nck, we found that the steady-state level of paxillin was significantly reduced in Nckβ knockdown, but not Nckα knockdown, neurons. Interestingly, Nckβ knockdown did not affect the paxillin level in glial cells and several other cell types of various tissue origins. Genetic silencing of paxillin blocked neuritogenesis, just like Nckβ knockdown. Reintroducing a nondegradable Nckβ into Nckβ short interfering RNA-expressing PC12 cells rescued paxillin from down-regulation and allowed the resumption of neuritogenesis. Forced expression of paxillin in Nckβ knockdown PC12 also rescued its capacity for neuritogenesis. Finally, Nckβ, but not Nckα, binds strongly to paxillin and treatment of the neurons with proteosome inhibitors prevented paxillin down-regulation in Nckβ knockdown neurons. Thus, Nckβ maintains paxillin stability during neuritogenesis.

The "phosphotyrosine > SH2/SH3 adapter > effector" signaling mechanism is used by many cell membrane receptors, in which SH2/SH3-containing adapter proteins link tyrosine-phosphorylated receptors to scores of common and cell type-specific downstream signaling events (18). The natural role for the SH2/SH3 adapter Nck is to connect the receptors to Rho family GTPase-mediated actin cytoskeleton assembly/disassembly, essential for cell morphology, motility, and differentiation (16). Therefore, the function of Nck is distinct from that of another well-characterized SH2/SH3 adapter protein, Grb2, which connects cell surface receptors to the Ras-GTPase pathway leading to DNA synthesis and cell proliferation (22).

There are two mammalian Nck genes, encoding Nck/Nckα/Nck1 and Grb4/Nckβ/Nck2. In humans, they are located on chromosome 3 and chromosome 2, respectively (4, 12). A simple answer is still lacking for the question of whether Nckα and Nckβ exist as mutually compensating partners or whether each of them has its own cell and/or tissue-specific function. It has been shown that mice deficient for either Nckα or Nckβ are viable, whereas the elimination of both Nckα and Nckβ results in lethality at embryonic day 9.5. Mouse embryonic fibroblast cells derived from the Nck double-knockout mice exhibited defects in cell migration in vitro (1). Therefore, the authors of this study concluded that Nckα and Nckβ are functionally redundant during mouse development. However, an increasing number of studies have also provided evidence that Nckα and Nckβ have rather nonoverlapping functions in various type of cells (5, 6, 14, 19, 21, 25). So far, no Nckα- or Nckβ-specific downstream target proteins have been identified, and it has obviously become critical to further understand the underlying mechanisms.

What tissue/cell model system should be used to study Nckα versus Nckβ? In fact, the most clearly demonstrated cell type-specific function of Nck is its role in mediating the signals for neuronal cell differentiation. *Drosophila* Nck, dreadlocks, is essential for axon guidance during eye development via a receptor to $p21^{rac/cdc42}$-activated kinase (Pak) to actin cytoskeleton pathway (8, 11). In mammalian cells, Nck plays a critical role in the bidirectional signals mediated by membrane-anchored ephrins and their Eph family tyrosine kinase receptors (23). One example is the reverse signaling by Ephrin-B3, which regulates cell-cell interactions during axon pathfinding and hindbrain segmentation (6). Another example is the Disabled 1-mediated Reelin signal that regulates neuronal placement by remodeling the actin cytoskeleton (19). Therefore, we turned to neuronal systems for answers. In this current study, we compared the importance of Nckα and Nckβ in two types of neuronal cells from rat: the pheochromocytoma cell line PC12 and primary cortical neurons. Our results show that Nckβ, but not Nckα, is essential for the induction and maintenance of neuronal cell differentiation, i.e., neuritogenesis. More importantly, we found that the mechanism for the Nckβ-mediated differentiation of neurons is through its ability to maintain the intracellular level of paxillin, a well-characterized protein that links plasma membrane receptors with actin cytoskeleton-orchestrated events. In contrast, Nckα has no effect on the intracellular levels of paxillin in neuronal cells. Moreover, the Nckβ-paxillin relationship in neurons was not found in rat glial cells and cells originating from a number of other tissues.

* Corresponding author. Mailing address: 1303 North Mission Road, Los Angeles, CA 90033. Phone: (323) 224-7058. Fax: (323) 224-7679. E-mail: wli@usc.edu.

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MATERIALS AND METHODS

PC12 cells were maintained in RPMI 1640 supplemented with 10% horse serum (GIBCO BRL) and 5% fetal bovine serum. Primary cortical neurons were prepared from embryonic day 18 rat brains as described previously (17) and cultured in tissue culture dishes containing NEUROBASAL medium (Life Sciences) supplemented with B27, 5 U/ml penicillin, 5 mg/ml streptomycin, 0.5 mM glutamine, and 25 μM glutamate (Gibco, Rockville, MD), at 37°C in 5% CO_2. Nerve growth factor (NGF; 2.5S) was purchased from Upstate (Lake Placid, NY). Rat-tail type I collagen and antipaxillin antibody (P13520) were purchased from BD Biosciences (Bedford, MA). Anti-Nckα- and anti-Nckβ-specific antibodies were generated as previously described by us (4). Anti-Pak1 (sc-882) and anti-FAK (c-20), anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and all horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). MG132 was purchased from Calbiochem. The inactive derivative, MG132-In, was synthesized by Synthetic biomolecules (San Diego, CA). The enhanced chemiluminescence Western blotting detection kit (RPN2106) was from Amersham (Buckinghamshire, United Kingdom). All other reagents and supplies, unless otherwise indicated, were from VWR (Bristol, CT) or SIGMA.

GFP, Nck, Nck RNA interference, paxillin, and paxillin RNA interference in lentiviral vectors. The lentivirus-derived vector, pRRLsinhCMV, had cDNAs encoding enhanced green fluorescent protein (EGFP) (at EcoRV), Nckα (at XbaI/PstI), or Nckβ (at XhoI/BamHI) inserted according to a procedure previously described (15). Chicken paxillin-EGFP cDNA (a gift from Alan F. Horwitz, University of Virginia, Charlottesville, VA) was subcloned into pRRLsinhCMV at XbaI and EcoRI. We used the siRNA Selection Program as described previously to identify possible target sequences (27). Two to three short interfering RNA (siRNA) sequences were cloned into the lentiviral siRNA delivery vector, FG-12, as previously described (20). The following siRNA sequences (sense) were selected against the rat Nck or paxillin cDNAs and used in the subsequent studies: GATGATAGCTTTGTTGATC (siNckα1), GAGAGAGAGGATGAA TTGT (siNckα2), GCATCTATGACCTCAACAT (siNckβ1), GAGGGCGACT TCCTCATTA (siNckβ2), GGCCCATCTTGGATAAAGT (siPaxillin-1), GGA CAACCCTACTGTGAAA (siPaxillin-2), and GGACAACCCTACTGTGAAA (siPaxillin-3).

Production of lentiviral stocks and infection. The pRRLsinhCMV and FG-12 constructs, together with packaging vectors pCMVΔR8.2 and pMDG, were used to cotransfect 293T cells as previously described (3). Typical viral titers were 1×10^6 to 7×10^6 transduction units/ml without concentration and 5×10^7 to 5×10^8 after ultracentrifugation, using GFP directly measured by fluorescence-activated cell sorter (FACS) analysis as the marker. Cell infection and the determination of infection efficiency, as monitored by EGFP expression, were carried out as previously described (15). Usually, the cells were subjected to biochemical and cell differentiation experiments five days after infection with FG-12 and forty-eight hours after infection with pRRLsinh-CMV.

Measurement of transgene expression or endogenous gene down-regulation. Nck and paxillin gene products were detected by immunoblotting of the lysates of infected cells ($>5 \times 10^5$ cells/experimental condition) using antibodies against the corresponding gene products. The lower part of the same blotting membrane was blotted with an anti-GAPDH antibody as the control for equal sample loading. ECL autographs with unsaturated exposure were used to assess the increases (over their corresponding GAPDH bands) by scanning densitometry. The results of one representative of three identical but independent experiments are shown (15).

Neurite outgrowth, gene rescue, and morphological analyses. PC12 cells (50,000) were seeded into each well of six-well plates which were precoated with 20 μg/ml of type I collagen and incubated overnight in RPMI 1640 with only 1% horse serum. NGF (100 ng/ml) was added to the cultures to induce neurite outgrowth. The neurite outgrowth of these cells was visualized daily for 4 consecutive days under a dark-field microscope (OLYMPUS IMT-2) with a 10× (for an overall view of cell population) or 20× (neurite or axon/dendrite tree structure) objective. The images were recorded in JEPEG by an attached charge-coupled-device camera using SCION Image software (version 6.1). Under each experimental condition, 80 to 120 cells were randomly selected from randomly selected microscopic fields, recorded, and analyzed. The neurite outgrowth of PC12 cells was also monitored by the ratio of the neurite length to the cell body diameter. Significant neurite outgrowth was arbitrarily defined as neurites with extensions greater than two times the cell body length.

To rescue the defect in PC12 cells expressing siNckβ, the cells were reinfected with virus carrying genes for Nckα, siNckβ-resistant Nckβ (Nckβ-r), or GFP-paxillin. Forty-eight hours after the infection, half of the cells were subjected to Western blotting analysis and the other half were used for the morphological neurite outgrowth assays.

Forty-eight hours after culturing the dissociated primary cortical neurons, we infected the neurons with lentiviruses carrying the indicated constructs. Five days after the infection, individual images were captured from randomly selected fields under a fluorescence microscope (TE-2000U Eclipse; Nikon). All the images were imported to Image J (NIH) for analyses of total dendrite length. Total dendrite length refers to the length of all dendrites and branches of a single neuron. Each experiment was repeated at least three times. All data are expressed as the means plus or minus the standard errors of the results of separate experiments. The differences between the means were determined by the Student *t* test for unpaired samples, and those with a *P* value of <0.05 were considered significant.

GST pull-down and coimmunoprecipitation assays. Purified glutathione *S*-transferase (GST), GST-Nckα, or GST-Nckβ bound to glutathione-agarose beads (5 μg each) was incubated overnight with postnuclear lysates (~0.5 to 0.7 mg of total proteins) of PC12 cells in lysis buffer (20 mM Tris base, 50 mM NaCl, 50 mM sodium pyrophosphate, 30 mM NaF, 100 μM orthovanadate, 5 μM zinc chloride, 2 mM iodoacetic acid, 1.0% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail). The beads were washed three times with the same lysis buffer, and the bound proteins were dissociated from the beads by boiling at 95°C for 5 min in 1× sample buffer. The supernatants were subjected to Western blotting analysis with antipaxillin antibodies. Total lysates (40 to 60 μg of total proteins) were included as the control.

To compare the in vivo interactions between Nckα or Nckβ and paxillin, we overexpressed Nckα or Nckβ in 293T cells by transfection. Lysates of the cells (4×10^6) were immunoprecipitated with antipaxillin antibodies. The immunoprecipitates were resolved in a sodium dodecyl sulfate gel, transferred onto a nitrocellulose membrane, and immunoblotted with either antipaxillin antibodies or anti-Nckα or anti-Nckβ antibodies, respectively. Likely due to the low affinities of the anti-Nckα and anti-Nckβ antibodies, we were unable to clearly detect endogenous Nck by antipaxillin antibody coimmunoprecipitation.

RESULTS

Nckβ and Nckα have distinct functions in NGF-induced neurite outgrowth. NGF induces PC12 cells to produce dramatic neurite outgrowth, which is the characteristic of sympathetic neurons. The initiation of the neurite outgrowth becomes detectable after 24 to 48 h of NGF exposure. However, it takes 3 to 4 more days for these cells to differentiate and form neurites that are several times longer than the width of the cell body (2, 9). Therefore, the clear and inducible phenotype of this neuronal cell line was used to determine whether Nckα and Nckβ have distinct or redundant functions. We genetically silenced the endogenous Nckα or Nckβ expression by using RNA interference. To ensure the specificity, two siRNA sequences against different regions in each rat Nck gene were designed and constructed into the lentiviral FG-12 system next to a GFP reporter gene (20). Since the FG-12 vector integrates into the host chromosome following infection, the siRNA expression becomes permanent in the host cells. This is particularly important for long-term biological assays, such as neurite outgrowth in neurons.

First, to test the infection efficiency of the FG-12 system in PC12 cells, the cells were infected with the control FG-12–GFP vector and subjected to FACS analyses after 48 h. As shown in Fig. 1A, 96% of the cells in culture expressed GFP. Next, we tested the effectiveness of siNckα and siNckβ on the down-regulation of their target genes. As shown in Fig. 1B, the two siRNA constructs against Nckα dramatically down-regulated the endogenous Nckα (panel a, lanes 3 and 4) in comparison to the levels of Nckα in FG-12 vector- or control siLacZ-infected cells (panel a, lanes 1 and 2). In contrast, as expected, siNckα had no effect on the endogenous levels of Nckβ (panel b, lanes 3 and 4 versus lanes 1 and 2). Likewise, the two siNckβ constructs significantly down-regulated the endogenous Nckβ (panel b, lanes 5 and 6) in comparison to the levels in cells infected with vector alone or

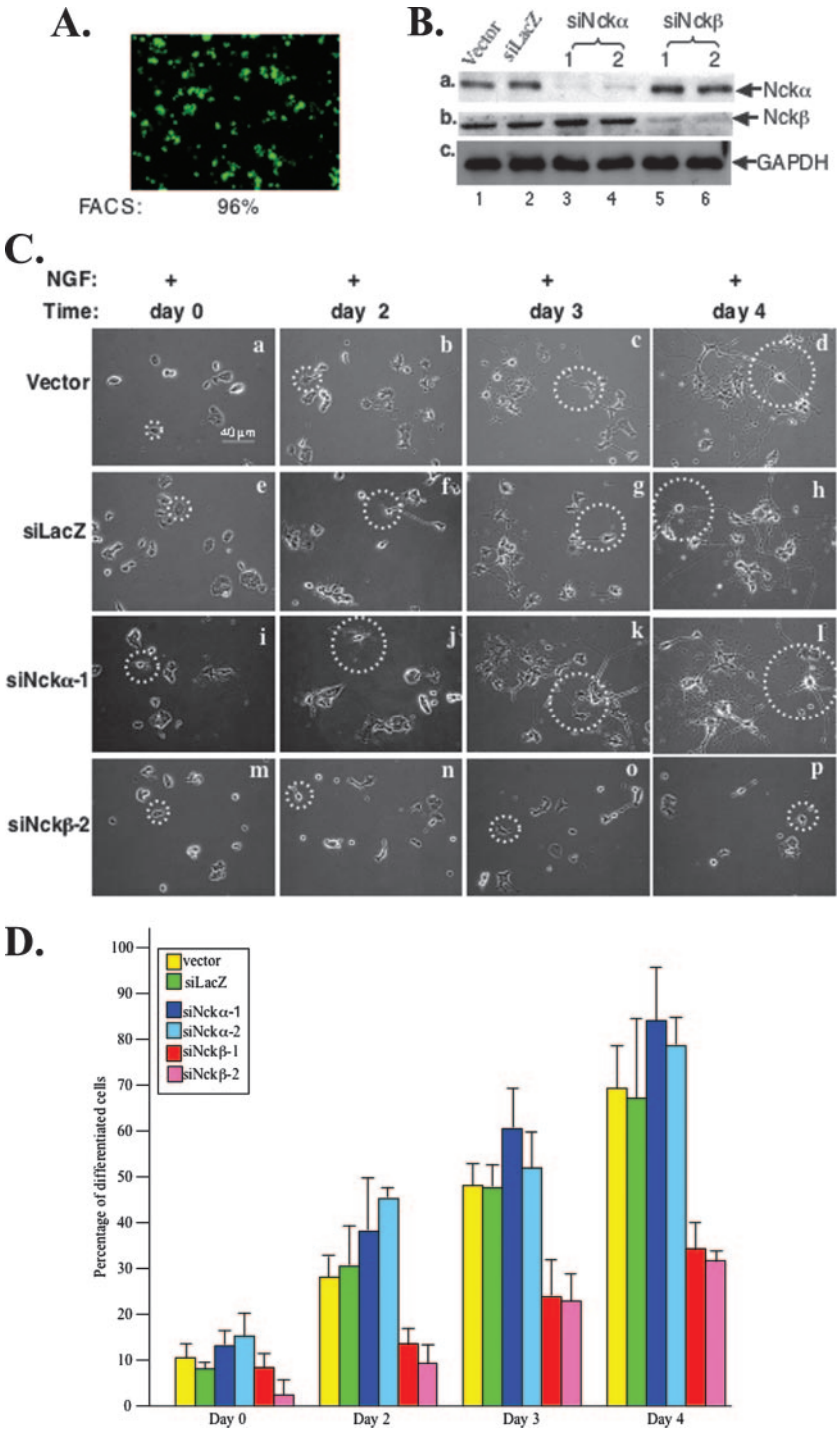

FIG. 1. Down-regulation of Nckβ, but not Nckα, blocks NGF-induced neurite outgrowth in PC12 cells. PC12 cells were infected individually with lentiviruses carrying empty vector (FG-12 with GFP alone), control siRNA against LacZ (siLacZ), two siRNA inserts against Nckα (siNckα1 and siNckα2), or two siRNA inserts against Nckβ (siNckβ1 and siNckβ2). (A) The expression of the GFP marker in the FG-12 vector showed a gene transduction efficiency of 96% by fluorescence microscopy and FACS analysis (green cells/total cells × 100%) of trypsin-suspended cells. (B) Five days after infection, the cells were lysed and subjected to Western blotting analysis with antibodies specific against Nckα (a), Nckβ (b), or GAPDH (c). The levels of Nck proteins were visualized by a horseradish peroxidase-conjugated secondary antibody followed by an enhanced-chemiluminescence reaction. (C) Following the above analyses, the cells were subjected to NGF-induced neurite outgrowth assays. Representative microscopic images of multiple cells and a single cell (as circled) under each indicated experimental condition are presented. (D) The percentage of the differentiated cells (averaged neurite extension from a single cell) under each indicated condition was based on more than 120 randomly selected cells per condition from randomly chosen microscopic fields. The values shown are the means ± standard errors of the means of the results of three independent assays; $P < 0.05$.

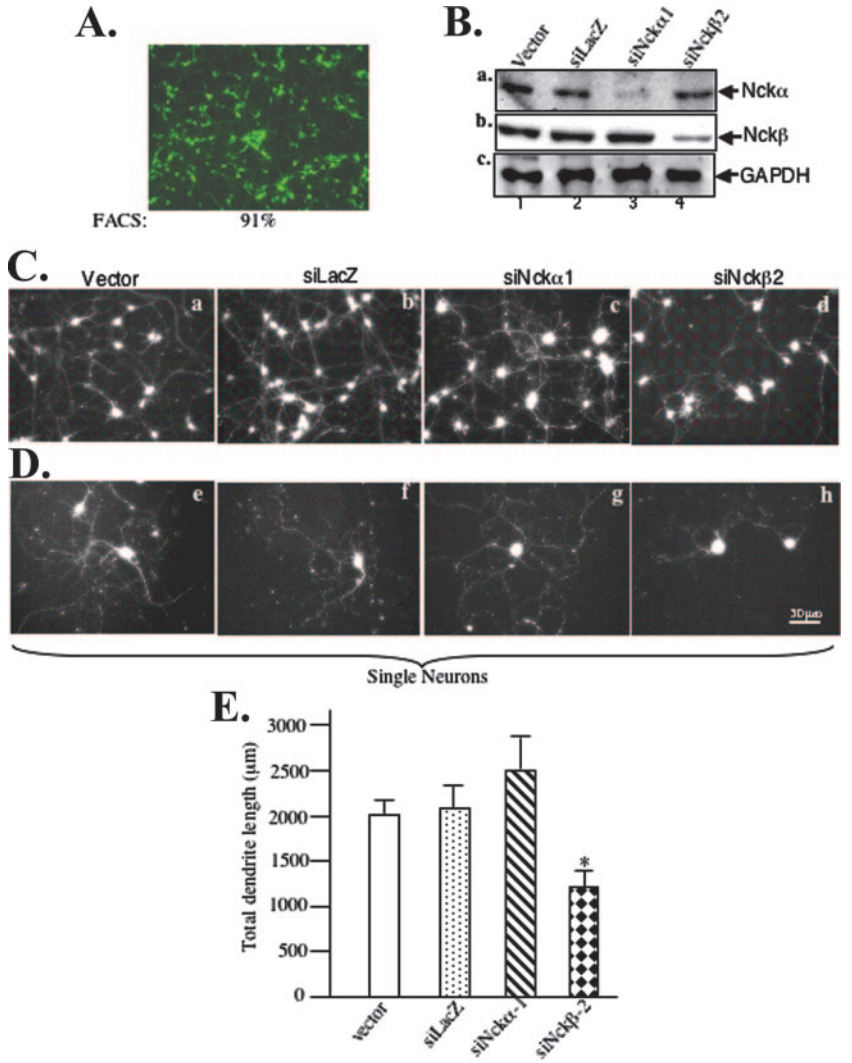

FIG. 2. Nckβ, but not Nckα, is critical for maintaining axonal and dendrite network in primary rat neurons. Primary rat cortical neurons in culture were infected with the vector alone, control siLacZ, siNckα1, or siNckβ2 construct. (A) Five days after infection, the gene transduction efficiency was determined by FACS measurement of GFP-positive cells in culture. (B) Lysates of the cells were subjected to Western blotting analysis with antibodies specific for Nckα (a), Nckβ (b), or GAPDH (c). (C and D) Fifteen randomly selected fields per experimental condition were analyzed under a fluorescence microscope, and representative images of multiple (C) and individual (D) cells at day 5 after infection are shown. (E) Results were quantitated by measuring total lengths of dendrites per neuron in 15 randomly selected areas or 80 neurons per condition (see Materials and Methods) and the means and standard deviations of the results were obtained. The values shown are the means ± standard errors of the means of data from three independent experiments. The value represented by the bar labeled with an asterisk is statistically significant over those of the three independent controls; $P < 0.02$. The values represented by bars not labeled with an asterisk are not statistically significant among themselves.

siLacZ control (panel b, lanes 1 and 2). However, the two siNckβ constructs did not affect endogenous Nckα levels (panel a, lanes 5 and 6). The results of immunoblotting duplicate membranes with anti-GAPDH antibodies showed that all lanes were loaded with similar amounts of total proteins (panel c).

PC12 cells infected with the control vector or siLacZ or siNck constructs were then subjected to experiments for determining the levels of NGF-stimulated neurite outgrowth. As shown in Fig. 1C, NGF induced dramatic neurite outgrowth in PC12 cells infected with the control vector (panels a to d) or the siLacZ construct (panels e to h). Interestingly, down-regulation of Nckα increased the basal level of neurite presence even in the absence of NGF (panel i versus panels a and e). In the presence of NGF, these cells showed earlier onset of induced neurite outgrowth (panel j versus panels b and f). Down-regulation of Nckβ did not alter the basal level of neurite outgrowth (panel m); however, it completely blocked NGF-stimulated neurite outgrowth over the time period (panels n to p). The second siRNA constructs (i.e., siNckα2 and siNckβ1) gave results similar to those for siNckα1 and siNckβ2, and the data are presented as the general quantitation of the neurite outgrowth under the various conditions shown in Fig. 1D. Thus, Nckβ is essential for NGF signaling to induce neurite outgrowth.

Nckβ is required for maintaining axonal and dendrite tree architecture in primary rat neurons. To investigate whether Nckβ plays a similar role in primary neurons, we infected isolated rat cortical neurons with the same set of siRNAs against Nck. As shown in Fig. 2A, the FG-12 system offered more than 90% gene

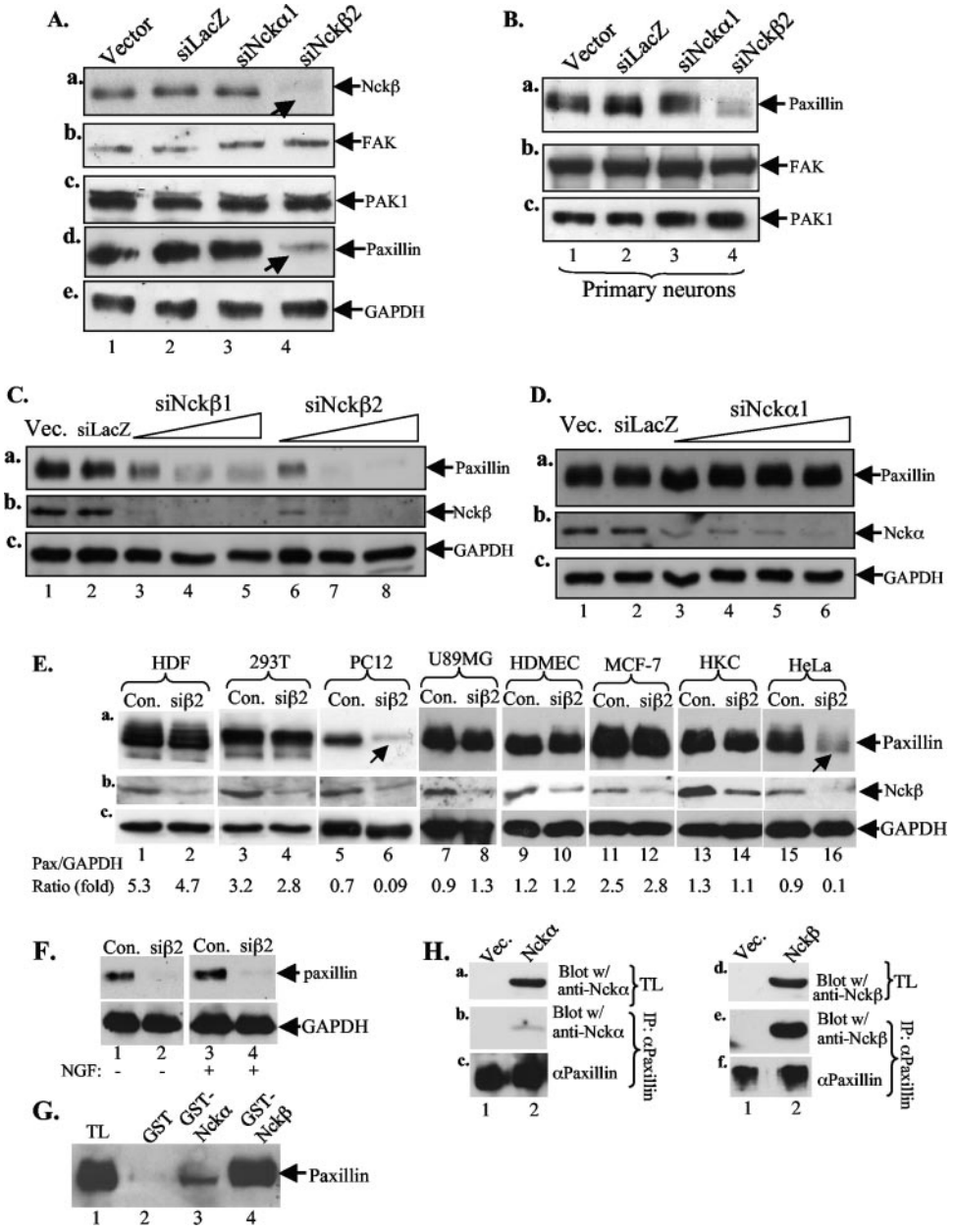

FIG. 3. Absence of Nckβ leads to paxillin down-regulation in neurons. (A) Effects of Nckβ knockdown on other components of the protein complex, i.e., paxillin, FAK, and Pak1. PC12 cells were infected with viruses containing the FG-12 vector or the control siLacZ, siNckα1, or siNckβ2 construct. Five days after infection, the lysates of the cells were blotted with antibodies against Nckβ, paxillin, FAK, Pak1, or GAPDH. (B) Experiments similar to those described for panel A were carried out with primary rat cortical neurons. (C and D) PC12 cells were infected with the FG-12 vector, control siLacZ, or increasing amounts (up to the same dilution of viruses used in the neuritogenesis assays) of viruses containing siNckβ1, siNckβ2, or siNckα1. Five days after infection, the lysates of the cells were subjected to Western blotting with antipaxillin (C, panel a, and D, panel a), anti-Nckβ (C, panel b), anti-Nckα1 (D, panel b), or anti-GAPDH (C and D, panel c) antibodies. (E) PC12 cells and nonneuronal cells of glial (U89MG), epithelial (MCF-7 and HeLa), endothelial (HDMEC), dermal (HDF), epidermal (HKC), and embryonic (293T) origins were infected with siNckβ2 (siβ2) and analyzed for paxillin down-regulation. The intensities of the paxillin (Pax) bands from underexposed films were quantitated by densitometry and compared with their corresponding GAPDH control bands; the ratios obtained are shown below the lanes. Con., control. (F) Down-regulation of paxillin in Nckβ knockdown cells in the absence or presence of NGF. Con., control; siβ2, siNckβ2. (G) GST-Nckα and GST-Nckβ (~5 μg) on beads were incubated with total lysates of the indicated cells (~800 μg of total proteins). The beads were washed and the bound proteins eluted by heating at 95°C for 5 min. The supernatants were subjected to Western blotting analysis together with total lysates (~40 μg total proteins) using antipaxillin antibodies. (H) Vector (Vec.), Nckα, or Nckβ cDNA was transfected into 293T cells. The immunoprecipitates of the cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with antipaxillin (c and f), anti-Nckα (b), or anti-Nckβ (e) antibodies. TL, total lysates; IP, immunoprecipate; αPaxillin, antipaxillin.

transduction efficiency in these fully differentiated neurons. When these neurons were infected with siNckα1 or siNckβ2, as shown in Fig. 2B, we detected down-regulation of the endogenous Nckα (panel a, lane 3) or Nckβ (panel b, lane 4), respectively, in comparison to the levels in the cells infected with the FG-12 vector or siLacZ control (lanes 1 and 2). When the axon and dendrite structures in these cells were analyzed under a fluorescence microscope, as shown in Fig. 2C, the neurons infected with either vector or siLacZ control maintained their constitutive axon and dendrite networks (panels a and b). Likewise, down-regulation of Nckα did not cause any significant changes in the cells' phenotype (panel c). However, down-regulation of Nckβ dramatically reduced the number of axons and dendrites per neuron and deteriorated the overall network architecture. When we visualized the effects of Nckβ knockdown on individual neurons, as shown in Fig. 2D, we observed that neurons infected with vector alone (panel e), siLacZ (panel f), or siNckα1 (panel g) still maintained the fully differentiated phenotype. However, neurons infected with siNckβ2 showed severely disrupted axon and dendrite networks (panel h). Therefore, Nckβ also has a unique role in the induction and maintenance of neuritogenesis in primary neurons. The quantitation of the axon and dendrite structure is shown in Fig. 2E.

Nckβ maintains the paxillin level in neuronal cells. To investigate the possible downstream defect of Nckβ knockdown, we screened a number of focal adhesion-associated proteins which have been previously shown to directly or indirectly associate with Nck (24, 26). As shown in Fig. 3A, down-regulation of Nckβ or Nckα did not alter the cellular levels of focal adhesion kinase (FAK) (panel b) or $p21^{Rac/Cdc24}$-activated kinase 1 (PAK1) (panel c). Interestingly, down-regulation of Nckβ (panel a, lane 4), but not Nckα (panel a, lane 3), significantly reduced the intracellular level of paxillin (panel d, lane 4 versus lane 3). GAPDH was used as the loading control (panel e). We confirmed a similar observation in primary neurons. As shown in Fig. 3B, down-regulation of Nckβ caused a concomitant down-regulation of paxillin (panel a, lane 4 versus lanes 1 and 2). In contrast, Nckα knockdown showed little effect (panel a, lane 3). As expected, down-regulation of Nckβ did not affect FAK or PAK in the cells (panels b and c, lane 4).

To further establish the above finding, we studied the effects of dose-dependent down-regulation of Nckβ and Nckα on paxillin levels. As shown in Fig. 3C, infecting PC12 cells with increasing titers of siNckβ1 or siNckβ2 virus resulted in a dose-dependent down-regulation of the endogenous paxillin (panel a, lanes 3 to 8 versus lanes 1 and 2). This down-regulation of paxillin correlated well with the degree of Nckβ knockdown (panel b, lanes 3 to 8 versus lanes 1 and 2). In contrast, as shown in Fig. 3D, dose-dependent down-regulation of Nckα (panel b, lanes 3 to 6) had little effect on the paxillin level (panel a), even though the down-regulation of Nckα was nearly complete (panel b, lane 6). In both experiments, GAPDH was used as the loading control (panel c).

Because paxillin is critical for mouse development (10), we wondered, if paxillin had been totally missing in $Nck\beta^{-/-}$ mice, how the mice would have survived. Therefore, we speculated that Nckβ may not control the stability of paxillin in all cell types. We tested this possibility by screening several types of cells from a variety of origins, together with PC12 cells. As shown in Fig. 3E, Nckβ knockdown caused, as expected, more than an 85% reduction of paxillin in PC12 cells (lane 6). In

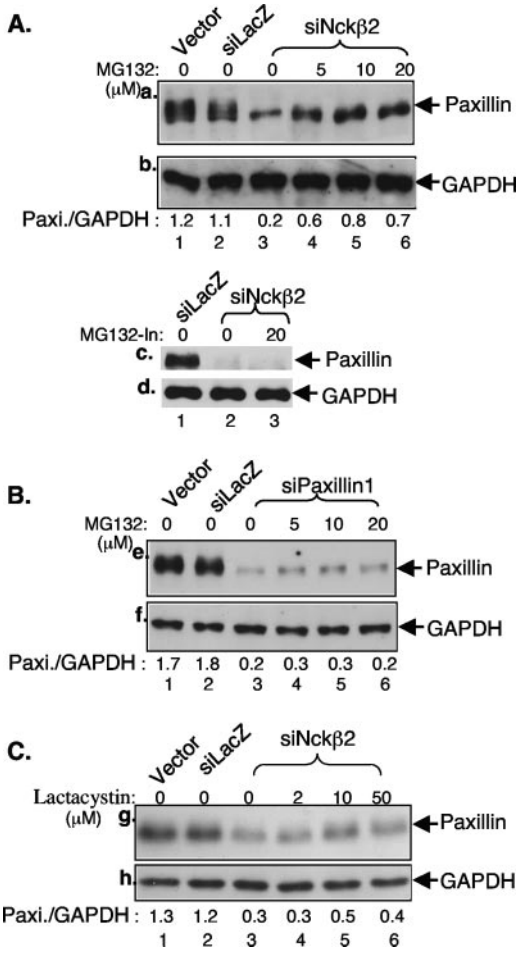

FIG. 4. Inhibition of proteasome-mediated protein degradation prevents paxillin down-regulation in siNckβ-expressing cells, but not in siPaxillin-expressing cells. Five days after infection of PC12 cells with siNckβ2 or siPaxillin, the cells were incubated with increasing concentrations of MG132, its inactive derivative (M132-In), or lactacystin for 12 h. (A and B) Lysates of the cells were analyzed by antipaxillin antibody blotting (a, c, and e). GAPDH was included as the sample loading control (b, d, and f). (C) Experiments similar to those described for panel A were carried out, except that MG132 was replaced with lactacystin. The paxillin levels were quantitated by densitometry, giving rise to ratios of paxillin (Paxi.) intensity compared with the control GAPDH intensity, shown below the lanes.

contrast, the absence of Nckβ did not affect the paxillin levels in primary human dermal fibroblasts (lanes 1 and 2), embryonic kidney epithelial cell line 293T (lanes 3 and 4), primary human dermal microvascular endothelial cells (lanes 9 and 10), human breast epithelial cancer cell line MCF-7 (lanes 11 and 12), or primary human epidermal keratinocytes (lanes 13 and 14). More intriguingly, we found that the paxillin level remained unchanged even in a glioma cell line, U89MG, after down-regulation of Nckβ (lanes 7 and 8). However, as in the neurons, down-regulation of Nckβ also caused a concomitant reduction of paxillin in the cervical epithelial cancer cells, HeLa (lanes 15 and 16). At this time, it remains to be tested whether this result was due to the transformed cellular context in HeLa cells. Therefore, it would be interesting to test primary epithelial cells of breast and cervix. Finally, the down-regula-

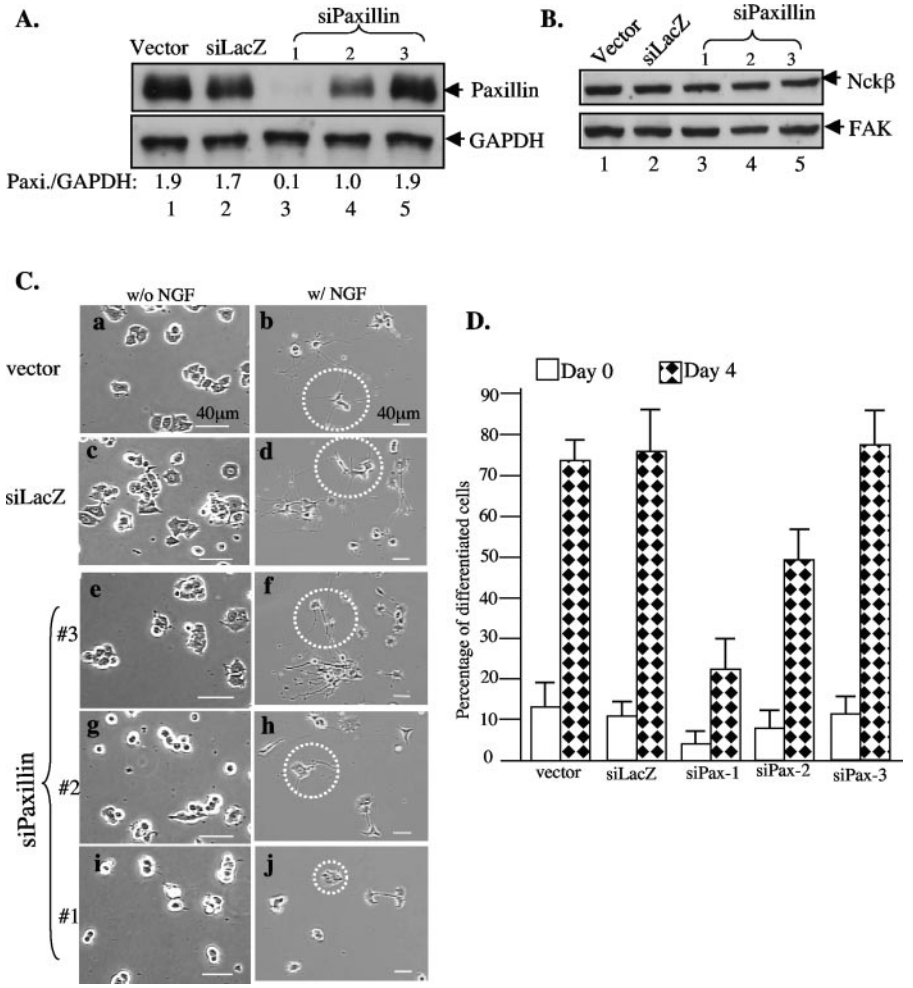

FIG. 5. The cellular paxillin level is critical for NGF-induced neurite outgrowth in PC12 cells. PC12 cells were infected with the FG-12 vector, the siLacZ construct, or one of three independent siRNA constructs against paxillin (siPaxillin-1, -2, and -3). (A) Five days after infection, cells were lysed and subjected to Western blotting. The upper portion of the membrane was immunoblotted with an antipaxillin antibody, and the lower portion of the same membrane immunoblotted with an anti-GAPDH antibody. The ratio of paxillin (Paxi.) intensity to GAPDH intensity quantitated by densitometry is shown below each lane. (B) Duplicate membranes were immonublotted with anti-FAK and anti-Nckβ antibodies. (C) Representative images of PC12 cells infected with the indicated siPaxillin or control constructs in the absence or presence of NGF (100 ng/ml of NGF for 3 days) are shown. We show the images of the cells not treated with NGF twice as large as those of the NGF-treated and differentiated cells for the purpose of a closer view of their morphology. The circled cell represents the morphology of the majority of the neurons under that condition. (D) The averaged percentage of more than 120 randomly selected cells per experimental condition was quantitated. The values shown are the means ± standard errors of the means of the results of three independent experiments; $P < 0.05$.

tion of paxillin remained the same in Nckβ knockdown PC12 cells cultured either in the presence or in the absence of NGF (Fig. 3F, lane 4 versus lane 2).

We then studied the possible mechanism by which Nckβ maintains, whereas Nckα does not, the steady-state paxillin level in neuronal cells. First, we carried out GST-Nckβ and GST-Nckα pull-down assays using lysates of PC12 cells. As shown in Fig. 3G, GST alone did not bind any detectable amount of paxillin (lane 2) with reference to that of the total lysate control (lane 1). GST-Nckα pulled down a small amount of paxillin (<0.1%). In contrast, GST-Nckβ pulled down significantly more paxillin (~10% of the total paxillin in the lysate, lane 4). Unfortunately, due to the low affinities of the anti-Nckα and anti-Nckβ antibodies in both Western blots and immunoprecipitations, we were unable to detect any endogenous Nck that otherwise might coimmunoprecipitate with paxillin. Therefore, as shown in Fig. 3H, we transiently overexpressed Nckα (panel a, lane 2) and Nckβ (panel d, lane 2) in 293T cells and coimmunoprecipitated them with antibodies against the endogenous paxillin (panels c and f). Clearly, significantly more Nckβ (panel e, lane 2) than Nckα (panel b, lane 2) was coimmunoprecipitated by antipaxillin antibodies.

Didier and colleagues showed that paxillin is a target for posttranslational modification by ubiquitination (7). Therefore, we asked if the reduction of paxillin in Nckβ-knockdown neuronal cells is due to protein degradation. We tested two proteasome inhibitors, MG132 and lactacystin, on paxillin down-regulation in PC12 cells expressing siNckβ. Among the three types of proteases in proteasomes, MG132 is known to inhibit chymotrypsin-like and calpain-like activities, whereas

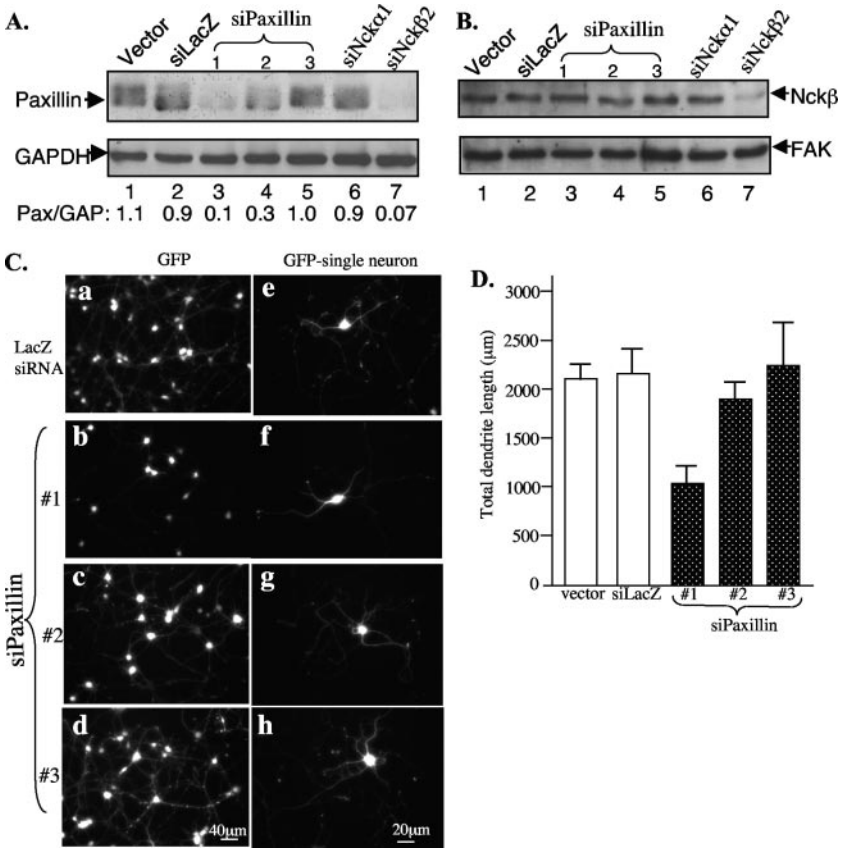

FIG. 6. Requirement for a physiological level of paxillin in maintaining dendrite tree structure in primary neurons. Primary rat neurons were infected with the FG-12 vector or the siLacZ, siNckα, siNckβ, or siPaxillin-1, -2, or -3 construct. (A) Five days after infection, cells were lysed and subjected to Western blotting. The upper portion of the membrane was immunoblotted with an antipaxillin antibody, and the lower portion of the same membrane immunoblotted with an anti-GAPDH antibody. The ratio of paxillin (Pax) to GAPDH is shown below each lane. (B) Duplicate membranes were immonublotted with anti-FAK and anti-Nckβ antibodies. (C) Representative images of the neurons infected with the indicated control siRNA or siPaxillin construct under a fluorescence microscope are shown. The left panels show multiple cells, and the right panels show single neurons. (D) The results were quantitated by measuring the total lengths of dendrites/neuron in 15 randomly selected areas per experimental condition. The values shown are the means ± standard errors of the means of the results of four independent experiments; $P < 0.05$.

lactacystin inhibits chymotrypsin-like and trypsin-like activities. As shown in Fig. 4A, MG132 treatment of Nckβ-knockdown PC12 cells partially (50 to 70%) restored the paxillin level (panel a, lanes 4 to 6 versus lane 3). In contrast, an inactive MG132 derivative (benzyloxycaronyl-leucinyl-leucinyl-leucinyl-amide) was unable to do the same (panel c, lane 3). As expected, MG132 was unable to rescue the down-regulation of paxillin caused by siRNA against paxillin itself (Fig. 4B, panel e, lanes 4 to 6 versus lane 3). Lactacystin showed much weaker rescuing effect. As shown in Fig. 4C, we detected a maximum of 15% to 20% restoration of the steady-state paxillin level following lactacystin treatments (panel g, lanes 5 and 6 versus lanes 1 and 2). These results suggest that Nckβ controls paxillin stability by a posttranslational mechanism in neurons.

Like Nckβ, paxillin is critical for neuritogenesis. We then investigated whether the failure of Nckβ knockdown PC12 cells and primary neurons to undergo and maintain neuritogenesis was due to the concurrent down-regulation of paxillin in the cells. To study this question, we constructed three siRNAs against paxillin (siPaxillin-1, siPaxillin-2, and siPaxillin-3). As shown in Fig. 5A, siPaxillin-1, siPaxillin-2, and siPaxillin-3 down-regulated paxillin either completely (lane 3 versus lanes 1 and 2), partially (lane 4 versus lanes 1 and 2), or undetectably (lane 5 versus lanes 1 and 2), respectively. In contrast, as expected, none of the three siRNAs against paxillin affected FAK or Nckβ in the same cells (Fig. 5B). Therefore, these siRNAs against paxillin could be utilized to determine the dose-dependent effects of paxillin down-regulation. Four days following NGF stimulation, as shown in Fig. 5C, a dramatic neurite outgrowth was detected in both vector- and siLacZ control-infected cells (panels b and d). However, as the paxillin level decreased, the cells gradually lost their ability to undergo neurite outgrowth (panels f, h, and j). The quantitation of the data is shown in Fig. 5D.

Similar results were obtained in primary rat neurons. As shown in Fig. 6A, the three paxillin siRNAs differentially down-regulated the paxillin levels (lanes 3 to 5) in comparison to the levels in cells infected with vector alone, the siLacZ control (lanes 1 and 2), or the siNckα and siNckβ controls (lanes 6 and 7). In contrast, none of the siPaxillins showed any effect on the Nckβ or FAK levels (Fig. 6B). Since a GFP gene is coexpressed with both the control vector and the vectors with inserted siPaxillins, we analyzed the axon and dendrite structures of the neurons under a fluorescence microscope. As

shown in Fig. 6C (left column, panels a to d), the extent of disruption is correlated with the degree of paxillin knockdown (panels b, c, and d versus panel a). The siPaxillin-1 construct almost completely demolished the axon and dendrite network (panel b versus panel a). When single neurons were visualized, as shown in Fig. 6C (right column, panels e to h), down-regulation of paxillin was seen to decrease the number of axons and dendrites per neuron in correlation with the degree of paxillin knockdown (panels f to h versus panel e). Thus, paxillin, like Nckβ, is essential for maintaining neuritogenesis.

Reintroducing Nckβ or paxillin rescues the neuritogenesis defect in Nckβ knockdown PC12 cells. If the role of Nckβ in neuritogenesis is to maintain paxillin stability, the defect in Nckβ-knockdown neurons should be correctable by reexpressing a siNckβ-resistant Nckβ or the wild-type paxillin in the cells. To test this possibility, we reintroduced a full-length engineered Nckβ (Nckβ-r) into siNckβ2-expressing PC12 cells. This engineered Nckβ is resistant to siNckβ2, because it contains three silent mutations in nucleotides 975 (A to G), 978 (G to C), and 981 (T to C) within the 19-bp (973 to 992) siNckβ2-targeted region. Expression of the wild-type Nckα was included as a control. As shown in Fig. 7A, successful expression of Nckβ-r in control and siNckβ-expressing PC12 cells was demonstrated by immunoblotting the membrane with anti-Nckβ antibodies (panel a, lanes 2 and 4). As expected, down-regulation of Nckβ caused a decreased paxillin level (panel b, lane 3 versus lanes 1). While coexpression of Nckβ-r did not affect paxillin levels in cells expressing the siLacZ control (lane 2 versus lane 1), it overrode siNckβ2-induced down-regulation of paxillin and restored the normal cellular level of paxillin (lane 4 versus lane 3). Immunoblotting of a duplicate membrane with an anti-PAK1 antibody was used as the loading control (panel c). In contrast, the overexpression of wild-type Nckα was unable to rescue siNckβ-induced down-regulation of paxillin (Fig. 7B, panel b, lane 4 versus lane 3). Overexpressed Nckα itself was demonstrated by immunoblotting the lower part of the same membrane with an anti-Nckα antibody (panel a, lanes 2 and 4).

We then analyzed the control and the PC12 cells reexpressing the Nck gene for neurite outgrowth in response to NGF. As shown in Fig. 8A, we found that NGF was able to induce neurite outgrowth in siLacZ control cells (panel b versus panel a) but not the cells expressing siNckβ (panel d versus panel c). However, coexpression of the Nckβ-r construct rescued the ability of the "Nckβ-less" and "paxillin-less" PC12 cells to undergo neurite outgrowth in response to NGF (panel h versus panel d). In contrast, overexpression of Nckα was unable to do the same in these cells (panel f versus panel d). We expected that reexpression of paxillin should bypass the Nckβ knockdown blockade of neuritogenesis in PC12 cells. As shown in Fig. 7C, the expression of a higher-molecular-weight GFP-paxillin fusion protein was detected in the infected cells (panel a, lanes 3 and 4), but not in the siLacZ control cells (lanes 1 and 2). As expected, the overexpressed paxillin was unable to rescue the siNckβ-induced down-regulation of Nckβ (panel b, lane 4 versus lane 2), confirming that paxillin acts downstream of Nckβ. When these cells were subjected to neurite outgrowth assays in response to NGF, as shown in Fig. 8A, the overexpression of GST-paxillin in the siNckβ-expressing cells restored the production of NGF-induced neurite outgrowth (panel j versus panel i). The quantitation of the data is shown in Fig. 8B. Taken together, these results establish the pathway of Nckβ leading to paxillin stability leading to neuritogenesis.

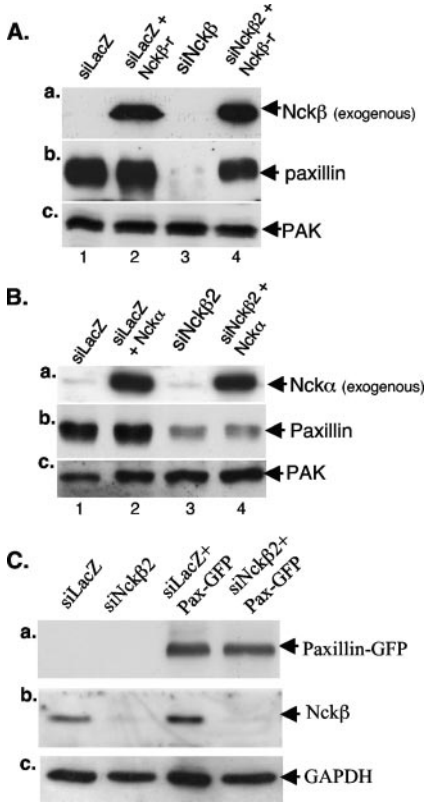

FIG. 7. Reexpression of siRNA-resistant Nckβ rescues paxillin in Nckβ knockdown cells. SiNckβ2-expressing PC12 cells were reinfected with pRRLsinh-CMV lentiviral vector carrying a siNckβ2-resistant Nckβ gene (Nckβ-r) (A) or the wild-type Nckα gene (B) or GFP-paxillin (C). Forty-eight hours following infection, lysates of the cells were analyzed by Western blotting with antibodies against paxillin, Nckβ, Nckα, Pak1, or GAPDH. (A) Overexpressed Nckβ-r (a), effect of the overexpressed Nckβ-r on paxillin levels (b), and anti-PAK blotting control (c). (B) Overexpressed Nckα (a), effect of overexpressed Nckα on paxillin levels (b), and anti-PAK blotting control (c). (C) Overexpressed GFP-paxillin (a), effect of the overexpressed GFP-paxillin level on endogenous Nckβ (b), and anti-GAPDH blotting control (c). These experiments were repeated three or four times.

DISCUSSION

Axonal growth and dendrite tree extension are key morphological features of neuronal differentiation. The process of neurite outgrowth defines neuronal shape, mediates neuronal pathfinding, and is essential for the eventual establishment of synaptic connections during development. Neurite outgrowth from the PC12 cell line originates from NGF binding to the receptor tyrosine kinase TrkA. This leads to a cascade of signal transduction and coordinated changes in the cytoskeleton, gene expression, and de novo protein synthesis (13). A number of previous studies of models from flies to mammals indicate that Nck adapters play an important role in extracellular cue-induced axon guidance and neuronal differentiation. The mechanism, however, remained elusive. In the current study,

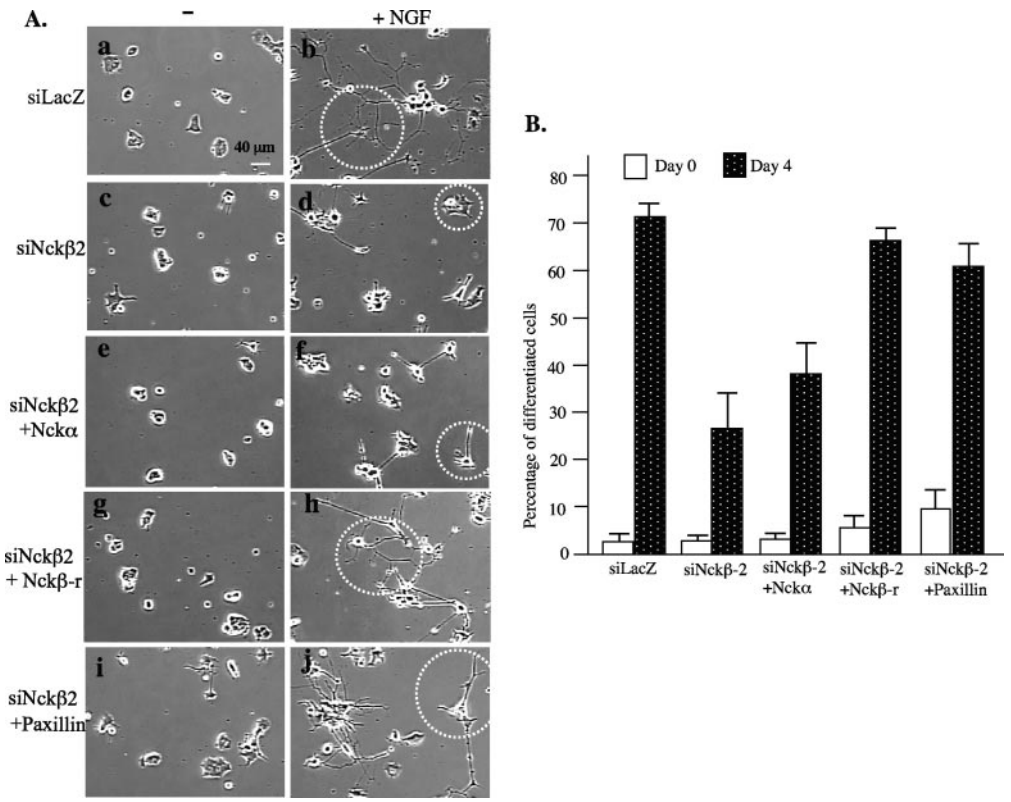

FIG. 8. Reexpression of siRNA-resistant Nckβ rescues NGF-induced neurite outgrowth in Nckβ knockdown cells. PC12 cells expressing siNckβ2 or control siRNA were reinfected with pRRLsinh-CMV carrying a siNckβ2-resistant Nckβ gene, a wild-type Nckα gene, or a GST-paxillin fusion gene. Forty-eight hours following infection, the cells were subjected to neurite outgrowth assays in response to NGF. (A) Reexpression of Nckβ-r rescued siNckβ2-expressing PC12 cells to reestablish the NGF-induced neurite outgrowth cellular morphology (panel h versus panel g). Reexpression of GFP-paxillin rescued siNckβ2-expressing PC12 cells to reestablish the NGF-induced neurite outgrowth (panel j versus panel i). Reexpression of Nckα had no rescuing effect. The circles indicate averaged neurite extension from a single cell. The values shown are the means ± standard errors of the means of the results of four independent assays; $P < 0.05$.

we have unveiled a previously unrecognized mechanism by which Nckβ, but not Nckα, is selected to mediate cell surface cues in supporting and/or maintaining neuritogenesis. In doing so, Nckβ binds and maintains the physiological level of paxillin, a critical focal adhesion molecule. Like Nckβ$^{-/-}$ neurons, neurons with paxillin knockdown failed to produce axons and dendrites. More intriguingly, Nckβ does not appear to affect paxillin stability in most of the other cell types studied. We conclude that Nckβ plays a critical role in neuronal differentiation.

Efforts were made to verify that the down-regulation of paxillin is specifically due to the down-regulation of Nckβ, rather than a cross-reactivity of Nckβ siRNAs with paxillin mRNA. First, neither of the two selective siRNA sequences has any significant homology with paxillin cDNA or any other existing rat mRNAs (according to an NCBI BLAST examination of RefSeq and UniGene databases). Second, two completely different Nckβ siRNA sequences showed similar effects on paxillin down-regulation, whereas none of the siRNAs against Nckα showed any effects on the paxillin levels. Third, reexpressing an engineered siRNA-resistant Nckβ (Nckβ-r) fully restored the cellular paxillin level. Finally, the reexpression of Nckβ-r or paxillin in Nckβ-down-regulated PC12 cells rescued the ability of the cells to undergo neurite outgrowth in response to NGF. We found that GST-Nckβ pulled down significantly more paxillin than GST-Nckα and antipaxillin antibodies coimmunoprecipitated more Nckβ than Nckα. These results suggest that Nckβ prevents paxillin degradation by binding, directly or indirectly, to paxillin.

The inhibition of protein degradation by two potent proteasome inhibitors, MG132 and lactacystin, showed differential effects on paxillin in PC12 cells expressing siNckβ. MG132 showed a much stronger protecting effect than lactacystin, suggesting that Nckβ controls paxillin stability via a posttranslational mechanism. Turner reported the formation of a Nck-Pak-PIX-PKL-paxillin-FAK multiple protein complex in rat brain lysate (24). However, we did not detect any significant down-regulation of Pak or FAK in the cells expressing siNckβ. Assuming the above complex also exists in PC12 cells, our finding indicates that Nckβ selectively controls the stability of certain components within the protein complex, such as paxillin, but not other components, such as FAK and Pak.

How does one integrate our findings with the observation that Nckβ$^{-/-}$ (Nckα$^{+/+}$) and Nckα$^{-/-}$ (Nckβ$^{+/+}$) mice are developmentally viable and normal (1)? Specifically, if Nckβ controls the paxillin stability in neurons, one might expect that Nckβ$^{-/-}$ (Nckα$^{+/+}$) mice would exhibit mental retardation and other neurological symptoms. There have been no answers

to these questions, since the Nckβ$^{-/-}$ (Nckα$^{+/+}$) and Nckα$^{-/-}$ (Nckβ$^{+/+}$) mice have not been evaluated for possible neurological pathology. In fact, the individual and noncompensating roles of Nckα and Nckβ are more evident in the central nervous system than other tissues in the mice (Tony Pawson, personal communication). Vaynberg and colleagues suggested an alternative explanation. They proposed that "chronic" alteration of Nck genes, such as in mice, is less harmful than an "acute" inhibition of the same gene in somatic cells (25). It may also depend upon the developmental stage of a given signaling pathway, which may only become indispensable in adult animals and tissues. For instance, embryonic fibroblast cell lines derived from Nckβ$^{-/-}$ or Nckα$^{-/-}$ mice both exhibited defects in migration in response to platelet-derived growth factor-BB (21). Therefore, the platelet-derived growth factor-BB signaling may be compensated during the embryonic stage of mouse development and become indispensable in fully differentiated somatic cells.

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REFERENCES

1. **Bladt, F., E. Aippersbach, S. Gelkop, G. A. Strasser, P. Nash, A. Tafuri, F. B. Gertler, and T. Pawson.** 2003. The murine Nck SH2/SH3 adaptors are important for the development of mesoderm-derived embryonic structures and for regulating the cellular actin network. Mol. Cell. Biol. **23:**4586–4597.
2. **Chao, M. V., and B. L. Hempstead.** 1995. p75 and Trk: a two-receptor system. Trends Neurosci. **18:**321–326.
3. **Chen, M., W. Li, J. Fan, N. Kasahara, and D. Woodley.** 2003. An efficient gene transduction system for studying gene function in primary human dermal fibroblasts and epidermal keratinocytes. Clin. Exp. Dermatol. **28:**193–199.
4. **Chen, M., H. She, E. M. Davis, C. M. Spicer, L. Kim, R. Ren, M. M. Le Beau, and W. Li.** 1998. Identification of Nck family genes, chromosomal localization, expression, and signaling specificity. J. Biol. Chem. **273:**25171–25178.
5. **Chen, M., H. She, A. Kim, D. T. Woodley, and W. Li.** 2000. Nckβ adapter regulates actin polymerization in NIH 3T3 fibroblasts in response to platelet-derived growth factor bb. Mol. Cell. Biol. **20:**7867–7880.
6. **Cowan, C. A., and M. Henkemeyer.** 2001. The SH2/SH3 adaptor Grb4 transduces B-ephrin reverse signals. Nature **413:**174–179.
7. **Didier, C., L. Broday, A. Bhoumik, S. Israeli, S. Takahashi, K. Nakayama, S. M. Thomas, C. E. Turner, S. Henderson, H. Sabe, and Z. Ronai.** 2003. RNF5, a RING finger protein that regulates cell motility by targeting paxillin ubiquitination and altered localization. Mol. Cell. Biol. **23:**5331–5345.
8. **Garrity, P. A., Y. Rao, I. Salecker, J. McGlade, T. Pawson, and S. L. Zipursky.** 1996. Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. Cell **85:**639–650.
9. **Greene, L. A., and A. S. Tischler.** 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73:**2424–2428.
10. **Hagel, M., E. L. George, A. Kim, R. Tamimi, S. L. Opitz, C. E. Turner, A. Imamoto, and S. M. Thomas.** 2002. The adapter protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. Mol. Cell. Biol. **22:**901–915.
11. **Hing, H., J. Xiao, N. Harden, L. Lim, and S. L. Zipursky.** 1999. Pak functions downstream of Dock to regulate photoreceptor axon guidance in Drosophila. Cell **97:**853–863.
12. **Huebner, K., K. Kastury, T. Druck, A. E. Salcini, L. Lanfrancone, G. Pelicci, E. Lowenstein, W. Li, S. H. Park, L. Cannizzaro, et al.** 1994. Chromosome locations of genes encoding human signal transduction adapter proteins, Nck (NCK), Shc (SHC1), and Grb2 (GRB2). Genomics **22:**281–287.
13. **Kaplan, D. R., and F. D. Miller.** 2000. Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol. **10:**381–391.
14. **Kempiak, S. J., H. Yamaguchi, C. Sarmiento, M. Sidani, M. Ghosh, R. J. Eddy, V. Desmarais, M. Way, J. Condeelis, and J. E. Segall.** 2005. A neural Wiskott-Aldrich syndrome protein-mediated pathway for localized activation of actin polymerization that is regulated by cortactin. J. Biol. Chem. **280:**5836–5842.
15. **Li, W., J. Fan, M. Chen, S. Guan, D. Sawcer, G. M. Bokoch, and D. T. Woodley.** 2004. Mechanism of human dermal fibroblast migration driven by type I collagen and platelet-derived growth factor-BB. Mol. Biol. Cell **15:**294–309.
16. **Li, W., J. Fan, and D. T. Woodley.** 2001. Nck/Dock: an adapter between cell surface receptors and the actin cytoskeleton. Oncogene **20:**6403–6417.
17. **O'Neill, K., S. Chen, and R. D. Brinton.** 2004. Impact of the selective estrogen receptor modulator, raloxifene, on neuronal survival and outgrowth following toxic insults associated with aging and Alzheimer's disease. Exp. Neurol. **185:**63–80.
18. **Pawson, T., and P. Nash.** 2003. Assembly of cell regulatory systems through protein interaction domains. Science **300:**445–452.
19. **Pramatarova, A., P. G. Ochalski, K. Chen, A. Gropman, S. Myers, K. T. Min, and B. W. Howell.** 2003. Nckβ interacts with tyrosine-phosphorylated disabled 1 and redistributes in Reelin-stimulated neurons. Mol. Cell. Biol. **23:**7210–7221.
20. **Qin, X. F., D. S. An, I. S. Chen, and D. Baltimore.** 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci USA **100:**183–188.
21. **Rivera, G. M., S. Antoku, S. Gelkop, N. Y. Shin, S. K. Hanks, T. Pawson, and B. J. Mayer.** 2006. Requirement of Nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. Proc. Natl. Acad. Sci. USA **103:**9536–9541.
22. **Schlessinger, J.** 1994. SH2/SH3 signaling proteins. Curr. Opin. Genet. Dev. **4:**25–30.
23. **Stein, E., U. Huynh-Do, A. A. Lane, D. P. Cerretti, and T. O. Daniel.** 1998. Nck recruitment to Eph receptor, EphB1/ELK, couples ligand activation to c-Jun kinase. J. Biol. Chem. **273:**1303–1308.
24. **Turner, C. E.** 2000. Paxillin and focal adhesion signaling. Nat. Cell Biol. **2:**E231–E236.
25. **Vaynberg, J., T. Fukuda, K. Chen, O. Vinogradova, A. Velyvis, Y. Tu, L. Ng, C. Wu, and J. Qin.** 2005. Structure of an ultraweak protein-protein complex and its crucial role in regulation of cell morphology and motility. Mol. Cell **17:**513–523.
26. **Vindis, C., T. Teli, D. P. Cerretti, C. E. Turner, and U. Huynh-Do.** 2004. EphB1-mediated cell migration requires the phosphorylation of paxillin at Tyr-31/Tyr-118. J. Biol. Chem. **279:**27965–27970.
27. **Yuan, B., R. Latek, M. Hossbach, T. Tuschl, and F. Lewitter.** 2004. siRNA Selection Server: an automated siRNA oligonucleotide prediction server. Nucleic Acids Res. **32:**W130–W134.