Nectin-1 binds and signals through the fibroblast growth factor receptor*

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Background: Nectin-1 is a cell-adhesion molecule important for the formation of synapses.

Results: The third Ig-module of nectin-1 (nectin-1 Ig3) induces neuronal differentiation and promotes neuronal survival through a direct interaction with FGF-receptor.

Conclusion: FGF-receptor is a downstream signaling partner of nectin-1

Significance: This mechanism of nectin-1 signaling is crucial for understanding its neuritogenic and survival promoting effects.

SUMMARY

Nectins belong to a family of immunoglobulin(Ig)-like cell-adhesion molecules (CAMs) comprising four members, nectin-1 through nectin-4. Nectins are involved in formation of the mechanical adhesive puncta adherentia junctions (PAJs) of synapses. Nectins share the same overall structural topology with an extracellular region containing three Ig modules, a transmembrane region, and a cytoplasmic region. In nectin-1, the first and second Ig-module in the extracellular region is necessary for the trans-interaction with nectin-3 and formation of cis-dimers, respectively. The function of the third Ig-module of nectin-1 remains unknown.

We here report the structure in solution of the third, membrane–proximal Ig module of mouse nectin-1 (nectin-1 Ig3) solved by means of nuclear magnetic resonance (NMR) spectroscopy. It belongs to the C1 set of the Ig superfamily. Nectin-1 Ig3 was produced as a recombinant protein and induced neurite outgrowth in primary cultures of hippocampal and cerebellar granule neurons (CGNs), an effect abolished by treatment with the fibroblast growth factor receptor (FGFR) inhibitor SU5402, or by transfection with a dominant-negative FGFR1 construct. We showed by surface plasmon resonance (SPR) analysis that nectin-1 Ig3 directly interacted with various isoforms of FGFR. Nectin-1 Ig3 induced phosphorylation of FGFR1c in the same manner as the whole nectin-1 ectodomain, and promoted survival of CGN induced to undergo apoptosis.

Finally, we constructed a peptide, nectide, by employing in silico modeling of various FGFR ligand binding-sites. Nectide mimicked all the effects of nectin-1 Ig3. We suggest that FGFR is a downstream signaling partner of nectin-1.

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Nectins belong to a family of immunoglobulin (Ig)-like cell-adhesion molecules (CAMs) comprising four members, nectin-1 through nectin-4. Nectins share the same overall structural topology with an extracellular region containing three Ig modules, a transmembrane region, and a cytoplasmic region (1). Via their extracellular regions nectins can homophilically (in cis) and heterophilically (in trans) interact with each other (2-4). Nectins participate in formation of cell-cell junctions, cooperatively with or independently of another family of CAMs namely cadherins (5,6). Nectins are involved in formation of mechanical adhesive sites in synapses termed puncta adherentia (PAJs) in the CA3 area of hippocampus, and interaction between nectin-1 and nectin-3 on the pre- and postsynaptic sides, respectively, initiates the formation of PAJs between axons and dendrites (7-9).

Nectin-1 is prominently expressed in the brain (10,11), among other places in the cerebellum and hippocampus (7,9,12). Nectin-1 is required for development of ectodermal structures, and mutation in the nectin-1 gene can cause mental retardation in severe cases (13), emphasizing the importance of nectin-1 in the developing CNS. In nectin-1, the first Ig-module in the extracellular region is necessary for the trans-interaction with nectin-3 (14) and the second Ig-module is involved in the formation of cis-dimers (15), but the function of the third Ig-module remains unknown.

We hypothesized that the function of the soluble third Ig-module of nectin-1 (nectin-1 Ig3) is stimulation of axon growth and promotion of survival of immature neurons. Furthermore we hypothesized that nectin-1 Ig3 mediates its neuritogenic effects through the fibroblast growth factor receptors (FGFRs), since the FGFRs play critical roles during development of CNS, and has been shown to interact directly with other neuronal CAMs (16-19). The FGFR1-3 are, among other regions, expressed in the cerebellum and hippocampus (19) overlapping the expression pattern of nectin-1 in the CNS. We expressed the third Ig module of nectin-1 as a recombinant protein in the Pichia pastoris expression system and used nuclear magnetic resonance (NMR) spectroscopy to solve its structure in solution. We here report that mouse nectin-1 Ig3 induces neurite outgrowth through binding to and activation of FGFR. It also promotes neuronal survival. The whole nectin-1 ectodomain, which includes Ig3, also activates FGFR. We identified an amino acid sequence motif in nectin-1 Ig3 involved in FGFR binding and activation. We show that a corresponding peptide termed nectide mimics the effects of nectin-1 Ig3. We suggest that FGFR is a downstream signaling partner of nectin-1.

EXPERIMENTAL PROCEDURES

Materials - The peptide termed nectide (WTTLNGSLPKGVEAQNR) corresponding to amino acids 282 through 299 of nectin-1 from mouse [National Center for Biotechnology Information (NCBI) Reference Sequence NP_067399] and the control peptide with the reverse sequence (TRNQAEVGKPLSGNLTTW) were synthesized as tetrameric dendrimers composed of four monomers coupled to a three-lysine-containing backbone by Schäfer-N (Copenhagen, Denmark). The recombinant ectodomain of human nectin-1 was obtained from R&D Systems, Catalog #2880-N1 (Abingdon, UK). An expression vector that encodes a dominant-negative form of FGFR1 with a deleted kinase domain (dnFGFR) was kindly provided by Dr. Jane Saffell (20). An expression vector that encodes the enhanced variant of the Aequorea Victoria green fluorescent protein (pEGFP-N1) was purchased from Clontech (Palo Alto, CA, USA). Recombinant human insulin-like growth factor 1 (IGF-1) was obtained from Invitrogen (Taastrup, Denmark). The FGFR inhibitor SU5402 was obtained from Calbiochem (Bad Soden, Germany).

Plasmid construction and cloning - The coding sequences of the combined Ig2-3 modules of FGFR1-3 isoforms “b” and “c” were amplified by using reverse transcription polymerase chain reaction (RT-PCR) with corresponding gene-specific primers and using Wistar rat brain RNA as a template. Briefly, to generate individual His-tagged Ig2-3 modules, the coding regions of the FGFR1-3 isoforms were amplified using...
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Primers that contain the His-tag coding sequence (16). The amplified fragments were cloned into a pPICZαC vector (Invitrogen) at the ClaI and NotI sites and sequenced. The cloning of the Ig2-3 modules has been described previously (16-19). All of the FGFR recombinant proteins contained the His-tag sequence AGHHHHHHE at the N-terminus.

Using PCR, a DNA fragment that encodes residues 241-335 of nectin-1 (NCBI accession no. NP_067399) and a C-terminal 6 x His tag was amplified. The fragment was subcloned into the ClaI/NotI site of the pPICZαC vector (Invitrogen). Recombinant plasmids were analyzed by restriction analysis and DNA sequencing. Before transformation of the P. pastoris strain KM71H, the plasmids were linearized by cleavage with the SacI restriction enzyme (New England Biolabs, Hitchin, UK).

mRNA pools from neurons isolated from mouse cerebellum were purified according to the manufacturer’s recommendations (Oligotex Direct mRNA mini kit, Qiagen Nordic-Denmark, Copenhagen, Denmark). Template DNA was made using 10 ng mRNA in a reverse transcriptase reaction (SuperScript III Reverse Transcriptase, Invitrogen).

Production of recombinant proteins - The FGFR constructs that code for Ig2-3 of FGFR1b, FGFR2c and FGFR3c were expressed in the KM71 or KM71H strain of Pichia pastoris (P.pastoris) (Invitrogen) according to the manufacturer’s instructions (16). Ig2-3 of FGFR1c was expressed in Drosophila Schneider 2 cells as previously described (21). The recombinant proteins were purified by affinity chromatography using Ni-NTA resin (Qiagen) or ion exchange chromatography and gel filtration. The recombinant rat full-length FGF1 (amino acids 1-155, Swiss-Prot P61149) was kindly provided by Dr. Artur Kochoyan (Copenhagen University, Denmark).

The third Ig module of the ectodomain of nectin-1 was produced as a recombinant protein in a P. pastoris expression system (Invitrogen). A protein-expressing KM71H clone was inoculated in 10 ml of buffered minimal glycerol (BMG) medium (100 mM potassium phosphate, 3.4 g/l yeast nitrogen base without ammonium sulfate, 10 g/l ammonium sulfate, 0.4 mg/l biotin, 10 g/l glycerol) and incubated at 30°C overnight. The culture was then spun down at 1500 × g for 5 min. The cell pellet was resuspended in 150 ml of BMG and incubated at 30°C overnight. The following day, the culture was spun down at 1500 × g for 5 min, and the cell pellet was resuspended in 2 l BMG with 100 µl of antifoam 204 (Sigma-Aldrich, Vallensbæk, Denmark). The culture was transferred to the autoclaved fermentor Biostat B (B Braun Biotech International, Allentown, PA, USA), and the fermentor ran at 30°C and pH 5 with 600 rpm stirring and 8 l/min filtered atmospheric air until an optical density of 8 at 600 nm was reached. The cells were spun down at 1500 × g for 5 min, and the cell pellet was resuspended in 2 l of buffered minimal methanol (BMM) medium (100 mM potassium phosphate, 3.4 g/l yeast nitrogen base without ammonium sulfate, 10 g/l ammonium sulfate, 0.4 mg/l biotin, 0.5 % v/v methanol) and induced for 24 h under the conditions described above. During induction, an additional 100 ml of 20% v/v methanol was added at a constant flow rate. After induction, the cells were spun down at 3000 × g for 5 min, the pH of the harvested supernatant was adjusted to 7.4, and the supernatant was filtered. The sample was loaded on 5 ml of Ni-NTA Superflow (Qiagen) and eluted in 10 ml phosphate-buffered saline (PBS) with 0.5 M imidazole. Endoglycosidase (20 µl, Endo Hf 1,000 units/µl, NEB) were added, and deglycosylation was performed for 24 h at room temperature. The solution was diluted in 500 ml PBS, and Ni-NTA purification was repeated as above. The sample was then desalted on a size PD-10 exclusion column (GE Healthcare, Hillerød, Denmark). The purity and identity of the protein were verified by sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE) and N-terminal sequencing.

NMR sample preparation, data recording and structure calculation - The 15N and 13C double-labeled protein was produced as above, with the exception that BMG was replaced with BMG2xL (100 mM potassium phosphate, 3.4 g/l yeast nitrogen base without ammonium sulfate, 4 g/l 15N-
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ammonium sulfate, 8 g/l potassium sulfate, 0.4 mg/l biotin, 10g/l $^{13}$C-glucose) and BMM was replaced with BMM2xL (100 mM potassium phosphate, 3.4 g/l yeast nitrogen base without ammonium sulfate, 4 g/l $^{15}$N-ammonium sulfate, 8 g/l potassium sulfate, 0.4 mg/l biotin, 1 % v/v $^{13}$C-methanol). The volumes were then reduced to 1 l and 300 ml for growth and induction, respectively. The sample was concentrated to 1.5 mM in NMR buffer (10 mM sodium phosphate, 100 mM NaCl, 0.03% w/v D20, 0.03% w/v Azide) in a total volume of 300 µl and transferred to a Shigemi tube (Shigemi, Allison Park, PA, USA). First, the $^{1}H$-$^{15}N$-HSQC, HNCO, HN(CA)CO, HN(CO)CA, HNCA, CBCA(CO)HN, and HN(CA)CO spectra were recorded for backbone assignment. These were followed by the recording of H(CC)NH, HCCH-TOCSY, and $^{15}N$-TOCSY-HSQC spectra for side chain assignment. Finally, $^{15}N$-NOESY-HSQC and $^{13}C$-NOESY-HSQC were recorded, and NOE signals were manually chosen.

All recorded data were processed and transformed using NMRpipe (22) and Pronto3D was used for assignment and NOE picking analyzed (23). The assignment of the chosen NOEs was performed using Cyana 2.2.1, which was also used for the primary structure calculation (24). Apart from the NOE constraints, a constraint that defines a disulfide bond between the only two cysteines was applied together with 150 torsion angle constraints calculated in TALOS (25) and 25 hydrogen bonds. A total of 100 structures were calculated, of which the 20 structures that scored the best on the CYANA target function were saved in a coordinate file. Finally, the structures were water-refined as described previously (26) and deposited in the Protein Data Bank (accession no. 2L7J) and Biological Magnetic Resonance Data Bank (accession no. 17358).

Surface plasmon resonance - Real-time biomolecular interaction termed surface plasmon resonance (SPR) analysis was performed using a BIAcore-2000 instrument (GE Healthcare) at 25°C using 10 mM sodium phosphate (pH 7.4), 150 mM NaCl (PBS) as running buffer. 2500 resonance units (RU) of nectin-1 Ig3, 500 RU of nectide and 2500 RU of FGF1 were immobilized by amine coupling at a flow rate of 5 µl/min on a CM5 chip activated by 35 µl activation solution followed by the injection of protein or peptide in 10 mM sodium acetate (pH 4.5 or 5.0). 35 µl ethanalamine-HCl was injected to deactivate the flow cell (FC) after an adequate level of immobilization was reached. Binding between the Ig2-3 fragments of various FGFR isoforms in the reported concentrations were measured at 25°C in the running buffer at a flow rate of 10-30 µl/min. After each binding cycle, the sensor chip was regenerated by an injection of 10 µl of 1 M NaCl. For each protein and peptide concentration, two injections were made in each experiment.

Curves were then analyzed by nonlinear curve fitting using the software package BIAevaluation, version 4.1 (BIAcore). Before fitting, curves were double-referenced, i.e. curves for binding to a blank reference chip (activated and deactivated) were subtracted from the matching curves for binding to nectin-1 Ig3, nectide or FGF1, and then a control curve obtained by injecting PBS alone was subtracted. Curves were fitted using a 1:1 interaction model, and the KD was calculated as $k_d/k_a$, where KD is the equilibrium dissociation constant and $k_a$ and $k_d$ are the association and dissociation rate constants, respectively.

Cell cultures - The fibroblastoid mouse cell line L929 (L-cells) was obtained from European Cell Culture Collection (Salisbury, UK) and maintained in Dulbecco’s modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM Glutamax, 100 U/ml penicillin, 100 µg/ml Streptomycin, and 2.5 mg/ml fungizone (Invitrogen).

Cerebellar granule neurons (CGNs) were prepared from postnatal 7-8 days Wistar rats (Charles River, Sulzfeld, Germany) essentially as previously described (27). Briefly, cerebella were isolated from the brain and kept in ice-cold modified Krebs Ringer solution, cleared of blood vessels and meninges, and roughly homogenized by chopping and thereafter trypsinization. The CGNs were washed in
the presence of DNAse1 and soybean trypsin inhibitor (Sigma-Aldrich).

Hippocampal neurons were isolated from Wistar rat embryos on embryonic day 19 or newborn rats as previously described (28). Hippocampal tissue was isolated from the brain in modified Krebs-Ringer solution, kept on ice, and treated as described above for the CGNs. All cell cultures were incubated at 37°C in a humidified atmosphere that contained 5% CO2. All of the animals were handled in accordance with the national guidelines for animal welfare.

**FGFR1c phosphorylation - TREX-293 cells (Invitrogen) stably transfected with human full-length FGFR1c with a C-terminal Strep II tag (IBA Biotec, Göttingen, Germany) (21) were maintained in DMEM with 200 µg/ml hygromycin, 10% fetal calf serum, 2 mM Glutamax, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). To determine the phosphorylation of human FGFR1c, 2 × 10⁶ cells were first starved overnight in medium without serum. The cells were then treated with nectin-1 Ig3, the whole ectodomain of nectin-1, nectide or the control protein (boiled ectodomain of nectin-1) or control peptide (Rev nectide) at various concentrations and subsequently lysed using 300 µl lysis buffer that contained 1% (v/v) NP-40, 0.5% (v/v) Triton X-100, 1% (v/v) deoxycholate, 1% (v/v) sodium dodecyl sulfate, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1% (v/v) protease inhibitors (Roche, Mannheim, Germany), and phosphatase inhibitors (Calbiochem inhibitor cocktail III; 1:100) in PBS. The protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA), and 500 µg of protein from each lysate were incubated with 15 µl agarose-coupled anti-phosphotyrosine antibodies (4G10-AC; Upstate Biotechnologies, Lake Placid, NY, USA) for 6 h at 4°C. The bound protein was washed and eluted with 180 mM phenylphosphate (Sigma-Aldrich). Equal amounts of purified protein were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Immunoblotting was performed using rabbit antibodies (diluted 1:2000) against the recombinant StrepII tag (IBA Biotech) and swine anti-rabbit IgG horseradish peroxidase conjugate (diluted 1:2000; DakoCytomation, Glostrup, Denmark) in 5% (w/v) non-fat dry milk. The immune complexes were developed by SuperSignal West Dura extended-duration substrate (Pierce, Rockford, IL, USA) and visualized and quantified using SynGene Gene Tool image analysis software (Synoptics, Cambridge, UK).

For the analysis of total FGFR expression, 30 µg of proteins from each original lysate were separated by SDS-PAGE and then analyzed by Western blotting using antibodies against the StrepII tag. The amount of actin (loading control) was visualized using rabbit polyclonal antibody (diluted 1:1000) against actin (Sigma-Aldrich).

**Neurite outgrowth assay -** The single-cell cultures were plated at a density of 12.5 × 10³ cells/cm² onto uncoated eight-well Permanox Lab-Tek Chamber Slides (NUNC, Roskilde, Denmark) in Neurobasal medium supplemented with 0.4% (w/v) bovine serum albumin (BSA; Sigma-Aldrich), 2% (v/v) B27 Neurobasal supplement, 2 mM Glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 mM HEPES (all from Invitrogen). Solutions with nectin-1 Ig3 or nectide with or without the SU5402-inhibitor (Calbiochem) were added at various concentrations to a total volume of 300 µl/cm² immediately after plating. The transfected CGNs were plated at a density of 62.5 × 10⁴ cells/cm² on a monolayer of L-929 cells, and the co-culture was grown for 24 h before analysis. All cell cultures were maintained at 37°C and 5% CO2. The cells were then fixed and immunostained for GAP-43 (Chemicon, AH Diagnostics, Aarhus, Denmark) to visualize the neurons. The immunostained cultures were recorded by computer-assisted fluorescence microscopy. At least 200 neurons for each group in each individual experiment were captured in a systematic series of fields of view as previously described (29). A Nikon Diaphot inverted microscope with a Nikon Plan 20× objective (Nikon, Tokyo, Japan) coupled to a video camera (Grundig Electronics, Nürnberg, Germany) was used for recordings. The average neurite length per cell was estimated using a stereological approach (29) and the Prima software package developed at the Protein Laboratory (Copenhagen, Denmark).
**Transfection of CGNs** - CGNs from postnatal 7-8 days Wistar rats were prepared as described above and were subsequently transfected by electroporation with a Nucleofector device and a Rat Neuron Nucleofactor™ Kit (Amaxa, Gaithersburg, MD, USA) according to the manufacturer’s recommendations. 3 × 10^6 CGNs were transfected with 3 µg of the dnFGFR1 expression vector and 3 × 10^6 control CGNs were transfected with 3 µg of an empty vector. All CGNs were co-transfected with 0.5 µg of the p-EGFP-N1 vector to visualize the transfected cells. The transfected CGNs were suspended in Neurobasal™-A medium supplemented with 2% (v/v) B-27, 2% (v/v) horse serum, and 4 mM Glutamax (Invitrogen) and seeded in two-well Permanox Lab-Tek Chamber Slides on 3 × 10^5/well L-929 cells suspended in DMEM medium with supplements as described earlier and plated 24 h prior to transfection.

**Survival assay** - Primary cultures of CGNs were plated on poly-L-lysine-coated eight-well Permanox Lab-Tek Chamber Slides at a density of 1 × 10^4 cells/cm^2 in Neurobasal-A medium (Gibco BRL) supplemented with 2% (v/v) B27, 1 mM (v/v) Glutamax, 100 units/ml penicillin, 100 µg/ml streptomycin, and 40 mM KCl. Cytosine-β-D-arabinofuranoside (Ara-C; Sigma-Aldrich) was added to a final concentration of 10 µM 22 h after plating to avoid the proliferation of glial cells. CGNs were allowed to differentiate for another 6 days at 37°C and 5% CO₂ and then washed in Basal Medium Eagle (BME; Invitrogen) containing only 5 mM KCl and supplemented with 1% (v/v) glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, 3.5 g D-glucose/L, and 1% (v/v) sodium pyruvate (Invitrogen) followed by 48 h of incubation in the same medium that then included nectin-1 Ig3 or nectide. Cell viability was estimated by staining with Hoechst 33258 for 25 min as previously described (30). Images of approximately 2000 CGNs were recorded for each group in each experiment in a systematic series of fields as described in the neurite outgrowth section above by computer-assisted fluorescence microscopy using a Nikon plan 20× objective and video camera (QImaging, Burnaby, British Columbia, Canada). Survival was estimated by comparing the number of living neurons with the total number of neurons (31).

**Statistical analysis** - Statistical analysis was performed using one-way repeated-measures analysis of variance (ANOVA) followed by either the Dunnett or Newman-Keuls multiple comparison *post hoc* test. ANOVA was performed using GraphPad Prism, version 4.02 (GraphPad, San Diego, CA, USA) or SAS, version 9.1 (SAS Institute, Cary, NC, USA).

**RESULTS**

The membrane proximal module of nectin-1 belongs to the C1-set subgroup of Ig domains - Using sequential assignment followed by side-chain assignment; chemical shifts were assigned to 91.4% of the atoms in nectin-1 Ig3. We used CYANA to assign 2020 manually prepicked NOE signals that yielded 1478 non-redundant distance constraints. From the assigned chemical shifts, 150 angle constraints were derived using the TALOS algorithm (25). Finally, NOE data supported 25 hydrogen bonds in the protein backbone. Using the data above in seven CYANA calculation cycles, we generated 100 nectin-1 Ig3 models, of which the 20 that had the lowest energy had an average root mean square deviation of 0.55 ± 0.15 Å and 1.37 ± 0.19 Å for the backbone and all heavy atoms, respectively (24). Each of the 20 lowest energy models was refined in a water-containing environment that yielded the final model, and the ensemble was uploaded to the Protein Data Bank (accession no. 2L7J; Table 1).

The model of nectin-1 Ig3 showed an Ig-like fold with a total of seven β strands (A-G) distributed on two sandwiched anti-parallel sheets connected by a disulfide bond. As for all of the other Ig-like modules, β strands B, C, E, and F form the core of the module, whereas the number and position of the remaining strands determine the Ig-like subgroup. In this case, the B and E strands are flanked by the A and D strands, respectively, whereas the G strand is aligned to the F strand (Fig. 1A). This makes nectin-1 Ig3 a C1-set subgroup Ig-like module (32).
40 of the 85 assigned H-N backbone signals showed double signals that originated from the same residue with a relative intensity of 40/60%. In the structure calculation, we included a signal that related to the high intensity of the double peaks only, disregarding the lower-intensity set of peaks. The origin of the low-intensity peaks remains unclear.

Examples of HNCACB-CBCA(CO)HN overlay and HSQC spectra of nectin-1 Ig3 are shown (Fig. 1C and Fig. 1D). The differences from peaks showing double signals measured in changes in chemical shifts are presented in Table 2. The crystal structure of the whole ectodomain of human nectin-1 has recently been solved (33) and this structure, together with mutation analysis, suggests a V-shaped homophilic dimer formed through the first Ig-module (Fig. 1B). The backbone structures of mouse nectin-1 Ig3 in solution and human nectin-1 Ig3 in the crystal have very similar folding topologies.

The third Ig module of nectin-1 induces neurite outgrowth in an FGFR activation-dependent manner - Several other neuronal CAMs, such as L1, NCAM, V-cadherin, neuroplastin, and neurofascin, are able to induce neuronal differentiation, reflected by neurite outgrowth, in immature neurons (19,34,35). We investigated whether nectin-1 Ig3 also has neuritogenic properties. Neurons were grown on plastic at low density to avoid any cell-cell contacts. Neurite outgrowth was stimulated by adding increasing concentrations of nectin-1 Ig3 and evaluated after incubation for 24 h. Nectin-1 Ig3 induced neurite outgrowth in hippocampal neurons and CGNs compared with control cultures grown in medium only (Fig.2A and Fig. 2B, respectively). In hippocampal neurons, a bell-shaped dose-response relationship was observed, with a maximal response of 190 ± 13% at a concentration of 10 µM nectin-1 Ig3. In CGNs, the response was lower but still statistically significant and reached a plateau with a maximal response of 140 ± 10% at a concentration of 0.47 µM nectin-1 Ig3.

The neuritogenic effect of various CAMs is at least partially dependent on FGFR activation (19,34,35). Therefore, we inhibited FGFR in CGNs to investigate the involvement of FGFR activation in nectin-1 Ig3-induced neurite outgrowth. First, CGNs were stimulated with nectin-1 Ig3 at a concentration of 0.87 µM and concomitantly treated with the specific FGFR inhibitor SU5402, which inhibits the tyrosine kinase activity of FGFR1 by interacting with the catalytic module of the receptor. Second, we transfected CGNs with a vector that encodes dominant-negative FGFR1 (dnFGFR1), in which the cytoplasmic kinase module is deleted (20). As a control, CGNs were transfected with an empty vector. Increasing concentrations of SU5402 significantly inhibited neurite outgrowth induced by nectin-1 Ig3 (Fig. 2C). Furthermore, treatment with 0.87 µM nectin-1 Ig3 in CGNs transfected with an empty vector resulted in a neurite outgrowth response of 130 ± 5% compared with untreated controls, an effect abrogated in CGNs transfected with dnFGFR1 (Fig. 2D). This indicates that activation of FGFR1 is an obligatory step for the neuritogenic effect of nectin-1 Ig3.

The Ig3 module of nectin-1 binds various isoforms of FGFR - The FGFR family consists of various isoforms (36-38) that have been shown to directly interact with neuronal CAMs, such as NCAM, L1, and neurofascin (16-19). To investigate whether this would be the case for nectin-1 Ig3 as well, we applied an SPR-based approach to test the binding of this module to various isoforms of FGFR, namely FGFR1b, FGFR1c, FGFR2c and FGFR3c. The second and third Ig3 modules of FGFRs are known to be responsible for ligand binding (39,40), and we tested the binding of nectin-1 Ig3 to the Ig2-3 modules of the FGFR isoforms.

We first wanted to validate the folding and integrity of various recombinantly produced FGFRs are known to be responsible for ligand binding (39,40), and we tested the binding of nectin-1 Ig3 to the Ig2-3 modules of the FGFR isoforms. We first wanted to validate the folding and integrity of various recombinantly produced FGFR fragments in solution by testing their binding to the cognate ligand FGF1, which is known for its promiscuity in relation to the binding to various FGFR isoforms. We covalently immobilized FGF1 on a sensor chip. Binding curves for FGF1 and various concentrations of the tested FGFR isoforms are shown in Fig. 3 (A, B, C and D). Consistent with previously published data (39), we found that the Kd values of the FGF1-FGFR interactions were

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Subsequently, nectin-1 Ig3 was covalently immobilized on a sensor chip, and the binding of various concentrations of the FGFR isoforms were tested. FGFR1b, FGFR1c, FGFR2c and FGFR3c all bound nectin-1 Ig3 with $K_D$ values within the range of $10^{-7} - 10^{-8}$ M (Table 3). Binding-curves were analyzed by nonlinear curve fitting and are shown in Fig. 3 (E,F,G and H).

The Ig3 module of nectin-1 does not bind to PDGFRα and PDGFRβ - The nectin3-afadin complex was recently shown to be involved in the platelet-derived growth factor (PDGF)-induced activation of phosphatidylinositol 3-kinase (PI3K)-Akt signaling for cell survival (41). We therefore investigated whether nectin-1 Ig3 directly interacts with PDGFRα and PDGFRβ as well. Using SPR, we first covalently immobilized the cognate ligand PDGF on a sensor chip as a positive control and then covalently immobilized nectin-1 Ig3 at the sensor chip as well. Both PDGFRα and PDGFRβ in solution bound PDGF, whereas no binding to nectin-1 Ig3 was detected.

Both the Ig3 module of nectin-1 and the whole nectin-1 ectodomain induce FGFR activation - To further validate FGFR as a downstream signaling partner of nectin-1, we investigated whether the nectin-1 Ig3-FGFR interaction resulted in FGFR phosphorylation, which has been shown for other neuronal CAMs (19-21). TREX-293 cells stably transfected with C-terminally Strep II-tagged full-length FGFR1c were stimulated with various concentrations of nectin-1 Ig3 or the whole nectin-1 ectodomain, and the degree of FGFR1c phosphorylation was determined by immunoprecipitation and Western blotting. Fig. 4A shows that nectin-1 Ig3 concentration-dependently induced FGFR1c phosphorylation, with maximal phosphorylation occurring at 0.09 μM. FGF2, which was used as a positive control, also induced FGFR activation in these cells. Fig. 4B shows that the whole nectin-1 ectodomain also induced FGFR1c phosphorylation with maximal phosphorylation occurring at 0.09 μM. The denatured (boiled) protein had not effect on receptor activation. To preclude the possibility that the observed differences in FGFR phosphorylation were caused by variations in nectin-1 Ig3-induced FGFR expression or in sample immunoprecipitation, we also analyzed the expression of total FGFR and actin in all of the samples. We found that all of the lysates contained similar amounts of total FGFR and actin (Fig. 4A and 4B).

The Ig3 module of nectin-1 promotes neuronal survival - FGFR is a known mediator of neuroprotection in the CNS (42). Therefore, we investigated whether nectin-1 Ig3 could promote neuronal survival. Primary cultures of CGNs were grown for 7 days in an elevated concentration of K+ (40 mM) followed by a reduction in the K+ concentration (5 mM) that caused the CGNs to undergo apoptosis (31,43). As shown in Fig. 4C, a reduction of the K+ concentration significantly induced neuronal cell death compared with a medium that contained high K+, an effect partially prevented by treatment with the known survival factor insulin-like growth factor 1 (IGF-1). Cell death induced by a reduction of the K+ concentration was partially prevented in CGNs treated with various concentrations of nectin-1 Ig3 (Fig. 4D). A plateau was reached at concentrations in the range of 0.017-0.43 μM. Thus, nectin-1 Ig3 has a survival-promoting effect.

The CDE strand-loop-strand region peptide nectide is a functional mimetic of the third Ig module of nectin-1 that targets FGFR - After demonstrating that nectin-1 Ig3 mediates biological effects through the binding and activation of FGFR, we determined which part of the module that might contribute to the FGFR interaction site. We aligned several previously identified FGFR binding motifs to the sequence of nectin-1 Ig3 and found that an 18-amino-acid motif that encompasses part of the C-strand, the entire D-strand, part of the E-strand, and the loops between these strands of nectin-1 Ig3 has partial homology with two previously identified FGFR binding motifs: the dekafin motif in FGF10 (44) and the FGL motif in NCAM (21) (Fig. 4E)
We termed this motif the nectide motif and investigated whether the nectide peptide could mimic the function of nectin-1 Ig3. Binding to various FGFR isoforms was first tested. As for nectin-1 Ig3, we used an SPR-based approach by covalently immobilizing nectide on a sensor chip and subsequently testing the binding of various FGFR isoforms. FGFR1b, FGFR1c, FGFR2c and FGFR3c all bound nectide with K_D values within the range of $10^{-7} - 10^{-8}$ M (Table 4) thus mimicking the binding between nectin-1 Ig3 and the FGFR isoforms. Binding-curves were analyzed by nonlinear curve fitting and are shown in Fig. 6 (A,B,C and D).

We then examined whether nectide could induce neuronal differentiation reflected by neurite outgrowth in primary CGNs. As shown in Fig. 7, nectide had a clear neuritogenic effect (Fig. 7A). Neurite outgrowth-inducing concentrations were higher for nectide than for the Ig3 module of nectin-1, indicating a higher potency of the module. However, the efficacy of the nectide-peptide was much higher than that of the Ig3 module. The control peptide with a reverse sequence (Rev nectide) had no effect on neurite outgrowth in CGNs (Fig. 7B), indicating that the effect of nectide was sequence-specific.

We then tested the effect of 9.77 µM nectide on neurite outgrowth in the presence of the FGFR inhibitor SU5402 and found that it inhibited nectide-induced neuritogenesis by 112%, 196%, and 311% at concentrations of 20, 40, and 80 µM, respectively (Fig. 7C). It was not possible to completely abrogate the nectide-induced neuritogenesis, since the SU5402 inhibitor became toxic to the CGNs at the high concentration (80 µM) (Fig. 7C).

We also compared the neuritogenic effect of 9.77 µM nectide in CGNs transfected with either an empty vector or dnFGFR1 and found that nectide-induced neurite outgrowth was abrogated when the CGNs were transfected with dnFGFR1 (Fig. 7D).

Nectide also activated FGFR in a similar manner as nectin-1 Ig3 in TREX-293 cells, although with much lower potency compared with the effect of nectin-1 Ig3 (Fig. 8A). As a negative control we tested if the control peptide with a reverse sequence (Rev nectide) could activate FGFR in a similar manner as nectide. The control peptide did not have any effect on FGFR1c phosphorylation (Fig. 8B). As for nectin-1 Ig3, we analyzed the expression of total FGFR1c and actin in all of the samples and found that all of the lysates contained similar amounts of total FGFR and actin, which excludes the possibility that the observed differences in FGFR phosphorylation are caused by variations in nectide-induced FGFR expression or sample immunoprecipitation (Fig. 8A and 8B).

Finally, we tested the effect of nectide on CGN survival and found that, similar to nectin-1 Ig3, the peptide promoted neuronal survival at a broad range of concentrations (0.04-3.26 µM) (Fig. 8C).

To conclude, the nectide-peptide mimics the effects of nectin-1 Ig3 on FGFR activation, neurite outgrowth, and neuronal survival. Higher concentrations of the peptide are required to obtain effects similar to nectin-1 Ig3.

**DISCUSSION**

Nectin-1 plays an important role during development of the CNS and mutation in the nectin-1 gene can cause mental retardation in severe cases (13). The extracellular region of nectin-1 consists of three Ig modules (1) with the first Ig module being involved in trans-interaction with nectin-3 (14) and the second Ig-module in the formation of cis-dimers (15), but the function of the third Ig-module is currently unknown. We hypothesized that the function of the third Ig-module of nectin-1 is stimulation of axon growth and promotion of survival of immature neurons through interaction with FGFR.

We solved the structure of nectin-1 Ig3 in solution and found that this module belongs to the C1-set subgroup of Ig-like domains. It is the first structural determination of the third module of nectin in solution. The C1 Ig module type consists of seven β-strands arranged into two antiparallel sheets: one contains strands A, B, D, and E, and the other contains strands G, F and C (see Fig. 1A). The C’ strand is absent; it is found only in molecules related to antigen recognition molecules such as the constant modules of the immunoglobulins,
the membrane proximal modules of major histocompatibility complex class I and class II antigens, and in β2 microglobulin (45,46). Recently, successful crystallization of the ectodomain of nectin-1 and nectin-2 has been reported, diffraction data for the heavy-atom derivatives have been prepared for phase determination (47) and the structure of the whole extracellular region of nectin-1 has been solved by X-ray crystallography (33). The backbone structures of mouse nectin-1 Ig3 in solution (see Fig. 1A) and human nectin-1 Ig3 in the crystal (see Fig. 1B) have very similar folding topologies.

We found that nectin-1 Ig3 induced neurite outgrowth in hippocampal neurons and CGNs, an effect abolished by the FGFR inhibitor SU5402 and expression of dnFGFR1 in CGNs. Nectin-1 Ig3 as well as the soluble ectodomain of nectin-1 induced FGFR phosphorylation. The neuritogenic effects of nectin-1 were observed when nectin-1 Ig3 was applied to sparsely seeded, single-cell cultures of dissociated primary neurons that were not involved in cell-cell contacts/adhesion, and the present results suggest that neurite outgrowth induction occurred through a direct interaction between the recombinant protein and FGFR-expressing neurons. Thus, the interaction in trans with neuronal FGFR likely triggered the neuritogenic response. There were some variations in the magnitude of the neurite outgrowth response induced by nectin-1 Ig3 in hippocampal neurons and CGNs, with the most marked response in hippocampal neurons at the concentration of 10 µM. This difference in neuritogenic response with lower sensitivity of CGNs has been observed before (48), and is probably explained by variations in the quantity of expressed FGFR isoforms in the two types of neurons, which might indicate an importance of the type and/or developmental stage of neurons.

Numerous CAMs such as NCAM, L1, N-cadherin, neurofascin, and neuropilin (19,34,35), which all signal through the activation of FGFR, also display neuritogenic properties. All of these CAMs have fairly different downstream signaling mechanisms that involve their specific cytoplasmic domains. The common feature is the use of FGFR signaling through a direct interaction with this receptor. Thus, the association with this receptor probably determines the neuritogenic properties of these neural CAMs.

We ex silico identified a sequence within nectin-1 Ig3 that binds to FGFR and termed this peptide sequence nectide. The binding of nectide to FGFR resembles that of nectin-1 Ig3, both qualitatively and quantitatively. Namely, it binds the isoforms of FGFR, stimulates receptor phosphorylation, induces a strong neuritogenic response in an FGFR activation-dependent manner, and promotes neuronal survival. However, the nectide peptide had a lower potency compared with the effects observed with the nectin-1 Ig3 protein. Peptides usually have no stabilized tertiary structure. Thus, high peptide concentrations are needed to mimic the effects of proteins that contain the same binding motifs. The difference in potency may also indicate that the peptide motif comprises only part of the authentic binding site of the protein.

In early studies, the function of nectin-1 and -3 has largely been associated with the mechanical adhesion site termed PAJs involved in formation of synapses in the hippocampus of CNS (7-9). Recent studies have revealed that nectin-1 and -3 participate in synaptic remodeling by the orchestrated regulation of sheddases including ADAM10 and γ-secretase in neurons (49,50). Nectin-1 shedding and presenilin-dependent secretase-mediated intramembrane cleavage occur at synapses in conditions promoting synaptic plasticity in the brain by regulating maintenance of dendritic spine density (50,51). Our study showed that the soluble nectin-1 ectodomain as well as nectin-1-Ig3 and nectide peptide phosphorylate FGFR in TREX-293 cells, and we suggest that shed nectin-1 can induce neurite outgrowth by the direct binding and activation of FGFRs. It is also possible that both the soluble ectodomain of nectin-1 and the membrane-bound nectin-1 can bind and activate FGFR. One can assume the conditions when nectin-3 molecules expressed on one cell ligate/cluster nectin-1 molecules carrying bound FGFR molecules expressed on the opposed cell thereby enriching FGFR concentration in a small volume and promoting its dimerization and activation.
Nectin-1 binds and signals through FGFR

leading to neuronal differentiation and survival in the developing synapse. A similar mechanism has previously been suggested for NCAM-induced FGFR phosphorylation (52).

Nectin-1 has three isoforms, α, β, and γ (1). Nectin-1γ is a secreted isoform, which does not contain the transmembrane and cytoplasmic region. At present no biological role is assigned to nectin-1γ. Our results suggest that under conditions promoting neuronal plasticity, nectin-1γ can be involved in the induction of neurite outgrowth (and probably also in changes in dendritic spine morphology) by the direct binding and activation of FGFRs. It has been suggested that the nectin intracellular domain containing a presumptive nuclear localization signal (RRRH) right after the transmembrane domain and released by γ-secretase may act either as a transcriptional stimulator or repressor (50). Thus, shed nectin-1 ectodomain may not only be related to the induction of neuritogenesis, but also indirectly to the regulation of gene expression through release of the presumptive nuclear localization signal.

Thus, we identified a novel FGFR ligand, nectin-1, and showed that the third Ig module of nectin-1 can directly bind and activate FGFR, thereby inducing neurite outgrowth and promoting neuronal survival. We also solved the structure of nectin-1 Ig3 and identified a peptide motif, nectide that mimics the functional properties of this module. Our data strongly suggest that the third Ig module of nectin-1 not only plays a structural role, but also has a specific function, namely interaction with and activation of FGFR, thus implicating this receptor as a signaling partner of nectin-1.

REFERENCES
Nectin-1 binds and signals through FGFR


FOOTNOTES

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1To whom correspondence should be addressed. E-mail: berezin@sund.ku.dk
2The abbreviations used are: Ig, immunoglobulin; CAMs, cell-adhesion molecules; PAJs, puncta adherentia junctions; nectin-1 Ig3, the soluble third immunoglobulin-domain of nectin-1; NMR, nuclear magnetic resonance; CGNs, cerebellar granule neurons; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; dnFGFR1, dominant negative FGFR1; SPR, surface plasmon resonance; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; IGF-1, insulin-like growth factor 1; Revnectide, reverse nectide.
3 Unpublished data; Bojesen, K., Christensen, C.
FIGURE LEGENDS:

FIGURE 1. Backbone models of mouse nectin-1 Ig3 calculated by NMR (A) and human nectin-1 Ig3 solved by X-ray crystallography (B). (A) A single model from an ensemble of 20 models is shown (PDB accession number 2L7J). Sheets are sandwiched with yellow strands A, B, E, and D on one side and green strands G, F, and C on the other side. This composition of strands makes the third Ig module of nectin-1 a C1-set subgroup Ig-module. (B) A model was made based on the crystal structure of the whole ectodomain of human nectin-1 (PDB accession number 3ALP (32)). (C) HNCACB-CBCA(CO)HN Overlay showing an example from the sequential assignment of the backbone of nectin-1 Ig3 with signals from CBCA(CO)NH spectrum in gray and CA and CB signals from HNCACB spectrum in green and red, respectively. (D) HSQC of nectin-1 Ig3. (C-D) For detailed chemical shifts please see BMRB Entry 17358.

FIGURE 2. Nectin-1 Ig3 (N1-Ig3) induces neurite outgrowth in an FGFR activation-dependent manner. (A-B) Cultures were grown for 24 h in the presence of 0.09, 0.43, 2.17, 10.87, and 54.37 µM nectin-1 Ig3. (A) Effect of nectin-1 Ig3 on neurite outgrowth in hippocampal neurons and (B) on CGNs. (A-B) Results from four or six independent experiments are expressed as percentage ± SEM of unstimulated controls set at 100%. *p < 0.05, **p < 0.01, ***p < 0.001 compared with untreated control. (C-D) Effects of the fibroblast growth factor receptor (FGFR) inhibitor SU5402 and expression of a dominant-negative FGFR1 on nectin-1 Ig3 (N1-Ig3)-induced neurite outgrowth. Cultures were grown for 24 h. (C) CGNs treated with different concentrations of SU5402 (20, 40, and 80 µM) and 0.87 µM nectin-1 Ig3 (black line) or medium only (dotted line). (D) CGNs transiently transfected with a dominant-negative construct of FGFR1 (D.N.) or an empty plasmid (Empty), plated on a monolayer of L-929 cells, and treated with 0.87 µM nectin-1 Ig3 (gray bars) or medium only (white bars). (C-D) Results from 6-8 independent experiments are expressed as percentage ± SEM of unstimulated control set at 100%. ***p < 0.001, compared with untreated control. + p < 0.05, ++ p < 0.01, +++ p < 0.001, compared with nectin-1 Ig3-treated cultures.

FIGURE 3. Surface plasmon resonance (SPR) analysis of the binding between the combined Ig2-Ig3 modules of various FGFR isoforms and FGF1 or nectin-1 Ig3 (N1-Ig3). FGF1 or nectin-1 Ig3 was immobilized on a sensor chip, and the FGFR isoforms FGFR1b, FGFR1c, FGFR2c and FGFR3c were injected at the specified concentrations. Binding is expressed as the differential response (RU, resonance units) between the binding to the sensor chip with immobilized FGF1 or nectin-1 Ig3 and a blank sensor chip. At least 2 independent experiments were carried out for each receptor isoform. (A-D) Fitting curves of binding between FGF1 and various concentrations of the FGFR isoforms. (E-H) Fitting curves of binding between nectin-1 Ig3 and various concentrations of the FGFR isoforms.

FIGURE 4. Effects of nectin-1 Ig3 (N1-Ig3) and FGF2 and nectin-1 ectodomain on FGFR1c phosphorylation and of nectin-1 Ig3 (N1-Ig3) on the survival of CGNs induced to undergo apoptosis. (A-B) TREX-293 cells transfected with FGFR1c that contain a C-terminal StrepII tag were treated with 0.3 nM FGF2, nectin-1 Ig3 (A) or ectodomain of nectin-1 (B) at the specified concentrations. FGFR1c was subsequently immunopurified using antibodies against anti-phosphotyrosine and analyzed by Western blotting using antibodies against the StrepII tag. Results of four independent experiments are expressed as percentage ± SEM of the amount of phosphorylated FGFR1c with phosphorylated FGFR1c in unstimulated cells set at 100%. *p < 0.05, **p < 0.01, ***p < 0.001, compared with untreated control. (C-D) CGNs were left to differentiate for 7 days in the presence of high potassium (40 mM) before apoptosis was induced by changing to a low-potassium medium (5 mM). Cell survival was estimated 48 h later. (C) Cultures grown in 40 mM KCl, 5 mM KCl, or 5 mM KCl and 50 ng/mL IGF-1. (D) Cultures grown in 5 mM KCl in the presence of 0.02, 0.09, 0.43, 2.17, and 10.87 µM nectin-1 Ig3. (C-D) Results from at least four independent experiments are
expressed as percentage ± SEM with unstimulated controls (5 mM KCl) set at 100%. **p < 0.01, ***p < 0.001, compared with untreated CGNs induced to undergo apoptosis.

FIGURE 5. Structure of the nectide motif. (A) Alignment of the sequences of various peptide ligands of FGFR. (B) Location of the nectide motif in nectin-1 Ig3.

FIGURE 6. Surface plasmon resonance (SPR) analysis of the binding between the combined Ig2-Ig3 modules of various FGFR isoforms and nectide. Nectide was immobilized on a sensor chip, and the FGFR isoforms FGFR1b, FGFR1c, FGFR2c and FGFR3c were injected at the specified concentrations. Binding is expressed as the differential response (RU, resonance units) between the binding to the sensor chip with immobilized nectide and a blank sensor chip. 1-2 experiments were carried out for each receptor isoform. (A-D) Fitting curves of binding between nectide and various concentrations of the FGFR isoforms.

FIGURE 7. Effect of nectide on neurite outgrowth in CGNs. Cultures were grown for 24 h. (A) Dose-response relationship of the effect of 0.04, 0.12, 0.36, 1.19, 3.26, 9.77, and 29.31 µM nectide. (B) The neurotrophic effect of 29.31 µM nectide or a reversed version of nectide (Rev nectide). (A-B) Results from four or six independent experiments are expressed as percentage ± SEM of unstimulated controls set at 100%. *p < 0.05, **p < 0.01, compared with untreated control. p < 0.05, compared with nectide-treated control. (C) Effect of the FGFR inhibitor SU5402 on nectide-induced neurite outgrowth. CGNs were treated with different concentrations of SU5402 (20, 40, and 80 µM) and 9.77 µM nectide (black line) or medium only (dotted line). (D) Effect of expression of a dominant-negative FGFR1 on nectide-induced neurite outgrowth. CGNs were transiently transfected with a dominant-negative construct of FGFR1 (D.N.) or an empty plasmid (Empty), plated on a monolayer of L-929 cells, and treated with 9.77 µM nectide (gray bars) or medium only (white bars). (C-D) Cultures were grown for 24 h. Results from 6-8 independent experiments are expressed as percentage ± SEM of unstimulated control set at 100%. **p < 0.01, ***p < 0.001, compared with untreated control. +p < 0.05, +++p < 0.001, compared with nectide-treated control.

FIGURE 8. Effects of nectide, Rev Nectide and FGF2 on FGFR1c phosphorylation and of nectide on the survival of CGNs induced to undergo apoptosis. (A-B) TREX-293 cells transfected with FGFR1c containing a C-terminal StrepII tag were treated with 0.3 nM FGF2 or nectide at the specified concentrations (A) or the negative control Rev nectide (B). FGFR1c was subsequently immunopurified using antibodies against anti-phosphotyrosine and analyzed by Western blotting using antibodies against the StrepII tag. Results of four independent experiments are expressed as percentage ± SEM of the amount of phosphorylated FGFR1c with phosphorylated FGFR1c in unstimulated cells set at 100%. **p < 0.01, ***p < 0.001, compared with untreated control. (C) CGNs were left to differentiate for 7 days in a high-potassium medium (40 mM) before apoptosis was induced by changing to a low-potassium medium (5 mM). Cell survival was estimated 48 h later. Cultures grown in 5 mM KCl in the presence of 0.001, 0.012, 0.04, 0.12, 0.36, 1.09, 3.26, and 9.71 µM nectide. Controls are the same as those described for nectin-1 Ig3 in Fig. 4C. Results from at least four independent experiments are expressed as percentage ± SEM with unstimulated controls (5 mM KCl) set at 100%. **p < 0.01, ***p < 0.001, compared with untreated CGNs induced to undergo apoptosis.
Table 1. Data collection and structural statistics of the third Ig module of nectin-1.

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Table 2. Resonance assignments of 15N HSQC spectra of nectin-1 Ig3.
The table presents the differences from peaks showing double signals measured in changes in chemical shifts. Chain A follows the peaks with the higher intensity of approximately 60% whereas Chain B follows the peaks of the lower intensity of approximately 40%. Part of the chain is identical and there is only one chain in this part of the sequence. G253, G256, N297 and S309 were not assigned (NA).

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Table 3. Binding affinities for the interaction between FGF1 and nectin-1 Ig3, respectively, and Ig2-3 modules of various FGFR isoforms.
FGF1 or nectin-1 Ig3 was immobilized on a sensorchip by amine coupling and Ig2-3 modules of FGFR isoforms were injected into the chip at various concentrations. The estimation of kinetic parameters was performed as described under Experimental Procedures. The equilibrium dissociation constant (K_D) of the complexes was calculated as k_d/k_a. For each concentration two injections were made in each experiment. All constants are expressed as mean ± SEM and \(\chi^2\) indicate the quality of the fit.

<table>
<thead>
<tr>
<th>Immobilized FGFR</th>
<th>FGF1 K_D ± SEM (M)</th>
<th>(\chi^2)</th>
<th>Nectin-1 Ig3 K_D ± SEM (M)</th>
<th>(\chi^2)</th>
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<td>FGFR1b</td>
<td>1.02×10^{-7} ± 0.10×10^{-7}</td>
<td>3.37</td>
<td>7.76×10^{-8} ± 5.84×10^{-8}</td>
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<tr>
<td>FGFR1c</td>
<td>2.07×10^{-7} ± 0.59×10^{-7}</td>
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<td>FGFR2c</td>
<td>2.07×10^{-7} ± 1.79×10^{-7}</td>
<td>1.43</td>
<td>1.79×10^{-8} ± 0.07×10^{-8}</td>
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<td>FGFR3c</td>
<td>7.74×10^{-8} ± 2.80×10^{-8}</td>
<td>15.68</td>
<td>1.18×10^{-7} ± 0.39×10^{-7}</td>
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Table 4. Binding affinities for the interaction between the nectide peptide and Ig2-3 modules of various FGFR isoforms.
Nectide was immobilized on a sensorchip by amine coupling and Ig2-3 modules of FGFR isoforms were injected into the chip at various concentrations. The estimation of kinetic parameters was performed as described under Experimental Procedures. The equilibrium dissociation constant (K_D) of the complexes was calculated as k_d/k_a. For each concentration two injections were made in each experiment. Constants calculated from more than one binding experiment are expressed as mean ± SEM and \(\chi^2\) indicate the quality of the fit.

<table>
<thead>
<tr>
<th>Immobilized FGFR</th>
<th>Nectide K_D ± SEM (M)</th>
<th>(\chi^2)</th>
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</thead>
<tbody>
<tr>
<td>FGFR1b</td>
<td>*2.01×10^{-8}</td>
<td>15.43</td>
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<td>FGFR1c</td>
<td>*1.72×10^{-7}</td>
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<td>FGFR2c</td>
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<td>FGFR3c</td>
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*Results from one binding experiment are shown
Fig. 3

A

FGFR1b (µM)
1: 3.81
2: 1.90
3: 0.95
4: 0.48
5: 0.24
6: 0.12

Binding to FGFR (RU)

Time (s)

0 50 100 150 200 250 300 350

B

FGFR1c (µM)
1: 15.23
2: 7.62
3: 3.91
4: 1.90
5: 0.48
6: 0.24

Binding to FGFR (RU)

Time (s)

0 50 100 150 200 250 300 350

C

FGFR2c (µM)
1: 1.90
2: 0.95
3: 0.48
4: 0.24
5: 0.12

Binding to FGFR (RU)

Time (s)

0 50 100 150 200 250 300 350

E

FGFR1b (µM)
1: 1.90
2: 0.95
3: 0.48
4: 0.24
5: 0.12
6: 0.06

Binding to N-αι3 (RU)

Time (s)

0 50 100 150 200 250 300 350

F

FGFR1c (µM)
1: 7.62
2: 3.81
3: 1.90
4: 0.95
5: 0.48

Binding to N-αι3 (RU)

Time (s)

0 50 100 150 200 250 300 350

G

FGFR2c (µM)
1: 0.95
3: 0.48
4: 0.24

Binding to N-αι3 (RU)

Time (s)

0 50 100 150 200 250 300 350
Fig. 4

A

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B

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Fig. 6

A

B

C

D

FGFR1b (µM)

1: 1.90
2: 0.95
3: 0.48
4: 0.24
5: 0.12
6: 0.06

FGFR2c (µM)

1: 1.90
2: 0.95
3: 0.48
4: 0.24

FGFR1c (µM)

1: 7.62
2: 3.81
3: 1.90
4: 0.95
5: 0.48

FGFR3c (µM)

1: 1.90
2: 0.95
3: 0.48
4: 0.24
Fig. 8

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C

Survival (% of control)

Nectide (μM)

0 0.001 0.01 0.1 1 10 100
Nectin-1 binds and signals through the fibroblast growth factor receptor
Kirsten Borup Bojesen, Ole Clausen, Kristian Rohde, Claus Christensen, Lanjun Zhang, Shizhong Li, Lene Kohler, Steen Nielbo, Janne Nielsen, Michelle Denise Gjorlund, Flemming M. Poulsen, Elisabeth Bock and Vladimir Berezin

J. Biol. Chem. published online September 5, 2012

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