THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 42, pp. 28533–28542, October 16, 2009

Analysis of Non-canonical Fibroblast Growth Factor Receptor 1 (FGFR1) Interaction Reveals Regulatory and Activating Domains of Neurofascin*

Received for publication, April 6, 2009, and in revised form, July 15, 2009 Published, JBC Papers in Press, August 7, 2009, DOI 10.1074/jbc.M109.004440

Katja Kirschbaum[‡], **Martin Kriebel**[‡], **Eva Ursula Kranz**[‡], **Oliver Pötz**[§], **and Hansjürgen Volkmer**[‡1]

From the Departments of [‡]*Molecular Biology and* [§]*Biochemistry, Naturwissenschaftliches und Medizinisches Institut an der Universität Tübingen, Markwiesenstrasse 55, 72770 Reutlingen, Germany*

Fibroblast growth factor receptors (FGFRs) are important for many different mechanisms, including cell migration, proliferation, differentiation, and survival. Here, we show a new link between FGFR1 and the cell adhesion molecule neurofascin, which is important for neurite outgrowth. After overexpression in HEK293 cells, embryonal neurofascin isoform NF166 was able to associate with FGFR1, whereas the adult isoform NF186, differing from NF166 in additional extracellular sequences, was deficient. Pharmacological inhibitors and overexpression of dominant negative components of the FGFR signaling pathway pointed to the activation of FGFR1 after association with neurofascin in neurite outgrowth assays in chick tectal neurons and rat PC12-E2 cells. Both extra- and intracellular domains of embryonal neurofascin isoform NF166 were able to form complexes with FGFR1 independently. However, the cytosolic domain was both necessary and sufficient for the activation of FGFR1. Cytosolic serine residues 56 and 100 were shown to be essential for the neurite outgrowth-promoting activity of neurofascin, whereas both amino acid residues were dispensable for FGFR1 association. In conclusion, the data suggest a neurofascin intracellular domain, which activates FGFR1 for neurite outgrowth, whereas the extracellular domain functions as an additional, regulatory FGFR1 interaction domain in the course of development.

The four known fibroblast growth factor receptors (FGFRs),[2] which are targeted by a large family of 22 fibroblast growth factor ligands, represent a highly diverse signaling system important for migration, proliferation, differentiation, and survival of many different cell types (1, 2). fibroblast growth factor activation of FGFR leads to the activation of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase Cγ (PLCγ), depending on the cellular system under study. Non-canonical FGFR interactions with NCAM, cadherins, and syndecan via extracellular domains were also described (1). However, the contribution of intracellular interactions of FGFR1 with further membrane co-receptors is poorly understood. Only cytosolic interaction between FGFRs and EphA4 have been described that are involved in mutual transphosphorylation (3).

The cell adhesion molecule neurofascin is important for cell-cell communication in the nervous system (4, 5). Neurofascin regulates many different functions in the brain, suggesting that it functions as a key regulator for both developing and differentiated neural cells. Different alternatively spliced neurofascin isoforms are expressed in different cells and at different times of development (6). Embryonal neurofascin NF166 is important for neurite outgrowth and guidance (7, 8). Recently, a role for neurofascin NF166 for early processes of inhibitory synaptogenesis at the axon hillock and for the positioning of inhibitory synapses at the axon initial segment has been proven (9, 10).

In the more developed nervous system, NF166 is replaced by NF186, which is inhibitory for neurite outgrowth (11). NF186 is linked to the cortical actin cytoskeleton via ankyrin$_G$ (12). Clustering of voltage-gated sodium channels both at axon initial segments and at the nodes of Ranvier is conferred by neurofascin NF186 (13, 14). A further cytosolic interaction partner is the PDZ molecule syntenin-1 (15).

Despite the well known functional importance of neurofascin in the nervous system, corresponding signaling pathways have not been investigated. In contrast, signaling by the related molecules NCAM and L1 have been studied with regard to the induction of neurite outgrowth in greater detail (for a review, see Refs. 16–18). Both NCAM and L1 induce neurite outgrowth through activation of FGFR1 (19–23). NCAM may further undergo lateral interactions with PrP (prion precursor protein) or GFRα, which is part of the glia-derived neurotrophic factor receptor (24, 25). In addition to FGFR1 interaction, both L1 and NCAM are connected to non-receptor tyrosine kinases. However, whereas NCAM employs the non-receptor kinase c-Fyn as an upstream component, L1 is linked to c-Src (26, 27). L1 converges with NCAM signaling upstream of the MAPK pathway at the level of Raf (18, 21, 28, 29). NCAM may induce alternative signaling pathways, including protein kinase A-dependent signaling or G-proteins (18, 30). NCAM signaling to the nucleus may include activation of CREB and c-Fos or NF-κB (29, 31, 32).

Here, we elucidate the molecular mechanisms of neurofascin-FGFR1 interaction for neurite outgrowth. We show that

* This work was supported by European Union 6th Framework Program Grant LSHM-CT-2005-512012 and by Bundesministerium für Bildung und Forschung Grant 0312114.

[1] To whom correspondence should be addressed. Tel.: 49-7121-51530-44; Fax: 49-7121-51530-16; E-mail: volkmer@nmi.de.

[2] The abbreviations used are: FGFR, fibroblast growth factor receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; NCAM, neural cell adhesion molecule; dnFGFR1, dominant negative FGFR1; EGFP, enhanced green fluorescent protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ANOVA, analysis of variance; CD, cytosolic domain; ED, extracellular domain; ERK, extracellular signal-regulated kinase.

both cytosolic and the extracellular domains are important for the association of FGFR1 with neurofascin. Although the cytosolic domain represents a critical determinant for FGFR1 activation, the extracellular sequences of neurofascin act as a regulator for FGFR1-dependent signal transduction in the course of development.

MATERIALS AND METHODS

Plasmids and Antibodies—cDNA expression vectors for chick neurofascin isoforms NF166 and NF186 as well as the NF166-CD and -ED mutants were described previously (7, 10). NF166 point mutants and COOH-terminally truncated variants were constructed with the help of a QuikChange mutagenesis kit (Stratagene, Heidelberg, Germany). All vectors were controlled by sequencing. Mouse FGFR1 (isoform IIIc) cloned in the plasmid pcDNA3.1 Myc-His (Invitrogen) was supplied by P. Doherty (22). An expression vector for dominant negative FGFR1 (dnFGFR1) was cloned by integrating mouse FGFR1 sequences encoding the extracellular and transmembrane domains into pEGFP-N1 (Clontech, Palo Alto, CA), yielding a dnFGFR1-EGFP fusion protein.

Rabbit anti-neurofascin antibodies for immunoprecipitation recognize a peptide epitope at the COOH terminus common to chick and rat neurofascin (gift from P. Brophy). Immunocytochemistry of chick neurons and immunoprecipitation of chick NF166-ED were carried out with a polyclonal anti-chick neurofascin antibody that recognized the extracellular domain of chick neurofascin (7). FGFR1 peptides derived from rat brain were detected by polyclonal antibodies (Abcam, Cambridge, UK), whereas a monoclonal antibody was applied for recombinant mouse FGFR1 (Upstate Technologies, Dundee, UK). Precipitation of recombinant, Myc-tagged FGFR1 was performed using the Myc-specific monoclonal antibody 9E10 (Roche Applied Science).

Cell Culture—Media and solutions for cell culture were purchased from Cambrex Corp. (East Rutherford, NJ). PC12-E2 cells (7, 21) were grown in a humidified atmosphere containing 10% CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated horse serum (Invitrogen), 5% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml G418 (PAA, Pasching, Austria). NIH/3T3 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Tectal neurons prepared from embryonal day 6 chick embryos were plated in serum-free Dulbecco's modified Eagle's medium, N2 supplement (Invitrogen), 0.5% (w/v) bovine serum albumin at a density of 60,000 cells/cm^2 on petriPERM dishes (Heraeus Instruments, Osterode, Germany) coated with neurofascin-Fc fusion protein or laminin (each 40 μg/ml in phosphate-buffered saline overnight at 4 °C). Cells were cultured at 37 °C (humidified atmosphere, 5% CO_2) for 24 h before fixation and immunostaining. Fc fusion proteins were prepared as described earlier (33). ImageJ software was used to determine neurite lengths by tracing immunostained neurites after labeling with an antibody specific for the A2B5 antigene (33). For quantification of outgrowth of tectal neurons, only one neurite was measured per cell. Only the longest neurite of an individual cell, which should be at least as long as one cell diameter, was considered. Neurites crossing neighboring neurites were excluded. In each experiment, between 100 and 200 neurites were measured per condition. Each experiment was reproduced three times with comparable outcome.

Using Lab-Tek™ 8-well chamber slides (NUNC, Wiesbaden, Germany), 5000 PC12-E2 cells were cultivated on a confluent monolayer of neurofascin-expressing NIH/3T3 in the presence of 100 ng/ml nerve growth factor for 18–20 h after co-transfection with the indicated expression vectors and an EGFP expression vector for detection of transfected cells, as published previously (8, 21). Subsequently, random pictures were taken with a light-amplifying camera (Hamamatsu Photonics) to determine the percentage of process-bearing cells. In detail, 10–15 non-overlapping micrographs with 20–50 individual cells were taken from PC12 cell cultures. Cells were examined whether at least one out of several neurites was longer of at least one cell diameter. Cells elaborating neurites were set in relation to the number of all cells observed in the individual micrograph. Each experiment was performed three times and resulted in comparable results.

Inhibitors were dissolved in DMSO and added to culture media of dissociated cells prior to plating to an end concentration of 20 μM for FGFR1 inhibitor SU5402, 10 μM for MEK inhibitor U0126, 25 μM for MEK inhibitor PD98059, 1 μM for PLC inhibitor U73122, 400 nM for protein kinase C (PKC) inhibitor calphostin C, 500 nM for PKC inhibitor bisindolylmaleimide I, and 10 μM for PI3K inhibitor Ly294002.

Immunocytochemistry—For the detection of neurofascin and FGFR1 on the surface of dissociated neurons grown in culture, cells were fixed with ice-cold phosphate-buffered saline, 4% (v/v) formaldehyde for 15 min at room temperature. After washing in phosphate-buffered saline and blocking with Dulbecco's modified Eagle's medium, 10% fetal calf serum, sections were exposed to mouse anti-neurofascin monoclonal antibody (10 μg/ml) and rabbit anti-FGFR1 polyclonal antibody (rabbit anti-Flg (4 μg/ml); Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies, which were detected by Cy3™-conjugated goat anti mouse or Cy2™-conjugated goat anti-rabbit secondary antibodies (Dianova, Hamburg, Germany). Double fluorescence images were taken with a Zeiss Axiovert fluorescence microscope.

Immunoprecipitation—HEK293 cells were plated at a density of 550,000 cells/10-cm Petri dish and transfected with 20 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen) after 68 h of cultivation. 24 h after transfection, cells were washed with ice-cold phosphate-buffered saline and lysed with 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% desoxycholate), including a protease inhibitor mixture (Roche Applied Science) for 15 min on ice. After sonification, debris was removed by centrifugation at 16,000 × *g* in an Eppendorf centrifuge. Protein concentrations were subsequently determined (BCA assay; Pierce). 1.5–2 mg of protein were mixed with 2 μg of antibodies (rabbit anti-neurofascin polyclonal antibodies or mouse anti-FGFR1 antibody 19B2 (Upstate-Millipore, Charlottesville, VA), or anti-Myc

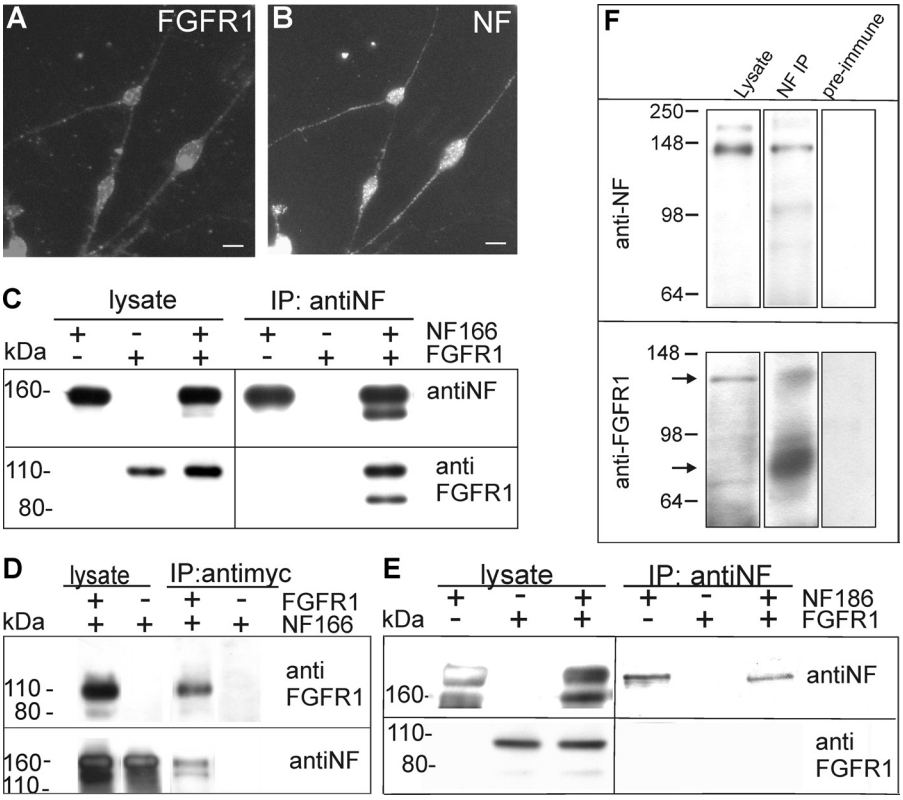

FIGURE 1. **Co-expression and interaction of FGFR1 and neurofascin.** *A* and *B*, double fluorescence labeling reveals co-expression of endogenous FGFR1 and neurofascin in chick primary tectal neurons. *Scale bar*, 10 μm. *C*, HEK293 cells were transfected with FGFR1 and neurofascin (NF166) expression vectors as indicated by −/+ in the *upper panel*. On the *left*, expression of FGFR1 and neurofascin is controlled in the lysate of transfected cells by Western blot analysis. On the *right*, a Western blot shows neurofascin- and FGFR1-specific peptides obtained after precipitation (*IP*) with neurofascin-specific antibodies. *Upper panels*, precipitated neurofascin bands detected with neurofascin-specific antibodies (*antiNF*). *Lower panels*, co-precipitated FGFR1 peptides detected by FGFR1-specific antibodies (*antiFGFR1*). *D*, Myc-tagged FGFR1 was precipitated with a Myc-specific antibody (9E10), and co-precipitated neurofascin was detected with neurofascin-specific antibodies. *Upper panels*, precipitated FGFR1 bands detected with FGFR1-specific antibodies. *Lower panels*, co-precipitated neurofascin peptides detected by neurofascin-specific antibodies. *E*, adult neurofascin NF186 does not interact with FGFR1. HEK293 cells were transfected with FGFR1 and NF186 expression vectors, as indicated in the *upper panel*. On the *left*, expression of FGFR1 and NF186 is controlled in the lysate of transfected cells by Western blot analysis. On the *right*, precipitated neurofascin and FGFR1 peptides are detected by Western blot analysis. *Top*, precipitated neurofascin bands detected with neurofascin-specific antibodies. *Bottom*, co-precipitated FGFR1 peptides detected by FGFR1-specific antibodies. *F*, co-precipitation of endogenous FGFR1 and neurofascin from rat brain lysate. Precipitation of neurofascin polypeptides from rat brain lysate and detection of neurofascin (*top*) or FGFR1 (*bottom*) is shown by Western blot analysis. Western blot analysis shows detection of neurofascin or FGFR1 in rat brain lysate (*lysate*), after immunoprecipitation of neurofascin with neurofascin-specific serum (*NF-IP*), or preimmune serum (*pre-immune*).

antibody 9E10 (Abcam, Cambridge, MA)) and 25 μl of a suspension (50%, v/v) of protein A-Sepharose CL-4B (Amersham Biosciences) or protein G-Sepharose (Sigma) for immunoprecipitation. Samples were shaken for 16 h at 4 °C before the beads were washed by five consecutive steps of centrifugation and resuspension in lysis buffer. Precipitated proteins and unprecipitated lysate samples were separated by SDS-PAGE and transferred to nitrocellulose membranes by Western blotting. Detection of antigen was carried out as described previously (4). Alternatively, neurofascin was precipitated from embryonal day 18 rat brain samples. 7 mg of brain protein was used for precipitation and detection of neurofascin and co-precipitated FGFR1.

RESULTS

FGFR1 Is Associated with Neurofascin—Co-expression of neurofascin and FGFR1 was tested with cultured chick tectal neurons by indirect immunohistochemistry. As shown in Fig. 1, *A* and *B*, both the cell bodies and the fibers of tectal neurons showed FGFR1 and neurofascin immunoreactivity in a double fluorescence labeling experiment. Co-immunoprecipitations were performed to examine possible complex formation of neurofascin and FGFR1 after transfection of embryonal neurofascin NF166 and FGFR1 expression plasmids. A neurofascin-specific antibody was used to precipitate neurofascin, and co-precipitation of FGFR1 was tested by immunostaining of Western blots. As shown in Fig. 1*C*, FGFR1 co-precipitated with neurofascin only if both neurofascin and FGFR1 were co-expressed in HEK293. Here, Western blot analysis revealed the expected band of ~110 kDa as well as the enrichment of a known FGFR1 peptide of lower molecular mass (80 kDa). FGFR1-specific polypeptides of ~110 and 80 kDa molecular mass have been described previously (34). The 80 kDa band may represent a soluble variant of FGFR1 generated by proteolytic cleavage (35). Likewise, expression of NF166 or NF186 also yielded two bands probably due to differential or incomplete glycosylation (36). *Vice versa*, neurofascin showed co-precipitation with FGFR1 in the reverse situation (Fig. 1*D*). Here, FGFR1 was precipitated with an antibody specific for a Myc tag fused to the COOH terminus of FGFR1 (22) to test for co-precipitation of neurofascin NF166. To show co-precipitation of endogenous neurofascin with FGFR1, lysates from embryonal rat brain were prepared (Fig. 1*F*). Neurofascin-specific bands were clearly detected in rat brain lysate and were also observed after immunoprecipitation with neurofascin-specific antibodies (*NF IP*). No signals were obtained in an immunoprecipitation experiment using preimmune serum. An expected 110 kDa band was observed for the high molecular mass variant of FGFR1 in rat brain lysate (*upper arrow* in the *lower half*). Upon precipitation of neurofascin, Western blot analysis revealed co-precipitation of the 110 kDa band of FGFR1 (*upper arrow* in the *lower half*) detected by the FGFR1 antibody. Enrichment of an 80 kDa band for FGFR1 was also observed (*lower arrow* in the *lower half*). This is in accordance with the findings in Fig. 1*C*. Here, the 80 kDa band is also missing in the lysate of FGFR1-overexpressing cells, whereas the additional 80-kDa variant of FGFR1 appears after co-precipitation. No co-precipitating

FGFR1-specific bands were observed after immunoprecipitation with preimmune serum. Comparable amounts of protein were loaded for HEK293 (10–30 μg; Fig. 1, *C–E*) and rat brain lysates (6 μg for neurofascin Western blot and 30 μg for the FGFR1 Western blot) (Fig. 1*F*). In comparison with overexpression in HEK293, the abundance of neurofascin and FGFR1 in brain lysates is much lower. 16 times more precipitated protein was applied to the FGFR1 in comparison with the neurofascin Western blot. In conclusion, our results show co-precipitation of endogenous neurofascin and FGFR1. NF186 represents an adult neurofascin isoform that contains additional membrane-proximal domains (7). Although FGFR1 co-precipitated with NF166, no co-precipitation was found with NF186 (Fig. 1*E*). This result indicates that co-precipitation is specific for the smaller embryonic neurofascin isoform NF166, because NF186 was not able to form complexes with FGFR1. Adult NF186 replaces embryonal NF166 after developmentally regulated alternative splicing. Therefore, differential expression of neurofascin isoforms may account for the regulation of FGFR1 interaction.

Pharmacological Inhibitors Targeting the FGFR1 Pathway Interfere with Neurofascin-dependent Neurite Outgrowth of Tectal Neurons—We have shown previously that cultured primary tectal neurons extend neurites on substrates of purified neurofascin via homophilic interactions with neurite-bound neurofascin (7, 8). The association of neurofascin with FGFR1 suggests a possible involvement of FGFR1 signaling in neurofascin-dependent neurite outgrowth. In order to test this hypothesis, we employed a panel of pharmacological inhibitors to interfere with endogenous FGFR1 signaling in neurite outgrowth assays using primary neurons.

Chick tectal cells were grown on a neurofascin substrate in the presence or absence of SU5402, a specific inhibitor of FGFR1. Quantification of neurite length indicated a partial decrease in mean neurite length to 40% of control levels after application of 20 μM SU5402. (Fig. 2*A*). SU5402 did not impair neurite outgrowth on a laminin substrate, which induces integrin-dependent mechanisms. The mitogen-activated protein kinase (MAPK) pathway, PI3K, PLCγ, and PKC are known signaling components acting downstream of FGFR1 (1, 21). As shown in Fig. 2*B*, MAPK/ERK kinase (MEK) inhibitors U0126 (10 μM) and PD98059 (25 μM) did not affect neurofascin-dependent neurite outgrowth of chick tectal neurons. This is in contrast to previous findings in which both NCAM and L1 were shown to require MAPK signaling pathways for neurite outgrowth (21, 28, 29, 37). On the other hand, U73122 (PLC), calphostin C (PKC), bisindolylmaleimide I (PKC), and Ly294002 (PI3K) significantly impaired neurite outgrowth of tectal neurons on a neurofascin substrate. Effects of SU5402, calphostin C, and bisindolylmaleimide I were also dose-dependent (data not shown). In summary, our data provide evidence for a contribution of FGFR and known FGFR downstream signaling components to neurofascin-dependent neurite outgrowth of tectal primary neurons.

Neurofascin Requires FGFR1 Signaling for Neurite Outgrowth in PC12-E2 Cells—To further corroborate the results obtained with primary neurons, PC12-E2 cells were applied, which are amenable to efficient transfection, a prerequisite for mutational

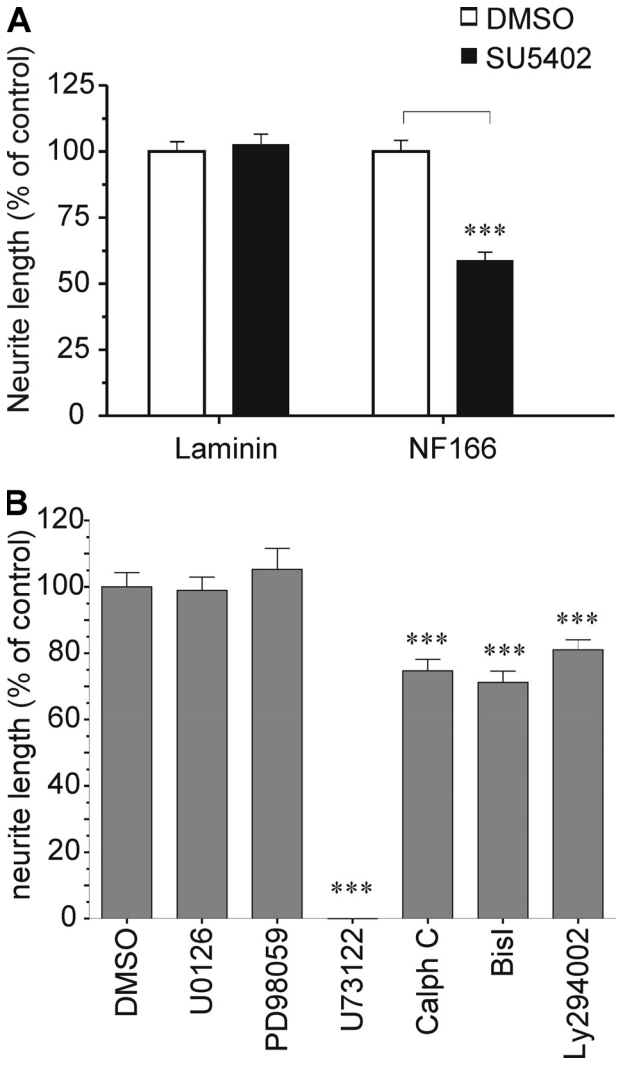

FIGURE 2. **Neurofascin-dependent neurite outgrowth requires FGFR1 activation.** *A*, quantification of neurite lengths of tectal neurons grown on a neurofascin or laminin substrate. Values on different substrates were set to 100% of DMSO-treated cultures. Mean neurite lengths of DMSO-treated cultures were 58 μm (laminin) and 64 μm (NF166). *p* values *versus* DMSO-treated controls were as follows: $p < 0.0001$ for neurofascin substrate (***), and $p = 0.8145$ for laminin substrate, ANOVA. *B*, tectal neurons grown on a neurofascin substrate were treated with a panel of inhibitors as indicated. Mean neurite length of DMSO-treated cultures was 98 μm on a neurofascin NF166 substrate. For inhibitor concentrations, see "Materials and Methods." *p* values *versus* DMSO controls (ANOVA) are as follows: U0126 ($p = 0.86$), PD98059 ($p = 0.40$), U73122 ($p < 0.0001$), calphostin C ($p < 0.0001$), bisindolylmaleimide ($p < 0.0001$), and Ly294002 ($p = 0.0003$). At least 100 individual neurites derived from each experimental setting out of one single culture plate were included in the calculation of each *bar*. One example experiment is shown, which was repeated at least twice with comparable outcomes. *Error bars*, S.E.

analysis. PC12-E2 cells represent a suitable model system to study neurofascin-dependent neurite outgrowth in a co-culture model (7, 8). PC12-E2 cells are exposed to a monolayer of NIH/3T3 cells for neurite outgrowth assays (Fig. 3). Neurofascin-dependent neurite outgrowth of PC12 cells relies on expression of neurofascin in both substrate NIH/3T3 cells and PC12 cells, whereas omission of neurofascin on either substrate cells or PC12 cells results in reduced neurite outgrowth (see Fig. 4*A*) (8).

Mock-transfected PC12-E2 or PC12-E2 cells expressing neurofascin were cultivated on either parental (control) or on NIH/

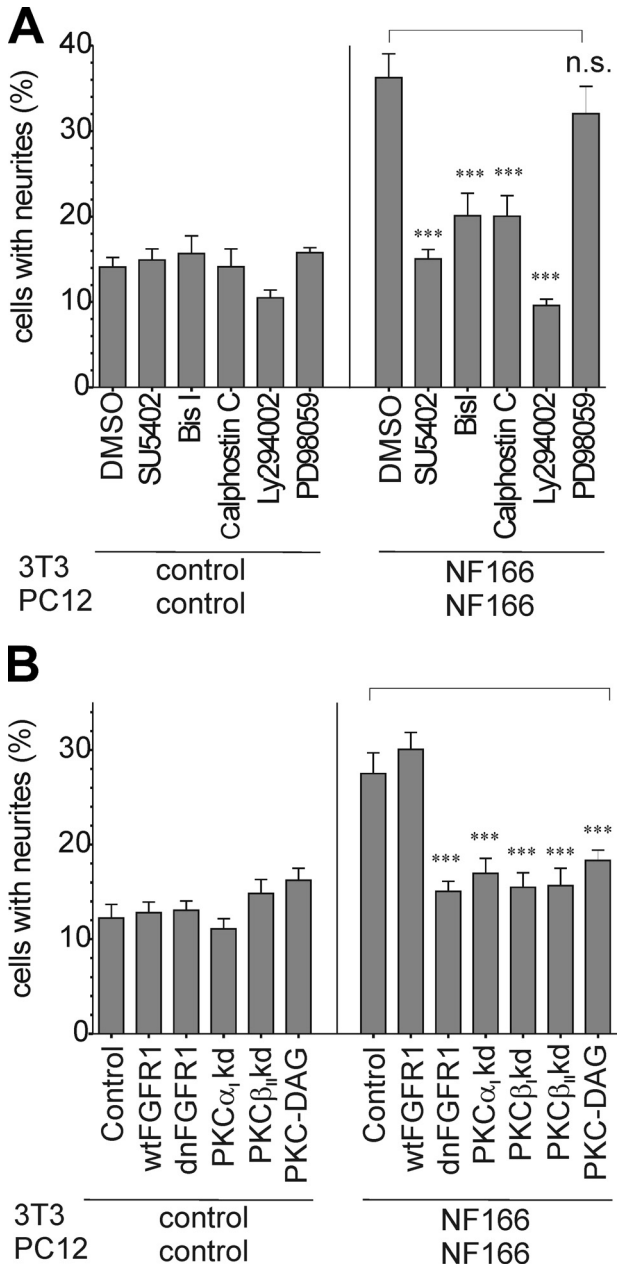

FIGURE 3. **Homophilic neurofascin interactions *in trans* require FGFR1 signaling in PC12-E2 cells.** *A*, co-cultures of parental PC12-E2 cells (control) or PC12-E2 cells transiently transfected with an NF166 expression vector were cultivated on parental (control) or NF166-expressing NIH/3T3 monolayers in the presence of inhibitors as indicated. For inhibitor concentrations, see "Materials and Methods." *B*, PC12-E2 cells were co-transfected with NF166 expression plasmid and with FGFR1 or a dominant negative mutant of FGFR1 (*dnFGFR1*) and kinase-dead mutants of PKC (*PKCaI kd* and *PKCbII kd* (where *kd* represents kinase-dead) and PKC-DAG (diacylglycerol binding domain only)), resulting in specific inhibition of neurofascin-dependent neurite outgrowth. *Error bars*, S.E.; $p > 0.0001$ (***); *n.s.*, not significant; ANOVA. At least 200 individual PC12 cells derived from each experimental setting out of one single culture plate were included in the calculation of each *bar*. One example experiment is shown, which was repeated at least twice with comparable outcomes.

3T3 cells expressing neurofascin NF166. In the presence of neurofascin isoform NF166 in both PC12-E2 cells and NIH/3T3 substrate cells, neurite outgrowth of PC12-E2 cells is increased in comparison with the control situation, indicating induction of neurofascin-dependent neurite outgrowth by homophilic interactions (7). As shown in Fig. 3*A* (*right half*), all inhibitors addressing FGFR1 signaling decreased neurite outgrowth ($p < 0.0001$, ANOVA) of NF166-transfected PC12-E2 cells exposed to a neurofascin-expressing NIH/3T3 monolayer with the exception of MEK inhibitor PD98059. Even on a control NHI/3T3 monolayer, neurite outgrowth was not impaired by MEK inhibitor PD98059, although MAPK pathways were shown to be important for neurite outgrowth of PC12 cells (38). However, our findings are in accordance with previous findings that neurite outgrowth of PC12 cells grown on parental NIH/3T3 cells is insensitive to MEK inhibition (21). Application of inhibitors SU5402, bisindolylmaleimide, calphostin C, and Ly294002 reduced neurite outgrowth of neurofascin-expressing PC12 cells to a level that was not significantly different from that obtained with untransfected control cells, indicating that neurofascin-dependent neurite outgrowth was completely abolished (p values for the comparison of NF166-transfected cells *versus* untransfected control: SU5402, $p = 0.95$; bisindolylmaleimide I, $p = 0.19$; calphostin C, $p = 0.08$; Ly294002, $p = 0.77$; ANOVA). The inhibitors did not significantly impair neurite outgrowth of untransfected cells in comparison with DMSO-treated controls (see Fig. 3*A*, *control*). Therefore, the results imply a complete reduction of specific neurite outgrowth by the inhibitors applied. In conclusion, the action of inhibitors applied in tectal neurons was reproduced in the PC12-E2 co-culture model, suggesting that PC12-E2 cells make use of FGFR1 signaling components.

For an independent approach and to rule out possible unspecific effects of pharmacological inhibitors, neurofascin was co-expressed with dominant negative mutants of signaling components, including an FGFR1 lacking the cytosolic domain (dnFGFR1), two protein kinase-dead mutants of either PKCα, PKCβ_I, or PKCβ_{II}, and a PKC mutant exclusively representing the diacylglycerol binding domain (19, 39). Expression of all mutants individually decreased neurofascin-dependent neurite outgrowth of PC12-E2 cells ($p < 0.0001$, ANOVA) to a similar extent, which was indistinguishable from the negative controls. In contrast, neurite outgrowth of PC12-E2 cultured on naive NIH/3T3 cells remained unaffected (Fig. 3*B*). Therefore, overexpression of dnFGFR1 completely inhibited neurofascin-dependent neurite outgrowth, similar to the application of FGFR1 inhibitor SU5402.

In conclusion, we have provided independent lines of evidence that neurofascin induces neurite outgrowth via FGFR1 signaling: 1) pharmacological inhibitors targeting FGFR1 signaling components and 2) overexpression of mutated FGFR1 and PKCs.

Dissection of Neurofascin Domains Required for Neurite Outgrowth—Neurofascin is a transmembrane protein composed of an extracellular and an intracellular domain linked by a transmembrane domain (4). To gain more mechanistic insight into possible mechanisms of non-canonical interaction with FGFR1, we included neurofascin deletion mutants lacking either the intracellular (NF166-ED) or the extracellular domain (NF166-CD; see Fig. 4*C*). Proper expression of mutants was previously published (10). After transfection of wild type NF166 into PC12-E2 cells grown on a monolayer of neurofascin-expressing NIH/3T3 cells, the percentage of PC12-E2 cells elabo-

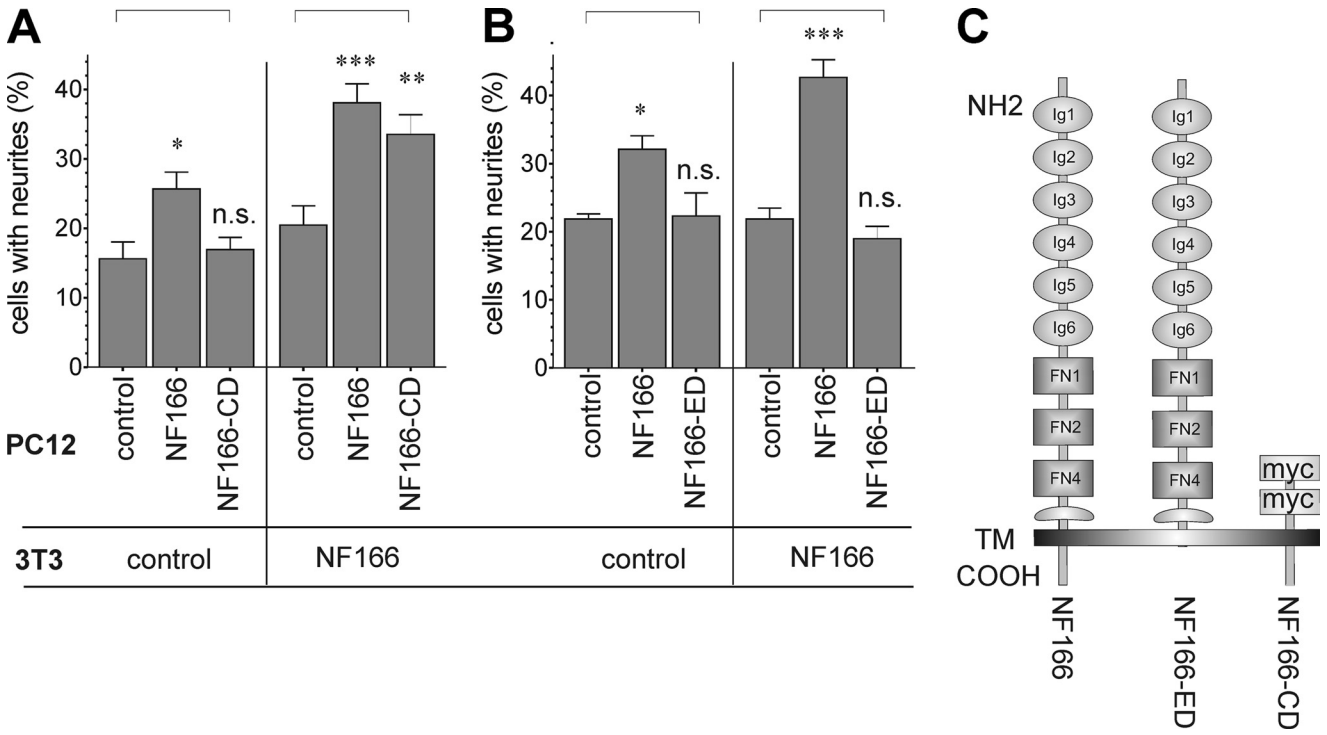

FIGURE 4. **The intracellular domain of neurofascin is necessary and sufficient to induce neurite outgrowth via FGFR1 signaling.** *A*, PC12-E2 cells transfected with NF166 or NF166-CD (cytosolic domain only) expression vector were cultivated on neurofascin-expressing NIH/3T3 monolayers (NF166) or parental NIH/3T3 cells (control), as indicated. *Error bars*, S.E. *p* values *versus* control were as follows: $p > 0.0001$ (***), $p = 0.0005$ (**), $p = 0.0094$ (*), or not significant (*n.s.*), ANOVA. *B*, PC12-E2 cells transfected with NF166 or NF166-ED (extracellular domain only) expression vector were cultivated on neurofascin-expressing NIH/3T3 monolayers or parental NIH/3T3 cells (control). *Error bars*, S.E. *p* values *versus* control were as follows: $p > 0.0001$ (***) or $p = 0.0061$ (*), ANOVA. *C*, the structure of the deletion mutants applied. In the case of NF166-CD, the extracellular domain of NF166 was replaced by a double Myc epitope. At least 200 individual PC12 cells derived from each experimental setting out of one single culture plate were included in the calculation of each *bar*. One example experiment is shown, which was repeated at least twice with comparable outcomes.

rating neurites was increased to about 40%, whereas only 25% were achieved in the absence of neurofascin overexpression as a control (Fig. 4*A*). Surprisingly, expression of NF166-CD was sufficient to induce neurite outgrowth on NF166-expressing substrate cells to an extent comparable with that observed for the wild type neurofascin (NF166, $p < 0.0001$; NF166-CD, $p = 0.0005$; ANOVA). Neurite outgrowth induced by NF166-CD could be blocked by inhibitors to FGFR and PI3K similar to wild type neurofascin NF166 (data not shown). NF166-CD did not significantly induce neurite outgrowth of PC12-E2 cells exposed to the parental NIH/3T3 cells. However, overexpression of NF166 in PC12 cells grown on parental NIH/3T3 cells increased neurite outgrowth only partially ($p = 0.0094$) in comparison with the situation with neurofascin expression both in substrate cells and PC12-E2 cells. In contrast, untransfected PC12-E2 cells grown on NF166-expressing NIH/3T3 extended neurites at a basal level, as observed for parental NIH/3T3 cells. Overexpression of NF166-ED lacking the cytosolic domains did not show increased neurite outgrowth in comparison with the control situation (Fig. 4*B*). Therefore, the cytosolic domain of neurofascin is both necessary and sufficient to induce neurite outgrowth.

The experiment shows that the cytosolic domain of neurofascin requires expression of neurofascin in the substrate monolayer for neurite induction. This finding suggests participation of an additional, unknown neuronal co-receptor of neurofascin and FGFR1.

Amino Acid Residues of the Cytosolic Domain of Neurofascin Important for Neurite Outgrowth—The cytosolic domain of wild type NF166 was successively truncated from the carboxyl terminus in order to define subdomains involved in neurite extension (Fig. 5*A*). The exact positions of the carboxyl-terminal truncations are indicated in Fig. 5*B* (see arrows for the respective last amino acid residue still present and naming of the corresponding neurofascin mutants). All neurofascin constructs were transfected into HEK293 cells for expression analysis. Our data indicate that all mutants are expressed with proteins of the expected size (Fig. 5*C*).

Neurofascin mutants were transfected into PC12-E2 cells to examine neurite outgrowth on a monolayer of wild type neurofascin-expressing NIH/3T3 cells. Removal of the first 10 amino acid residues from the carboxyl terminus (NF166-Δ100) completely abolished neurofascin-dependent neurite outgrowth, indicating that essential amino acid residues are located at the carboxyl terminus of neurofascin. Closer inspection of the cytosolic domain revealed that a serine residue is located at position 100, which is conserved with L1 and which may represent a target for L1 phosphorylation by ERK2 (see Fig. 5*B*, *boldface letters*) (28). Therefore, we constructed point mutations by replacing serine 100 by alanine or leucine. Since L1 amino acid residue Ser-56 was also shown to be targeted by ERK2, we constructed mutations NF166-S56A and NF166-S56L as well as double point mutants NF166-S56A/S100A and NF166-S56L/S100L. Western blot analysis and immunocytochemistry con-

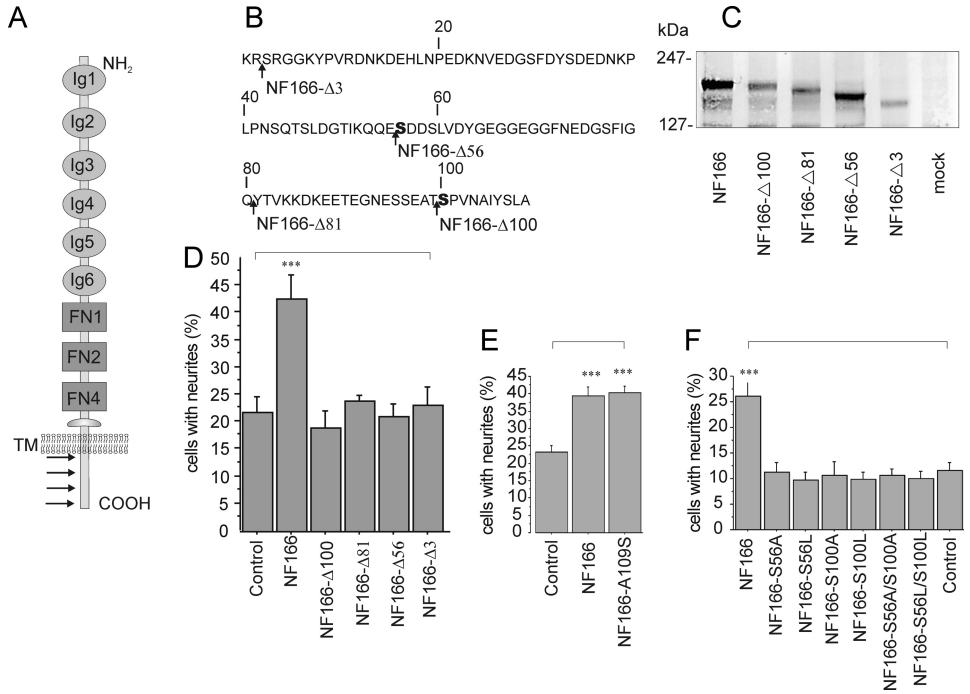

FIGURE 5. **Cytosolic neurofascin serines residues 56 and 100 are important for neurite outgrowth.** *A* and *B*, overview of COOH-terminally truncated mutants of neurofascin with the precise positions indicated in *B*. *C*, Western blot analysis of mutated neurofascin proteins expressed after transfection of HEK293 cells with plasmid vectors expressing deletion mutants indicated in *B*. Mutants were named according to the first omitted amino acid residue after carboxyl-terminal truncation. *D*, neurite outgrowth of PC12-E2 cells transfected with wild type (NF166) or mutated neurofascin expression vectors on monolayers of NF166-expressing NIH/3T3 cells indicates that neurite outgrowth is abolished after removal of the 10 COOH-terminal amino acid residues of neurofascin. *p* values (ANOVA) in comparison to control are as follows: NF166 ($p < 0.0001$), NF166-Δ100 ($p = 0.64$), NF166-Δ81 ($p = 0.78$), NF166-Δ56 ($p = 0.41$), NF166-Δ3 ($p = 0.27$). *E*, neurite outgrowth assays as in *D* with PC12-E2 cells transfected with NF 166 and NF166-A109S, indicating that syntenin-1 binding is not required for neurite outgrowth. ($p < 0.0001$ (***), ANOVA). *F*, application of point mutants that exchange serines 56 and 100 for alanine or leucine either individually or in double point mutants reveals that serines 56 and 100 are required for neurite outgrowth independently. *p* values in comparison to control (ANOVA) are as follows: NF166 ($p < 0.0001$), NF166-S56A ($p = 0.83$), NF166-S56L ($p = 0.24$), NF166-S100A ($p = 0.56$), NF166-S100L ($p = 0.3$), NF166-S56A/S100A ($p = 0.57$), and NF166-S56L/S100L ($p = 0.34$). At least 200 individual PC12 cells derived from each experimental setting out of one single culture plate were included in the calculation of each bar. One example experiment is shown, which was repeated at least twice with comparable outcome. *Error bars*, S.E.

firmed proper expression and location of the mutants (data not shown; for Western blot of NF166-S56A/S100A, see Fig. 6*C*). Neurite outgrowth assays showed that mutations of both of these serine residues reduced the number of cells elaborating neurites to control levels, as observed in the absence of neurofascin-dependent neurite outgrowth (Fig. 5*F*). Accordingly, the double mutant was also inactive. In contrast, mutation A109S, which is deficient in interaction with the PDZ protein syntenin-1 (15), did not impair neurite outgrowth in comparison with wild type NF166, indicating that syntenin-1 is not required for neurofascin-dependent neurite outgrowth (Fig. 5*E*).

In summary, mutational analysis provided further evidence that the cytosolic domain of neurofascin represents an essential domain for neurite outgrowth. Mutation of Ser^{56} and Ser^{100} points to a possible involvement of phosphorylation required for neurite outgrowth.

Neurofascin Domains Important for FGFR1 Complex Formation—Mutational analysis and functional assays imply a crucial role of the intracellular domain for the induction of neurite outgrowth. We next asked whether the differential behavior of intra- and extracellular domains can be explained in terms of FGFR1 interactions. NF166-ED and NF166-CD were co-expressed with wild type FGFR1 or dominant negative FGFR1 in HEK293 cells. NF166-CD was precipitated, and association with FGFR1 was monitored by Western blotting. As shown in Fig. 6*A*, FGFR1 was co-precipitated with NF166-CD, whereas NF166-CD did not co-precipitate with dnFGFR1 lacking the cytosolic domain as expected. The band for dnFGFR1 migrates at a higher molecular mass than that of wild type FGFR1, because the cytosolic domain of FGFR1 was replaced by EGFP. In a further experiment, neurofascin NF166-ED, which lacks the intracellular domain, was co-precipitated with FGFR1 (Fig. 6*B*). The results indicate that two independent sites for FGFR1 interaction coexist in the neurofascin protein both in the intra- and extracellular domain.

Analysis of neurofascin point mutants (Fig. 5*F*) revealed crucial Ser-56 and -100 residues for neurite outgrowth. We therefore asked whether the inactive neurofascin double point mutant NF166-S56A/S100A is able to form complexes with FGFR1. A co-precipitation experiment conducted as described above revealed that the NF166-S56A/S100A mutation does not interfere with FGFR1 complex formation (wild type NF166 is depicted in Fig. 6*C* as +, whereas the point mutant NF166-S56A/S100A is indicated as *PM*). Therefore, association of neurofascin with FGFR1 is independent of the neurite outgrowth-promoting activity of neurofascin. Activity of FGFR1 is required for neurofascin-dependent neurite outgrowth (Figs. 2 and 3). Hence, we examined association of neurofascin with FGFR1 in the presence of FGFR inhibitor SU5402. The co-precipitation experiment showed that FGFR1 co-precipitates with neurofascin in cells exposed to SU5402 (Fig. 6*C*), indicating that, in contrast to neurite outgrowth induction, FGFR1 activity is not required for neurofascin-FGFR1 association.

DISCUSSION

Our results indicate that FGFR1 interacts with embryonal neurofascin NF166 both with the extra- and intracellular domain, whereas adult NF186 is deficient in FGFR1 interaction. The interaction between NF166 and FGFR1 occurs in the absence of FGFR1 activation and neurite outgrowth induction. For the induction of neurite outgrowth, neurofascin requires FGFR1 activation and FGFR1 downstream components. The

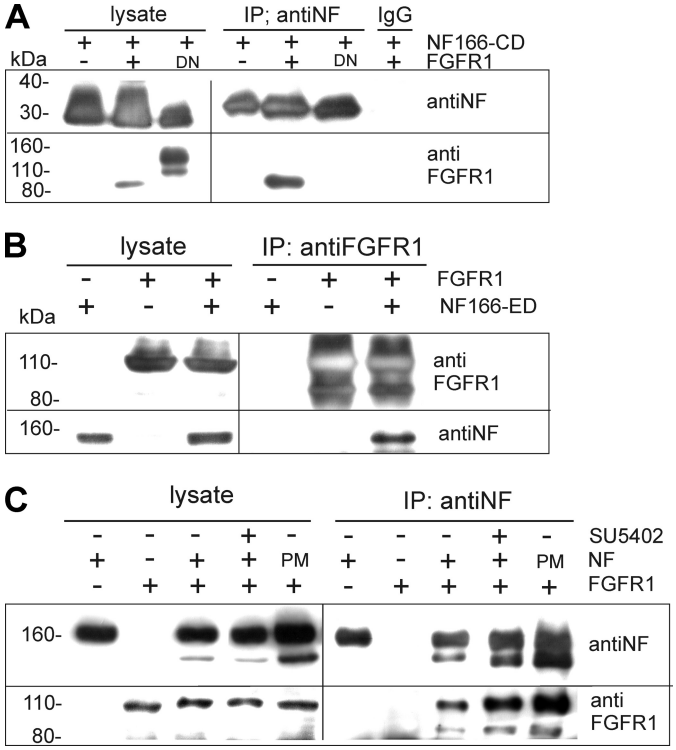

FIGURE 6. **The extra- and intracellular domains of neurofascin interact with FGFR1 independently.** *A*, HEK293 cells were transfected with expression vectors for NF166-CD and either FGFR1 or dominant negative FGFR1 as indicated by − (omitted), + (included), or *DN* (dominant negative variant of FGFR1) in the *upper panel*. On the *left*, expression of FGFR1 peptides and NF166-CD is controlled in the lysate of transfected cells by Western blot analysis. On the *right*, Western blot analysis shows peptides obtained after immunoprecipitation (*IP*) with neurofascin-specific antibodies. *Top*, precipitated NF166-CD bands. *Bottom*, co-precipitated FGFR1. *B*, experimental set-up as in *A* with expression of NF166-ED as well as FGFR1 and precipitation using antibodies specific for FGFR1. *C*, same set-up as in *A*. Expression of wild type neurofascin NF166 is indicated as +, whereas mutant NF166-S56A/S100A is indicated as *PM*. FGFR1 co-precipitates with wild type NF166 and with the inactive double point mutant NF166-S56A/S100A (*PM*) and in the presence of FGFR1 inhibitor SU5402 (20 μM), indicating that interaction between FGFR1 and neurofascin occurs in the absence of FGFR1 activation.

neurofascin cytosolic domain by itself is both necessary and sufficient to promote neurite outgrowth. To this end, the presence of two cytosolic serine residues of neurofascin at positions 56 and 100 are important.

Neurofascin is expressed on neuronal fibers in development and is also heavily expressed in the adult brain (40). Neurofascin is found in many differentially spliced variants that are subject to developmental regulation (6). Although embryonal neurofascin NF166 is required for neurite outgrowth and early mechanisms of inhibitory synapse formation, the adult neuronal isoform NF186 is inhibitory for neurite outgrowth and is implicated in the stabilization of protein complexes present in nodes of Ranvier or at axon-glia contacts (7, 9–11, 41, 42). In the course of neuronal development, axonal growth cones encounter target regions, stop growing, and form stable contacts for synapse formation. Molecular mechanisms are required that balance motility like, for example, migration of growth cones against static contact stabilization. FGFR1 has evolved as a crucial receptor involved in neurite outgrowth-promoting signal cascades induced by several cell adhesion molecules like NCAM, L1, and N-cadherin (19, 21–23). For L1 and N-cadherin, it is unknown how adhesion and neurite outgrowth are regulated to switch between motile mechanisms and contact stabilization. In the case of NCAM adhesion, it is widely accepted that modification with polysialic acid modulates NCAM-dependent adhesion and outgrowth (43). For neurofascin, interactions are regulated by the alternatively spliced fifth FN III-like repeat, which inhibits, for example, axonin-1 or homophilic neurofascin interactions and neurite outgrowth upon expression of "adult" NF186 (7). Therefore, expression of alternatively spliced neurofascin isoforms may represent a key mechanism to regulate the transition from motility to contact stabilization in the nervous system. Here, we show an additional regulatory mechanism, which relies on impaired interactions of neurofascin NF186 with FGFR1.

Previous findings suggest that neurofascin-dependent neurite outgrowth of primary tectal cells as well as transfected PC12 cells relies on homophilic interactions of neurofascin in *trans* (8). Our findings in this report imply a crucial contribution of the cytosolic domain of neurite-bound receptor neurofascin for neurite outgrowth and signaling. On the other hand, a purified substrate of Fc-neurofascin, which lacks intracellular domains, efficiently induces neurite outgrowth (see Fig. 2). Therefore, the cytosolic domain of substrate-bound neurofascin is dispensable.

FGFR1 interaction with the cell adhesion molecules L1, and NCAM was shown to be mediated through extracellular interactions (19, 23, 44–46). Our data indicate accordingly that neurofascin interacts with FGFR1 via extracellular interactions. However, the intracellular domain is necessary and sufficient to induce neurite outgrowth by FGFR1 signaling. A contribution of both domains may be explained by a hypothetical model that claims an activating cytosolic domain, the activity of which is regulated by interactions of the extracellular domain. Our results imply that complex assembly and disassembly of neurofascin and FGFR1 occur according to a three-step model. 1) Both intra- and extracellular domains contribute to neurofascin-FGFR1 complex formation, independent of FGFR1 activity and neurite outgrowth promotion. This view is supported by co-precipitation of FGFR1 and neurofascin after inactivation of neurofascin signaling either by mutation or inhibition of FGFR1 signaling. 2) Homophilic interactions of neurofascin in *trans* and heterophilic interactions with FGFR1 in *cis* are involved in the activation of FGFR1 downstream signal transduction for neurite outgrowth. Involvement of FGFR1-dependent signaling is complemented by previous observations that homophilic neurofascin interactions in *trans* are a prerequisite for neurofascin-dependent neurite outgrowth (8). 3) At the end of neuronal development, the association of FGFR1 with neurofascin is down-regulated by alternative splicing of the extracellular domain of neurofascin, which gives rise to NF186 (7).

Our data indicate the involvement of the IgCAM neurofascin in FGFR1 signaling for the promotion of neurite outgrowth. The finding is in accordance with the induction of FGFR1 signaling, as shown for IgCAMs NCAM and L1, the latter of which is closely related to neurofascin (47). The involvement of neurofascin in FGFR1 signaling further strengthens the view that the FGFR1 functions upstream of a central pathway used by different IgCAMs as well as non-IgCAM receptors like, for

instance, N-cadherin (22). Besides FGFR1 signaling, numerous studies on the function of NCAM led to the description of multiple outgrowth-promoting interactions with further cell surface receptors, including, for example, RPTPα, L1, and GFRα (25, 48–50). Whether other receptors than FGFR1 are employed by neurofascin, accordingly, remains open, although the partial inhibition of neurite outgrowth of primary tectal neurons cultivated on a neurofascin substrate may indicate such a possibility. On the other hand, we have previously shown that neurofascin substrates may induce neurite outgrowth both via homophilic interactions with neurite-bound neurofascin and via heterophilic interactions with the co-receptor Nr-CAM (8, 33). The signaling pathways activated by Nr-CAM are unknown and may account for the partial effects of FGFR1 inhibitors observed in tectal neurons. In contrast, inhibition of FGFR1 by dominant negative variants of FGFR1 signaling components or pharmacological inhibitors completely reduced neurofascin-dependent neurite outgrowth of PC12 cells, indicating that this test system allows for the examination of receptor neurofascin functions in the absence of induction of further signaling pathways.

NCAM may be located both inside and outside of lipid rafts depending on the specific isoform (17, 51). Upon activation, NCAM-140 and NCAM-180 were observed to be redistributed to lipid rafts. The relocation of these two transmembrane isoforms of NCAM to lipid rafts may rely on palmitoylation (52). Although FGFR interaction and activation are thought to occur outside lipid rafts, association with non-receptor tyrosine kinase c-Fyn and Ras stimulation may occur inside lipid rafts. It is presently unclear whether similar mechanisms are involved in neurofascin-dependent signaling. However, it was suggested that palmitoylation of neurofascin may contribute to the recruitment of neurofascin to lipid rafts, indicating an interesting possibility for the regulation of neurofascin functions (36).

Both extra- and intracellular domains of neurofascin account for an association with FGFR1. Previous reports mainly addressed the function of the extracellular domains of L1 and NCAM interacting with FGFR1, both of which have been shown to interact with FGFR1 directly (23, 46). Accordingly, our co-precipitation studies also imply association of the extracellular domain of neurofascin with FGFR1. In contrast, it is presently unclear whether the intracellular domains of NCAM and L1 are associated with FGFR1 independently of the extracellular domain as in the case of neurofascin. The intracellular domain of NCAM is associated with c-Fyn, FAK, spectrin, and RPRTα, whereas L1 may perform signaling via the non-receptor tyrosine kinases c-Src (26, 27, 48, 53).

Our results indicate that the cytosolic domain by itself is sufficient to induce neurite outgrowth of PC12 cells. This mutant is not able to undergo interactions with the extracellular domain of FGFR1 as shown in Fig. 6*A*. Interestingly, the neurite outgrowth-promoting activity is not observed on a monolayer of parental NIH/3T3 cells, suggesting a requirement of substrate neurofascin for the neurite outgrowth-promoting activity of the neurofascin cytosolic domain expressed in PC12 cells in the absence of extracellular homophilic interactions. This was a surprising finding, because the cytosolic domain lacking extracellular domains may not confer extracellular interactions with substrate neurofascin. A possible explanation could be the expression of endogenous neurofascin in PC12 cells. However, we have previously shown that PC12 cells are devoid of neurofascin (8). Therefore, an additional unknown heterophilic receptor may account for extracellular interactions with substrate-bound neurofascin needed to form a functional signaling complex together with NF-CD and FGFR1. We have observed association of NF-CD with FGFR1 in HEK293 cells, which may be interpreted as a possibility of an interaction independent of the unknown receptor. However, this point remains unclear, because it is not known whether the unknown receptor is expressed in HEK293 cells.

Downstream of receptor interactions, several signaling pathways were shown to be addressed by NCAM, including MAPK signaling, PLCγ and concomitant PKC activation in the FGFR1 pathway, and G-protein signaling (21, 29, 30). Analysis of these pathways with regard to a contribution of neurofascin provides evidence for mechanisms shared with NCAM, whereas obvious differences were also observed. In accordance with the activation of FGFR1, neurofascin-dependent neurite outgrowth relies on the activity of PLC and PKC essentially as shown for NCAM (21). In the case of NCAM, PKC activation is linked to the MAPK pathway via Raf-1. Alternatively, NCAM may also induce the MAPK pathway after stimulation of c-Fyn, focal adhesion kinase, and Ras. Both pathways ultimately lead to the induction of neurite outgrowth after MEK and ERK activation. Remarkably, Erk activation via PKC appeared to be important for NCAM-dependent neurite outgrowth (21). MAPK signaling is also essential for L1-induced neurite outgrowth (28, 37). Furthermore, the cytosolic domain of the L1 subgroup of IgCAMs, including neurofascin, L1, and NrCAM, are highly conserved (5). In particular, neurofascin serine residues 56 and 100 were also found in L1 and NrCAM. In the case of L1, both serine residues were reported to function as a target of Erk2 phosphorylation *in vitro*, which is in accordance with a function of MAPK signaling (28). Similar to neurofascin, both residues were also involved in the promotion of neurite outgrowth; however, our experiments clearly show that the MAPK pathway is not involved in neurofascin-dependent neurite outgrowth, which was not impaired by MEK inhibitors PD98059 and U0126. This apparent difference between L1 and neurofascin is not easy to reconcile and requires further examination of the protein kinases important for the phosphorylation of L1 and neurofascin. Instead of Erk, PKC or downstream kinases may contribute to the phosphorylation of cytosolic neurofascin serine residues crucial for neurite outgrowth. On the other hand, neurite outgrowth may be induced independently of the MAPK pathway by the PLC-mediated yield of arachidonic acid acting on calcium channels, as suggested previously (44, 54).

Phosphorylation of tyrosine 81 represents a further mechanism to control neurofascin functions (55). Interaction of adult neurofascin NF186 with ankyrin is abolished by tyrosine 81 phosphorylation, which provides enhanced lateral mobility of neurofascin. Ankyrin binding is important for neurofascin functions in the adult brain like, for instance, the stabilization of cell-cell contact (56). Serine 56, which is crucial for neurite outgrowth by cell-cell interaction of embryonal NF166 according to our results, was suggested to be part of the ankyrin rec-

ognition site defined in adult NF186. Therefore, serine 56 may also contribute to the regulation of ankyrin binding (57). However, a possible contribution of serine 56 to the regulation of neurofascin-ankyrin interactions remains to be investigated.

Acknowledgments—We thank S. Radziwill for technical assistance and C. Kazmaier for antibody purification. We acknowledge E. Bock and co-workers for providing expression vectors for dominant negative PKC variants and P. Doherty for FGFR1 expression vectors. P. Brophy kindly supplied neurofascin antibodies. B. Schmid contributed to the construction of the dnFGFR1 expression vector.

REFERENCES

1. Murakami, M., Elfenbein, A., and Simons, M. (2008) *Cardiovasc. Res.* **78,** 223–231
2. Ornitz, D. M., and Itoh, N. (2001) *Genome Biol.* **2,** REVIEWS3005
3. Yokote, H., Fujita, K., Jing, X., Sawada, T., Liang, S., Yao, L., Yan, X., Zhang, Y., Schlessinger, J., and Sakaguchi, K. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 18866–18871
4. Volkmer, H., Hassel, B., Wolff, J. M., Frank, R., and Rathjen, F. G. (1992) *J. Cell Biol.* **118,** 149–161
5. Volkmer, H. (2001) in *Cell Adhesion* (Beckerle, M. C., ed) pp. 1–29, Oxford University Press, Oxford
6. Hassel, B., Rathjen, F. G., and Volkmer, H. (1997) *J. Biol. Chem.* **272,** 28742–28749
7. Pruss, T., Kranz, E. U., Niere, M., and Volkmer, H. (2006) *Mol. Cell Neurosci.* **31,** 354–365
8. Pruss, T., Niere, M., Kranz, E. U., and Volkmer, H. (2004) *Eur. J. Neurosci.* **20,** 3184–3188
9. Ango, F., di Cristo, G., Higashiyama, H., Bennett, V., Wu, P., and Huang, Z. J. (2004) *Cell* **119,** 257–272
10. Burkarth, N., Kriebel, M., Kranz, E. U., and Volkmer, H. (2007) *Mol. Cell Neurosci.* **36,** 59–70
11. Koticha, D., Babiarz, J., Kane-Goldsmith, N., Jacob, J., Raju, K., and Grumet, M. (2005) *Mol. Cell Neurosci.* **30,** 137–148
12. Davis, J. Q., and Bennett, V. (1994) *J. Biol. Chem.* **269,** 27163–27166
13. Davis, J. Q., Lambert, S., and Bennett, V. (1996) *J. Cell Biol.* **135,** 1355–1367
14. Jenkins, S. M., and Bennett, V. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 2303–2308
15. Koroll, M., Rathjen, F. G., and Volkmer, H. (2001) *J. Biol. Chem.* **276,** 10646–10654
16. Doherty, P., Williams, G., and Williams, E. J. (2000) *Mol. Cell Neurosci.* **16,** 283–295
17. Ditlevsen, D. K., Povlsen, G. K., Berezin, V., and Bock, E. (2008) *J. Neurosci. Res.* **86,** 727–743
18. Maness, P. F., and Schachner, M. (2007) *Nat. Neurosci.* **10,** 19–26
19. Saffell, J. L., Williams, E. J., Mason, I. J., Walsh, F. S., and Doherty, P. (1997) *Neuron* **18,** 231–242
20. Rønn, L. C., Doherty, P., Holm, A., Berezin, V., and Bock, E. (2000) *J. Neurochem.* **75,** 665–671
21. Kolkova, K., Novitskaya, V., Pedersen, N., Berezin, V., and Bock, E. (2000) *J. Neurosci.* **20,** 2238–2246
22. Sanchez-Heras, E., Howell, F. V., Williams, G., and Doherty, P. (2006) *J. Biol. Chem.* **281,** 35208–35216
23. Kulahin, N., Li, S., Hinsby, A., Kiselyov, V., Berezin, V., and Bock, E. (2008) *Mol. Cell Neurosci.* **37,** 528–536
24. Santuccione, A., Sytnyk, V., Leshchyns'ka, I., and Schachner, M. (2005) *J. Cell Biol.* **169,** 341–354
25. Paratcha, G., Ledda, F., and Ibáñez, C. F. (2003) *Cell* **113,** 867–879
26. Beggs, H. E., Baragona, S. C., Hemperly, J. J., and Maness, P. F. (1997) *J. Biol. Chem.* **272,** 8310–8319
27. Beggs, H. E., Soriano, P., and Maness, P. F. (1994) *J. Cell Biol.* **127,** 825–833
28. Schaefer, A. W., Kamiguchi, H., Wong, E. V., Beach, C. M., Landreth, G., and Lemmon, V. (1999) *J. Biol. Chem.* **274,** 37965–37973
29. Schmid, R. S., Graff, R. D., Schaller, M. D., Chen, S., Schachner, M., Hemperly, J. J., and Maness, P. F. (1999) *J. Neurobiol.* **38,** 542–558
30. Hansen, R. K., Christensen, C., Korshunova, I., Kriebel, M., Burkarth, N., Kiselyov, V. V., Olsen, M., Ostergaard, S., Holm, A., Volkmer, H., Walmod, P. S., Berezin, V., and Bock, E. (2007) *J. Neurochem.* **103,** 1396–1407
31. Jessen, U., Novitskaya, V., Pedersen, N., Serup, P., Berezin, V., and Bock, E. (2001) *J. Neurochem.* **79,** 1149–1160
32. Krushel, L. A., Cunningham, B. A., Edelman, G. M., and Crossin, K. L. (1999) *J. Biol. Chem.* **274,** 2432–2439
33. Volkmer, H., Leuschner, R., Zacharias, U., and Rathjen, F. G. (1996) *J. Cell Biol.* **135,** 1059–1069
34. Boget, S., Cereser, C., Parvaz, P., Leriche, A., and Revol, A. (2001) *Eur. J. Endocrinol.* **145,** 303–310
35. Groth, C., and Lardelli, M. (2002) *Int. J. Dev. Biol.* **46,** 393–400
36. Ren, Q., and Bennett, V. (1998) *J. Neurochem.* **70,** 1839–1849
37. Schmid, R. S., Pruitt, W. M., and Maness, P. F. (2000) *J. Neurosci.* **20,** 4177–4188
38. Vaudry, D., Stork, P. J., Lazarovici, P., and Eiden, L. E. (2002) *Science* **296,** 1648–1649
39. Kolkova, K., Stensman, H., Berezin, V., Bock, E., and Larsson, C. (2005) *J. Neurochem.* **92,** 886–894
40. Rathjen, F. G., Wolff, J. M., Chang, S., Bonhoeffer, F., and Raper, J. A. (1987) *Cell* **51,** 841–849
41. Ratcliffe, C. F., Westenbroek, R. E., Curtis, R., and Catterall, W. A. (2001) *J. Cell Biol.* **154,** 427–434
42. Koticha, D., Maurel, P., Zanazzi, G., Kane-Goldsmith, N., Basak, S., Babiarz, J., Salzer, J., and Grumet, M. (2006) *Dev. Biol.* **293,** 1–12
43. Hildebrandt, H., Muhlenhoff, M., and Gerardy-Schahn, R. (2008) *Neurochem. Res.*, in press
44. Doherty, P., and Walsh, F. S. (1996) *Mol. Cell Neurosci.* **8,** 99–111
45. Cambon, K., Hansen, S. M., Venero, C., Herrero, A. I., Skibo, G., Berezin, V., Bock, E., and Sandi, C. (2004) *J. Neurosci.* **24,** 4197–4204
46. Kiselyov, V. V., Skladchikova, G., Hinsby, A. M., Jensen, P. H., Kulahin, N., Soroka, V., Pedersen, N., Tsetlin, V., Poulsen, F. M., Berezin, V., and Bock, E. (2003) *Structure* **11,** 691–701
47. Williams, E. J., Furness, J., Walsh, F. S., and Doherty, P. (1994) *Neuron* **13,** 583–594
48. Bodrikov, V., Leshchyns'ka, I., Sytnyk, V., Overvoorde, J., den Hertog, J., and Schachner, M. (2005) *J. Cell Biol.* **168,** 127–139
49. Bodrikov, V., Sytnyk, V., Leshchyns'ka, I., den Hertog, J., and Schachner, M. (2008) *J. Cell Biol.* **182,** 1185–1200
50. Heiland, P. C., Griffith, L. S., Lange, R., Schachner, M., Hertlein, B., Traub, O., and Schmitz, B. (1998) *Eur. J. Cell Biol.* **75,** 97–106
51. Leshchyns'ka, I., Sytnyk, V., Morrow, J. S., and Schachner, M. (2003) *J. Cell Biol.* **161,** 625–639
52. Little, E. B., Edelman, G. M., and Cunningham, B. A. (1998) *Cell Adhes. Commun.* **6,** 415–430
53. Ignelzi, M. A., Jr., Miller, D. R., Soriano, P., and Maness, P. F. (1994) *Neuron* **12,** 873–884
54. Williams, E. J., Doherty, P., Turner, G., Reid, R. A., Hemperly, J. J., and Walsh, F. S. (1992) *J. Cell Biol.* **119,** 883–892
55. Garver, T. D., Ren, Q., Tuvia, S., and Bennett, V. (1997) *J. Cell Biol.* **137,** 703–714
56. Tuvia, S., Garver, T. D., and Bennett, V. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 12957–12962
57. Zhang, X., Davis, J. Q., Carpenter, S., and Bennett, V. (1998) *J. Biol. Chem.* **273,** 30785–30794