Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 322 (2004) 186–196
BBRC
www.elsevier.com/locate/ybbrc

Tyrosine 734 of NCAM180 interferes with FGF receptor-dependent signaling implicated in neurite growth

Simone Diestel[a], Christine Laurini[a], Otto Traub[b], Brigitte Schmitz[a,*]

[a] Department of Biochemistry, Institute of Physiology, Biochemistry and Animal Health, Katzenburgweg 9a, 53115 Bonn, Germany
[b] Department of Molecular Biology, Genetics Institute, University of Bonn, Römerstr. 164, 53117 Bonn, Germany

Received 1 July 2004

Abstract

The cytoplasmic domain of the neural cell adhesion molecule (NCAM) contains multiple phosphorylation sites. We report here that in addition to serine and threonine residues a tyrosine of the NCAM180 isoform is phosphorylated as shown by phosphoamino acid analysis. Exchange of the only cytoplasmic tyrosine at position 734 of human NCAM180 (NCAM180-Y734F) to phenylalanine resulted in increased neurite outgrowth of NCAM180-Y734F transfected B35 neuroblastoma cells compared to NCAM180-wt transfectants on poly-L-lysine as substrate. As demonstrated by inhibitor studies the increased neurite outgrowth was due to higher FGF receptor 1 and ERK1 activity in NCAM180-Y734F cells, indicating that tyrosine residue 734 plays a role in signal transduction mediated by the FGF receptor. On an NCAM expressing monolayer of COS-7 cells the Y734F mutation also influences FGF receptor 1 dependent neurite outgrowth, but under these conditions additional mechanisms seem to be responsible for the increased neurite length observed for NCAM180-Y734F transfected cells.

The neural cell adhesion molecule (NCAM) is a member of the immunoglobulin (Ig) superfamily that mediates Ca^{2+} independent cell–cell adhesion. NCAM is widely expressed in neurons throughout the central and peripheral nervous system [1], where it plays a pivotal role in neural development and synaptic plasticity [2–4]. Alternative splicing results in expression of three major isoforms, the 180- and 140-kDa transmembrane forms and a 120 kDa isoform that is attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor [5,6]. The NCAM-140 and NCAM-180 kDa isoforms differ from each other in additional 261 amino acids in the cytoplasmic tail of the NCAM-180 kDa isoform encoded by an alternatively spliced exon. The three isoforms differ in their functions and expression patterns. NCAM140 is primarily localized in migratory growth cones of immature neurons, whereas NCAM180 is mainly expressed in postsynaptic densities of mature neurons and at sites of cell–cell contact [7]. The 261 amino acid insert of NCAM180 is responsible for the interaction of this isoform with the cytoskeletal linker protein spectrin [8,9]. Sytnyk et al. [10] showed that NCAM180 is one of the initial proteins that stabilize intracellular organelles at nascent synapses through the linker protein spectrin. The NCAM120 isoform is mainly expressed in glial cells [11].

The extracellular domain of NCAM mediates homophilic NCAM–NCAM or heterophilic binding to other glycoproteins implying that NCAM has different functional activities as a result of time and space dependent expression of NCAM itself and its binding partners [12].

An early study showed serine and threonine phosphorylation of chick NCAM [13]. Later it has been

* Corresponding author. Fax: +49 228 737938.
E-mail address: schmitz@uni-bonn.de (B. Schmitz).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.07.100

demonstrated by several groups that homophilic and heterophilic extracellular interactions of NCAM can activate several intracellular signaling pathways implicated amongst others in NCAM mediated neurite outgrowth.

One of these pathways is FGF receptor dependent and mediates neurite outgrowth through activation of phospholipase C-γ and diacylglycerol (DAG) lipase leading to release of inositol-trisphosphate (IP_3) and arachidonic acid. These events result in Ca^{2+} entry into cells through activation of N- and L-type Ca^{2+} channels and an IP_3 induced Ca^{2+} release from intracellular stores (reviewed in [14]). Recently it has been shown that the interaction of NCAM with the FGF receptor is mediated directly by the fibronectin type III repeats of NCAM and the Ig modules 2 and 3 of the FGF receptor 1 [15]. The activated FGF receptor is additionally able to activate the MAPK pathway via Ras, Raf, MEK, and ERK1/2 [16].

In addition to the above described FGF receptor signaling cascade which can be activated by NCAM140 and NCAM180 it has become clear in the last years that NCAM140 is able to activate the MAPK pathway in a fyn/FAK dependent manner. Beggs et al. demonstrated that a fraction of the nonreceptor tyrosine kinase fyn is constitutively associated with the NCAM140 isoform. Clustering of NCAM at the cell surface by NCAM-specific antibodies resulted in an autophosphorylation of associated fyn molecules, leading to recruitment of the focal adhesion kinase (FAK) to the complex and autophosphorylation of FAK [17]. Subsequently, the MAPKs ERK1 and ERK2 become activated leading to phosphorylation of CREB and induction of c-fos [18].

NCAM is also capable of activating the PI3K/Akt pathway and PKC mediated mechanisms [19,20]. The transcription factor NF-κB has been identified as another downstream effector molecule of NCAM [21].

The cytoplasmic domain of NCAM has been shown to be palmitoylated at four cysteine residues near the transmembrane domain thus providing a second plasma membrane anchor which is responsible for its lipid raft association [16,22]. The localization in different membrane subcompartments seems to be important for NCAM mediated signal transduction pathways. Niethammer et al. [16] presented a model in which lipid raft associated NCAM triggers the MAPK pathways upon homophilic activation via the raft associated kinase fyn, whereas the non-lipid raft fraction of NCAM activates the FGF receptor leading to an intracellular Ca^{2+}-elevation and also to an ERK1/2 activation [16,23].

Here we show that the NCAM180 isoform is tyrosine phosphorylated and that exchange of tyrosine 734 to phenylalanine of human NCAM180 plays a major role in the FGF receptor dependent NCAM mediated non-stimulated neurite outgrowth and a minor role in stimulated neurite outgrowth.

Materials and methods

Reagents. The human NCAM180 cDNA was a gift from F. Howell (London, UK). Hybridoma cells producing either monoclonal antibody 123C3 against human NCAM or monoclonal antibody H28 against murine NCAM were kindly provided by R. Michalides (Amsterdam, The Netherlands) and C. Goridis (Marseille, France), B35 neuroblastoma cells by P. Maness (North Carolina, USA), and COS-7 cells transfected with the human NCAM140 full length cDNA (COS/140) by R. Gerardy-Schahn (Hannover, Germany). The FGF receptor 1 inhibitor PD 173074 was a gift from S. Skaper (GlaxoSmithKline, Harlow, UK). The primers were purchased at MWG Biotech (Ebersbach, Germany), the eukaryotic expression plasmid pCDNA3 and LipofectAMINE PLUS were from Invitrogen (Karlsruhe, Germany). Pfu polymerase was from Stratagene (La Jolla, USA), TriStar reagent was from Hybaid AGS (Heidelberg, Germany), and AMV reverse transcriptase and the MEK inhibitor U0126 were from Promega (Mannheim, Germany). Geneticin was obtained from PAA Laboratories (Cölbe, Germany) and Opti-MEM I was from Invitrogen (Karlsruhe, Germany). [^{32}P]Orthophosphate was from Amersham Biosciences. Phospho-specific ERK1/2 antibodies were from Cell Signaling Technology (Frankfurt, Germany), ERK1 and ERK2 antibodies were from BD Biosciences (Heidelberg, Germany) and fyn antibodies from Santa Cruz (California, USA). Secondary goat anti-mouse, anti-rat or anti-rabbit antibodies conjugated to horseradish peroxidase or Cy3 were from Dianova (Hamburg, Germany). Nitrocellulose and PVDF membranes were from Schleicher & Schüll (Dassel, Germany), the TLC (thin-layer-chromatography)-cellulose plates were from Macherey–Nagel (Düren, Germany), and the Bio-Rad D_c Protein Assay was from Bio-Rad (München, Germany). RIPA-lysis buffer and phosphate-free RPMI 1640 medium were purchased from CC Pro (Neustadt/W, Germany), Roti-Block was from Carl Roth (Karlsruhe, Germany), and SuperSignal Substrate Kit was from Perbio Science (Bonn, Germany). DiI was obtained from Molecular Probes (California, USA). Permafluor was from Beckman–Coulter (Krefeld, Germany). All other reagents were of the highest quality and obtained from Sigma (Taufkirchen, Germany).

Construction of mutated NCAM180 cDNA. For cloning the wild type human NCAM180 cDNA into the mammalian expression vector pcDNA3 a primer pair was designed to frame the coding region of NCAM with *Bam*HI linkers (forward: 5′-CGC GGA TCC ATG CTG CAA ACT AAG GAT CTC-3′, reverse: 5′-CGG GAT CCC GTC ATG CTT TGC TCT CGT TCT C-3′). A PCR using these primers, human NCAM180 cDNA (without VASE exon) as a template, and Pfu polymerase (2.5 U/μL) to exclude amplification errors was performed. Site-specific mutagenesis was carried out using the overlap extension PCR method with pcDNA3/NCAM180-wt as a template. To create the NCAM180-Y734F mutant the sense primer 5′-GGA CAT CAC CTG CT***T*** CTT CCT GAA CAA GTG TGG-3′ and the antisense primer 5′-CCA CAC TTG TTC AGG AAG ***A***AG CAG GTG ATG TCC-3′ were used (sequence differences to NCAM180-wt are given in bold letters). The coding regions of both constructs (NCAM180-wt- and NCAM180-Y734F-cDNA) were checked for correctness by direct DNA sequencing (MWG Biotech, Ebersbach, Germany).

Cell culture conditions and transfection of B35 cells. The B35 neuroblastoma cell line was derived from rat CNS [24]. The cells express very low levels of endogenous NCAM as checked by indirect immunofluorescence (not shown). B35 cells and COS-7 cells (further termed COS cells) were maintained in DMEM (4500 mg glucose/L) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, Neuro-2a neuroblastoma cells in RPMI 1640 medium

with 10% fetal calf serum and 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were grown at 37 °C in a moist atmosphere of 5% CO_2. B35 and Neuro-2a cells were plated on 0.01% poly-L-lysine (pLL) coated plastic dishes.

For transfection B35 cells were grown to 60% confluence and transfected with 10 μg plasmid DNA and LipofectAMINE PLUS reagent according to manufacturer's recommended conditions. Forty-eight hours after transfection the cells were grown in geneticin-supplemented selective medium (500 μg/mL). Individual clones were picked and cultivated in selective medium for further analysis.

Phosphoamino acid analysis. Neuro-2a cells were grown on pLL coated plastic dishes to 70–80% confluence and incubated in phosphate-free RPMI 1640 medium supplemented with 10% FCS (dialyzed against 10 mM Tris, pH 7.5, 0.15 M NaCl) for 1 h. Labeling was carried out in the presence of 100 μCi/mL [^{32}P]orthophosphate for 4 h. After washing twice with PBS (4 °C), cells were lysed in RIPA-lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, and pepstatin (each 1 μg/mL) as well as 10 mM Na_3VO_4 and 40 mM NaF to inhibit tyrosine phosphatases and subjected to immunoprecipitation with H28 antibody followed by SDS–PAGE and transfer onto PVDF membranes. The band corresponding to NCAM180 was excised and hydrolyzed with 5.7 N HCl for 30 min. The amino acids were separated two-dimensionally on TLC-cellulose plates and identified by autoradiography and by visualization of co-migrating unlabeled phosphoamino acids with 0.2% ninhydrin [25].

Indirect immunofluorescence analysis. Cells were grown on pLL coated coverslips to 70–80% confluence, washed twice with PBS, fixed with 4% formaldehyde for 10 min at room temperature, and washed again. After blocking with 5% horse serum in PBS for 15 min the cells were incubated with protein G–Sepharose purified 123C3 antibody (45 μg/mL in blocking solution) for 30 min at room temperature. After three washing steps with blocking solution, incubation with Cy3-conjugated secondary antibody (goat anti-mouse, 1:500 in blocking solution) was carried out for another 30 min at room temperature. Finally, the cells were washed three times with PBS, once briefly dipped into demineralized water, and embedded in Permafluor.

Western blot analysis. Cells were grown on pLL coated plastic dishes to 70–80% confluence and harvested in RIPA-lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After 20 min incubation at 4 °C cell lysates were centrifuged at 20,000*g* and 4 °C. The protein concentration was determined using the Bio-Rad D_c Protein Assay according to the manufacturer's instructions. Lysates were heated in sample buffer 5 min at 95 °C, submitted to SDS–PAGE, and transferred to nitrocellulose membranes. The blocking, washing, and antibody incubation steps were all carried out with Roti-Block at room temperature. After blocking for 2 h, incubation with 123C3 antibody (2.25 μg/mL) was performed overnight followed by two washing steps. The first antibody was detected by incubation of the membrane with horseradish peroxidase-conjugated goat anti-mouse antibodies (1:5000). After four further washing steps bound antibodies were visualized by enhanced chemiluminescence using the SuperSignal Substrate Kit. The MAPK activation was analyzed under the same conditions using phospho-ERK1/2 specific antibodies (1:2000). Membranes were stripped and reprobed in the same manner with ERK1 and ERK2 specific antibodies (1:5000, respectively) to detect the total amount of ERK protein.

Reverse transcription-PCR. Cells were grown to 70–80% confluence and total RNA was isolated using TriStar reagent according to the manufacturer's instructions. Two microgram of RNA template was incubated at 42 °C with AMV reverse transcriptase to generate cDNA using 20 pmol of the FGF receptor 1- or β-actin-specific reverse primer, respectively. The following PCR amplification was performed on cDNA obtained by reverse transcription with 20 pmol of each primer in the volume of 50 μL. The FGF receptor 1-forward primer: 5′-GGC GTG CAC ACA GGA TGG TCC-3′ and FGF receptor 1-reverse primer: 5′-CGA GCT AGG CCA AAG TCT GCG-3′ were used to amplify a 290 bp fragment of the cDNA corresponding to the FGF receptor 1 mRNA. As an internal standard intron spanning β-actin specific primers were used (β-actin-forward primer: 5′-CGT GGG CCG CCC TAG GCA CCA G-3′ and β-actin-reverse primer: 5′-TTG GCC TTA GGG TTC AGG GGG G-3′) which amplify a cDNA fragment of 243 bp and additional 330 bp if the sample is contaminated with genomic DNA. The following amplification program was used to amplify the cDNAs: initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C denaturation of 1 min, 58 °C annealing for 1 min, and 72 °C extension for 90 s. The final extension was for 10 min at 72 °C. PCR-products were separated on 2% agarose gels.

Neurite outgrowth assays. For measurement of cell autonomous neurite outgrowth the B35 cells were plated on pLL coated plastic dishes as single cells (7.5×10^3 cells/60 mm dish). Neurite outgrowth on monolayers of COS cells transfected with a full length NCAM-140 cDNA (COS/140) and on untransfected COS cells, respectively, was determined by using B35 cells labeled with DiI prior to plating. NCAM expression of COS/140 cells was confirmed by indirect immunofluorescence analysis (not shown). For labeling with DiI the cells were washed twice with $HBSS^-$ and incubated with 10 μM DiI in isotonic glucose solution for 20 min, then rinsed twice with isotonic glucose solution, treated with 0.05% trypsin, and plated as single cells on the COS or COS/140 monolayer, respectively.

Twenty-four hours after seeding of the B35 cells the cultures were rinsed once with $HBSS^-$ and the medium was replaced by Opti-MEM I containing 1 mM dibutyryl cAMP (dbcAMP), dissolved in DMSO. The MEK inhibitor U0126 and the FGF receptor I inhibitor PD 173074 were also dissolved in DMSO and added 3 hours after seeding of the B35 cells. Control cells always received the same concentration of DMSO. Measurement was performed 22–24 h. after changing the medium to Opti-MEM I. Only neurites longer than 5 μm and not in contact with other B35 cells were measured using the KS100 software (Zeiss, Oberkochen, Germany).

Isolation of lipid raft microdomains. Cells were grown to 70–80% confluence, washed twice with PBS (4 °C), lysed in extraction buffer (25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 2% Triton X-100), and incubated on ice for 30 min. The extracts were adjusted to 40% sucrose by adding equal volumes of 80% sucrose in extraction buffer and placed into an ultracentrifuge tube. A step gradient of 35%, 30%, 25%, 20%, and 10% sucrose was layered on top of the lysate. The gradients were centrifuged for 7 h at 218,000*g* (Beckman, SW 60 Ti rotor, 4 °C). The fractions were harvested and an acetone precipitation was carried out. The pellets were solubilized in sample buffer and subjected to Western blot analysis as described above. As a marker protein for lipid rafts fyn was used (antibody concentration in Western blot analysis: 1:1000).

Results

Phosphoamino acid analysis of NCAM180-wt

To investigate the phosphorylation of NCAM180, NCAM was immunoprecipitated from Neuro-2a cells with monoclonal NCAM antibody H28, the 180 kDa band corresponding to NCAM180 excised from the membrane and submitted to phosphoamino acid analysis. Staining of co-migrating reference phosphoamino acids revealed [^{32}P]phosphate labeled serine, threonine, and tyrosine residues (Fig. 1), indicating that the only cytoplasmic tyrosine residue at position 734 might be phosphorylated.

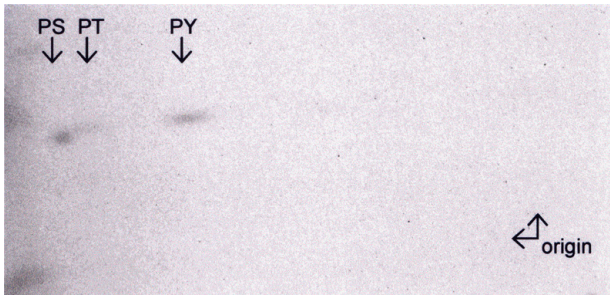

Fig. 1. NCAM180 is phosphorylated at tyrosine in addition to serine and threonine. The autoradiograph of the phosphoamino acid analysis of NCAM180-wt is shown. PS, phospho-serine; PT, phospho-threonine; and PY, phospho-tyrosine, the experiment was repeated twice.

Exchange of the cytoplasmic tyrosine residue and expression of NCAM180-wt and NCAM180-Y734F in B35 neuroblastoma cells

In order to analyze whether phosphorylation of the single cytoplasmic tyrosine residue (tyrosine 734) of human NCAM180 has a functional role, it was exchanged for phenylalanine. After stable transfection of B35 cells with the cDNAs of NCAM180-wt or NCAM180-Y734F, respectively, the expression of NCAM180-wt and NCAM180-Y734F was determined by indirect immunofluorescence and Western blot analysis. As shown in Figs. 2A and C NCAM180-wt and NCAM180-Y734F are expressed at the plasma membrane. Non-transfected B35 cells showed no antibody reactivity (Fig. 2E). Western blot analysis revealed the expected bands at 180 kDa for wild type and mutant proteins. Clones with nearly identical expression levels were chosen for further experiments (Fig. 2G).

Neurite outgrowth of NCAM180-wt and NCAM180-Y734F expressing cells on pLL as substrate

To study whether tyrosine 734 of NCAM180 plays a role in the extension of neurites we first analyzed neurite outgrowth on pLL as substrate which is thought to allow only unstimulated, i.e., cell autonomous neurite outgrowth to take place in contrast to neurite outgrowth stimulated, e.g., by homophilic trans interactions of NCAM or on outgrowth-promoting substrates like laminin. Under these conditions the cells transfected with NCAM180-Y734F cDNA exhibited significant longer neurites (162 ± 9%) compared to NCAM180-wt expressing cells (100 ± 4%, Figs. 3A and B) after 2 days in culture in the absence of dbcAMP.

It is known that B35 cells develop a more neuronal phenotype in the presence of 1 mM dbcAMP as indicated by the expression of several neuronal markers and enhanced neurite formation [24]. The application of dbcAMP led to a significant increase in neurite length of NCAM180-wt expressing cells (179% compared to control cells without dbcAMP). In contrast, the presence of dbcAMP led only to a small significant increase of NCAM180-Y734F expressing cells compared to NCAM180-Y734F transfectants without dbcAMP (123 ± 2% compared to 100 ± 4%) and was not significantly different from NCAM180-wt expressing cells in the presence of dbcAMP (112 ± 2% compared to 100 ± 2%).

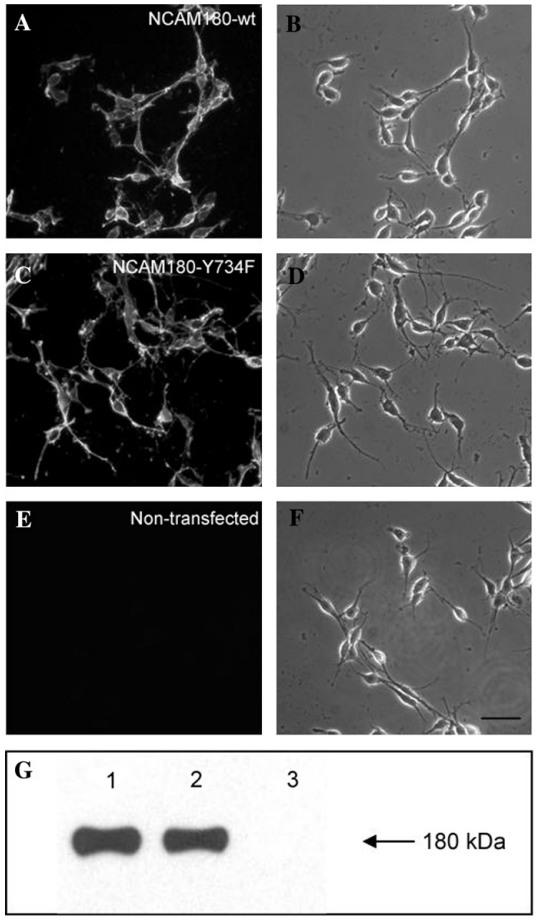

Fig. 2. Expression of NCAM180-wt and NCAM180-Y734F proteins after transfection of B35 cells with the respective cDNAs using monoclonal NCAM antibody (123C3). (A,C,E) show the indirect immunofluorescence micrographs, (B,D,F) the corresponding phase contrast images; the bar corresponds to 20 μm. (G) Western blot analysis of NCAM180-wt (1), NCAM180-Y734F (2), and non-transfected B35 cells (3). In each lane 20 μg of protein was applied.

To exclude clonal variability the neurite length of two other clones with a similar expression level of NCAM180-Y734F was measured with the same results (not shown).

Effect of FGF receptor and MAP kinase activity on cell autonomous neurite outgrowth

It has been shown earlier that NCAM180 in addition to NCAM140 is capable of activating the MAP kinases ERK1 and ERK2 through activation of the

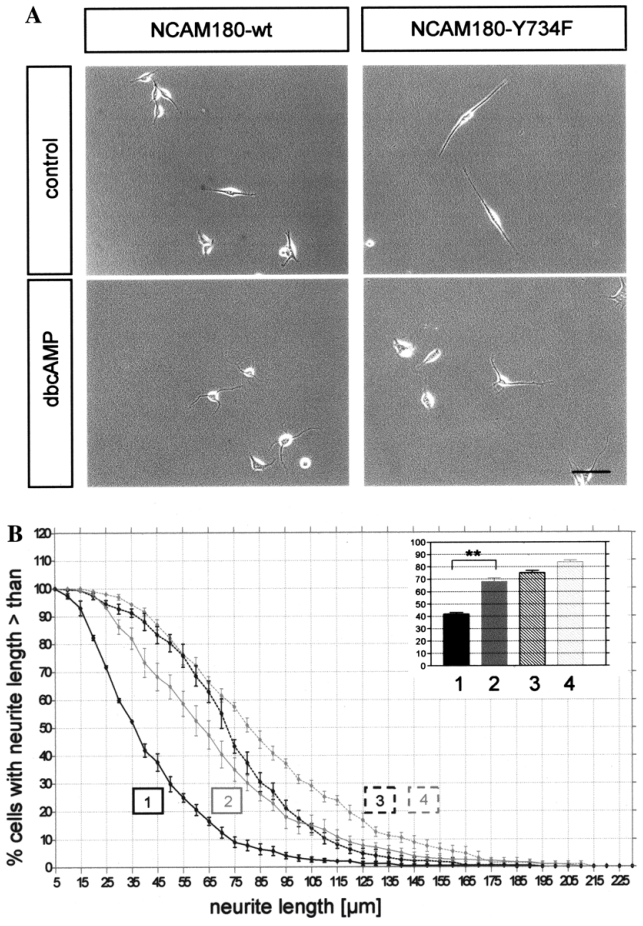

Fig. 3. Neurite length of NCAM180-Y734F transfected B35 cells on pLL is increased compared to NCAM180-wt transfected cells. Single cells were plated on pLL and neurite length of non-differentiated cells or cells differentiated with 1 mM dbcAMP was measured. (A) Representative cells plated on pLL. The bar corresponds to 40 µm. (B) Distribution of neurite length of (1) NCAM180-wt, (2) NCAM180-Y734F expressing cells without dbcAMP treatment, (3) NCAM180-wt, and (4) NCAM180-Y734F expressing cells after dbcAMP treatment. The inset shows the mean neurite length in micrometer, data are given in means ± SEM calculated from five independent experiments with about 100 neurites measured in each experiment. $^{**}p < 0.005$, using a paired t test. The experiment was repeated with two other clones with the same result.

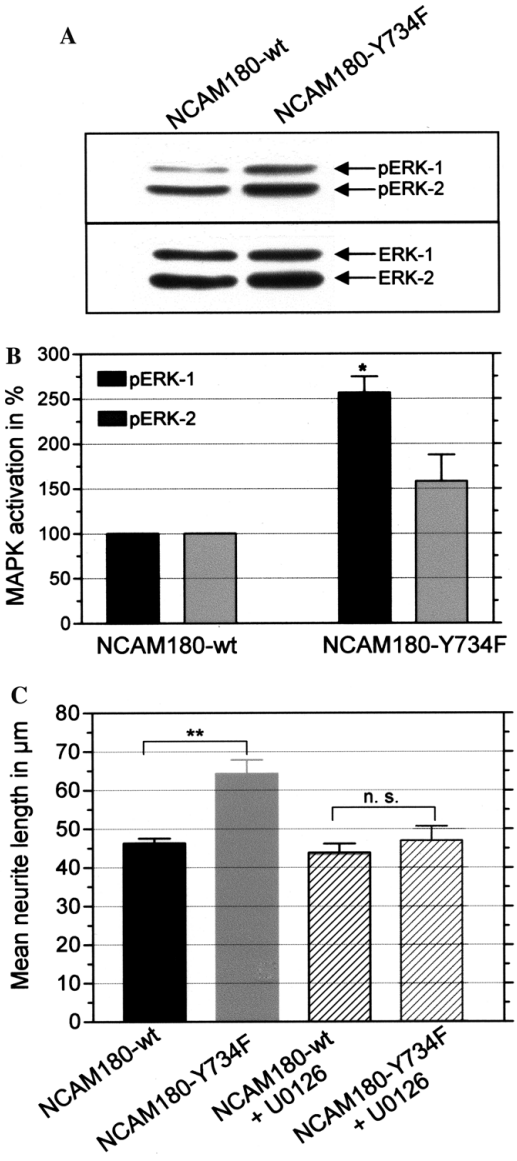

Fig. 4. ERK1/2 activity in NCAM180-Y734F expressing cells is increased compared to NCAM180-wt expressing cells. (A) Western blot analysis of cell lysates. In each lane 50 µg of protein was applied and incubated with antibodies against active ERK1/2, which recognize dually phosphorylated ERK1 and ERK2 (pERK1 and pERK2). The same membrane was stripped and reprobed with ERK antibodies recognizing total levels of ERK1 and ERK2 proteins. (B) Densitometric quantification of ERK1/2 (A). The signal intensities of phospho-ERK1/2 were normalized to the signal intensities of total ERK1/2. Data are given in means ± SEM relative to NCAM180-wt calculated from three different experiments. The asterisk denotes statistical significance ($^{*}p < 0.05$). (C) Application of the MEK inhibitor U0126 (10 µM) reduces the neurite length of NCAM180-Y734F transfected cells plated as single cells on pLL as substrate. Data were calculated from five different experiments with about 100 neurites measured in each experiment and presented in means ± SEM, $^{**}p < 0.005$, n.s., not significant.

FGF receptor after triggering the cells with polyclonal NCAM antibodies [16]. Furthermore, other groups reported effects of MAP kinase activity on neurite outgrowth due to different stimuli [26–28].

In order to investigate ERK1/2 activity of NCAM180-wt and NCAM180-Y734F expressing cells Western blot analysis was performed with antibodies specific for activated, i.e., phosphorylated ERK1/2. The NCAM180-Y734F expressing B35 cells exhibited a significant higher ERK1-activity (256 ± 18%, means ± SEM) compared to NCAM180-wt expressing cells (100%, Figs. 4A and B). Furthermore, in the presence of the MEK inhibitor U0126 the intrinsic neurite outgrowth of the NCAM180-Y734F expressing cells was reduced to the level of the NCAM180-wt transfectant that differed not significantly from the neurite length without the inhibitor (Fig. 4C). These data

indicate that the longer neurites measured in the NCAM180-Y734F mutant may be related to the observed significantly increased ERK1-activity.

As shown by several groups the FGF receptor activates the MAP kinase pathway [29–31] and since the only known possibility of NCAM180 to activate these MAP kinases is via activation of the FGF receptor [16] we performed a reverse transcription-PCR to determine the endogenous expression of the FGF receptor 1 in B35 cells. As shown in Fig. 5A the FGF receptor 1 is expressed in NCAM180-wt and in NCAM180-Y734F transfected cells. Application of the specific inhibitor of FGF receptor 1 signaling, PD 173074, to NCAM180-wt or NCAM180-Y734F expressing cells, respectively, revealed a concentration dependent reduction of the neurite length for the NCAM180-Y734F transfected cells, while no difference in neurite length was observed for NCAM180-wt expressing cells in the presence of PD 173074 compared to the control situation in the absence of the inhibitor (Fig. 5B). Neurite outgrowth studies in the presence of U0126 or PD 173074 were only carried out in the absence of dbcAMP because the difference in neurite outgrowth between NCAM180-wt and NCAM180-Y734F expressing cells was observed under these conditions. To exclude the possibility that the mutant NCAM180-Y734F exhibits an altered subcompartmental localization compared to NCAM180-wt, lipid raft microdomains were isolated. There was no detectable difference observed in the lipid raft localization of NCAM180-Y734F compared to NCAM180-wt (not shown).

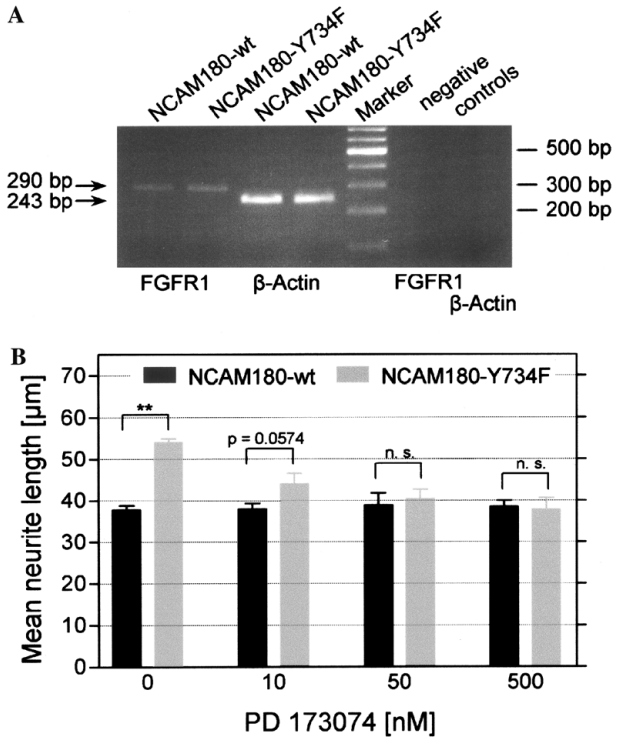

Fig. 5. The FGF receptor 1 is expressed in B35 cells and implicated in the enhanced neurite outgrowth of NCAM180-Y734F expressing cells on pLL as substrate. (A) Reverse transcription was performed using specific FGFR1 or β-actin primers, respectively, followed by a PCR with specific FGFR1 primers to detect mRNA expression of the FGF receptor 1 (290 bp) or with specific β-actin primers for amplification of β-actin as an internal standard (243 bp). Negative controls were carried out under the same conditions, but without addition of RNA to the reverse transcription assay. (B) Concentration dependent reduction of neurite length of NCAM180-Y734F transfected B35 cells after application of PD 173074 in the absence of dbcAMP. Data were calculated from four independent experiments with about 100 neurites measured in each experiment and presented in means ± SEM, $^{**}p < 0.01$, n.s., not significant).

Neurite outgrowth of NCAM180-wt and NCAM180-Y734F expressing B35 cells on NCAM expressing COS cells

In order to answer the question whether the tyrosine residue 734 also plays a functional role in NCAM-stimulated neurite outgrowth, NCAM180-wt and NCAM180-Y734F transfectants were plated on a monolayer of COS or COS/140 cells, respectively. COS cells have been described as NCAM-negative [32]. NCAM180-wt transfected cells exhibited the same neurite length on control COS cells as well as on COS/140 cells in the absence of dbcAMP, whereas NCAM140-wt expressing B35 cells exert significantly longer neurites on COS/140 cells compared to control COS cells (not shown) indicating the functionality of this system. Also, in the presence of dbcAMP the same increase in neurite length was observed on COS and on COS/140 cells. As on pLL the NCAM 180-Y734F expressing cells exhibit on monolayers of COS as well as COS/140 cells a similar increase in neurite length compared to NCAM180-wt transfected cells (161 ± 7% on COS and 147 ± 8% on COS/140 cells, means ± SEM) when not differentiated with dbcAMP. Again, after addition of dbcAMP neurite length of NCAM180-Y734F was indistinguishable from that of NCAM180-wt cells under the same conditions (Fig. 6).

To evaluate the functional role of the FGF receptor 1 in neurite outgrowth after trans interaction between NCAM we applied the specific inhibitor PD 173074 at a concentration of 50 nM to the B35/COS co-culture system under the same conditions as described for the analysis of neurite outgrowth on pLL. Incubation with the inhibitor resulted in decreased neurite outgrowth of NCAM180-Y734F leading to similar neurite length as that of NCAM180-wt transfectants when the cells were plated on control COS cells. In contrast, neurites of NCAM180-Y734F cells on a COS/140 monolayer remained significantly longer than the neurites of NCAM180-wt expressing cells under the same

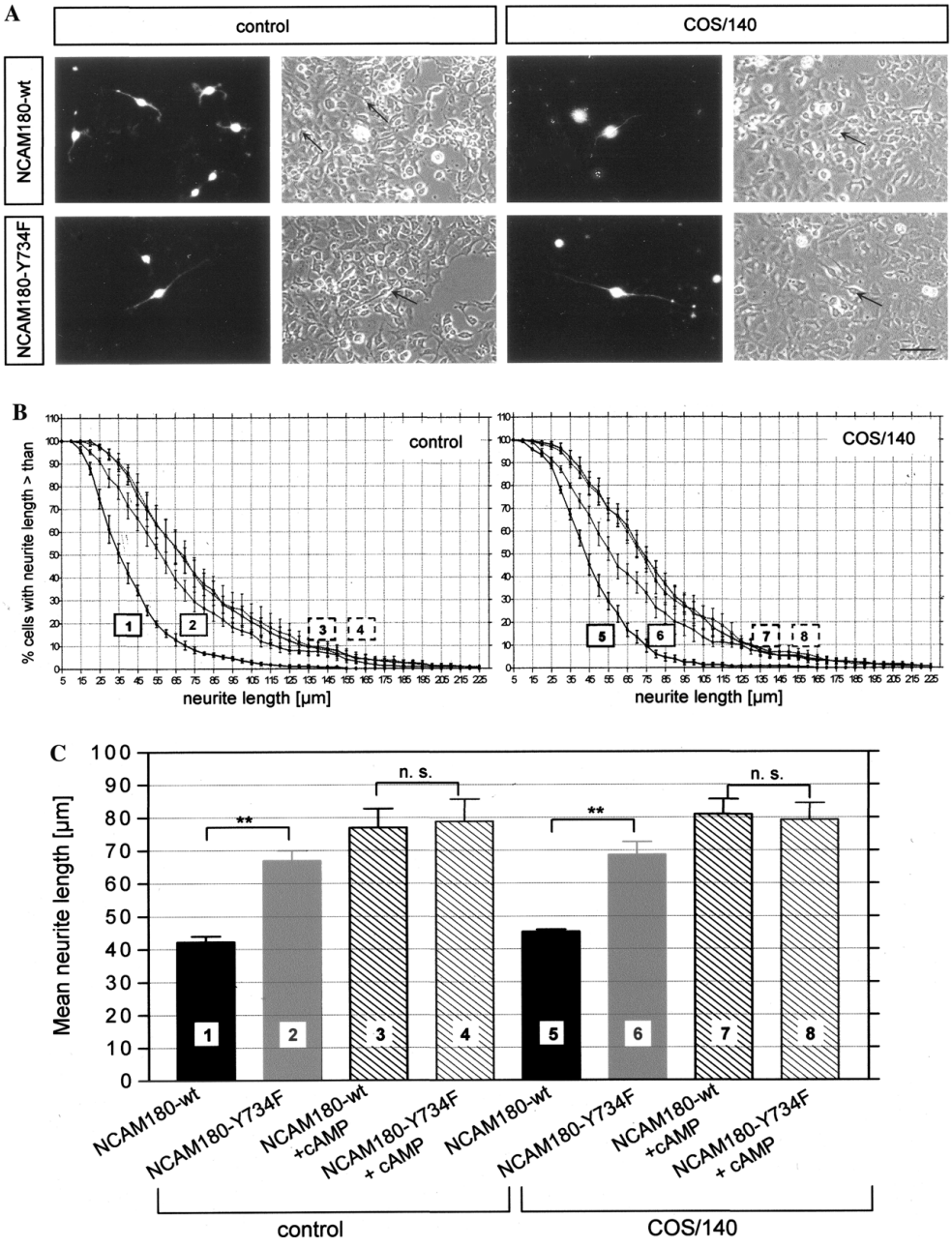

Fig. 6. Neurite length of NCAM180-Y734F expressing cells plated on parental (control) or NCAM140-expressing COS cells (COS/140) is increased compared to NCAM180-wt expressing cells. (A) Shows representative DiI labeled B35 cells transfected either with NCAM180-wt- or NCAM180-Y734F-cDNA, plated on control or COS/140 cells, respectively. Arrows indicate the DiI labeled B35 cells, bar corresponds to 40 μm. (B) Distribution of neurite length of B35 cells plated on control (1–4) or COS/140 cells (5–8) with or without dbcAMP (1, 5: NCAM180-wt; 2, 6: NCAM180-Y734F; 3, 7: NCAM180-wt + dbcAMP; and 4, 8: NCAM180-Y734F + dbcAMP). (C) Mean neurite length in micrometer, data were calculated from four independent experiments with about 100 neurites measured in each experiment and presented in means ± SEM, $^{**}p < 0.005$, using a paired t test, n.s., not significant; numbers in the bars indicate the same as described in (B).

conditions (117 ± 5% versus 100 ± 2%). On the other hand, they were not significantly shorter than those of the same cells in the absence of the inhibitor. The results indicate that in addition to activation of the FGF receptor additional mechanisms play a role in neurite outgrowth of NCAM180-Y734F expressing cells in neurite outgrowth on COS/140 cells (Fig. 7).

Discussion

Sorkin et al. [13] had demonstrated phosphorylation of chick NCAM at serine and threonine residues using embryonic brain homogenates. Later studies showed that NCAM is phosphorylated by casein kinase I and glycogen synthase kinase 3 (GSK-3) [33]. In recent years

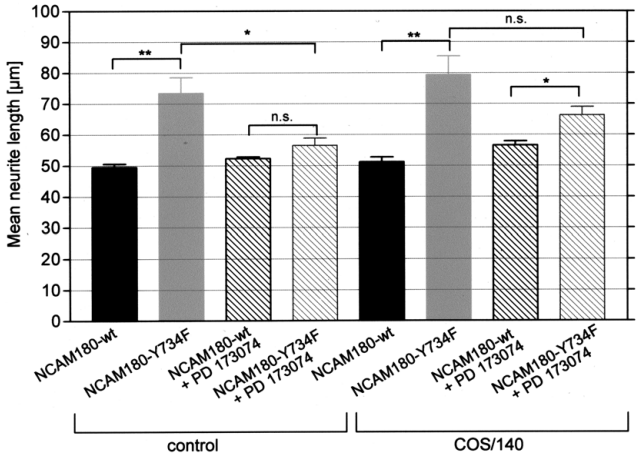

Fig. 7. Activation of the FGF receptor 1 is not solely responsible for the increase in neurite outgrowth of NCAM180-Y734F expressing B35 cells on COS/140 cells. Mean neurite length of NCAM180-wt or NCAM180-Y734F transfected B35 cells plated on parental (control) or NCAM140-expressing COS cells was determined in the absence or presence of the FGF receptor 1 inhibitor PD 173074. Data were calculated from four independent experiments with about 100 neurites measured in each experiment and presented in means ± SEM, $^{**}p < 0.005$, $^{*}p < 0.05$ using a paired t test, ns., not significant.

it has been shown that NCAM is capable of activating several signal transduction pathways which include tyrosine phosphorylation (for review see [14]). This knowledge as well as the fact that phosphotyrosine is very sensitive to phosphatases which can cleave phosphate residues within minutes during isolation procedures [34], but that no phosphatase inhibitors were used in the former studies led us to reinvestigate the phosphorylation of NCAM. With the same approach we had previously provided evidence for the tyrosine phosphorylation of the cell adhesion molecule L1 [35].

The only cytoplasmic tyrosine residue is located near the transmembrane domain in the sequence DITCYFL which is identical in mouse, rat, and human NCAM [36] and highly homologous to the sequence in chicken NCAM [37], suggesting an essential role of this region in NCAM function.

Our study demonstrates for the first time tyrosine phosphorylation of the 180 kDa isoform of NCAM in addition to the earlier described serine and threonine phosphorylation. Computer analysis revealed no consensus sequence for any kinase for the sequence comprising this position, indicating that either there might exist a yet unknown motif for a known kinase or any unknown kinase might phosphorylate this tyrosine.

The phosphoamino acid analysis shown here was carried out with NCAM from Neuro-2a cells and confirmed earlier observations with NCAM isolated from early postnatal cerebellar neurons [38]. For unknown reasons it proved, however, to be difficult to perform the phosphoamino acid analysis of NCAM isolated from NCAM180-wt expressing B35 cells. We therefore are at present preparing a phosphopeptide mapping analysis by mass spectrometry to identify the tyrosine phosphorylated peptide sequence. Nevertheless, the effects resulting from the exchange of tyrosine 734 for phenylalanine provide evidence for the functional importance of this single tyrosine residue.

In order to study whether this mutation would influence neurite outgrowth B35 cells were transfected with NCAM180-Y734F or NCAM180-wt cDNA and plated on pLL as substrate. Significantly longer neurites of the cells transfected with the mutant compared to those transfected with NCAM180-wt cDNA indicate that the tyrosine residue at position 734 in the cytoplasmic tail of NCAM180 plays a role in the non-stimulated cell autonomous neurite outgrowth. The fact that the difference in neurite length is only seen in undifferentiated B35 cells (without application of dbcAMP) insinuates that the non-phosphorylated form of NCAM180 may be relevant in not yet terminally differentiated neurons.

It has been described earlier that only the NCAM140 isoform exerts a neuritogenic effect when hippocampal neurons were plated on pLL as a substrate [16]. This neuritogenic effect was also seen without stimulation of the cells with an NCAM-Fc-fragment, which was suggested to be due to proteolytic processing of all NCAM isoforms leading to a soluble NCAM fragment [39]. Another explanation may be that membrane-bound NCAM140 can stimulate neurite outgrowth already to a certain extent without homophilic trans interaction. Whereas NCAM140 is regarded as a promotor of neurite outgrowth it has been proposed that NCAM180 rather acts as a stabilizer of cell–cell-contacts. This result is in agreement with our observation that NCAM180-wt transfected B35 cells do not form significantly longer neurites than control B35 cells (not shown). NCAM180 is known to establish a link to the cytoskeleton through its interaction with brain spectrin [9] which is suggested to play a role in the growth cone during leading edge protrusion [40]. One possible explanation for the extended neurites of the NCAM180-Y734F expressing cells is that interactions with cytoskeletal components are altered/reduced. However, co-precipitations of spectrin and NCAM180-wt or NCAM180-Y734F, respectively, revealed no difference in the interaction between wild type and mutant NCAM180 with spectrin (data not shown). Whether NCAM180 specific interactions with other proteins are affected as shown by Büttner et al. [41] remains to be seen.

Our results demonstrate that the activity of ERK1, even without antibody triggering, is significantly increased in NCAM180-Y734F transfected cells supporting the view that the mutation affects signal transduction rather than the interaction with cytoskeletal components. Furthermore, specific inhibition of either MEK1/2 or the FGF receptor 1 reduced neurite length of NCAM180-Y734F cells to the level of

NCAM180-wt cells. Interestingly, for PC12 cells the IC_{50} value of PD 173074 for FGF-2 mediated neurite outgrowth has been calculated as 9 nM [42]. Application of PD 173074 at a concentration of 10 nM to NCAM180-wt or NCAM180-Y734F expressing cells, respectively, revealed a difference of 48% in neurite length, if the difference in neurite length between NCAM180-wt and NCAM180-Y734F transfectants without PD 173074 is set to 100%. Higher concentrations of the inhibitor (50 nM) led to practically no difference in neurite length, indicating that the increase in neurite length of NCAM180-Y734F cells compared to NCAM180-wt cells is exclusively mediated by the activated FGF receptor 1. That the FGF receptor 1 is expressed in B35 cells was not surprising, because neurons of the developing nervous system predominantly express FGF receptor 1 [43]. The pivotal role of the FGF receptor in NCAM stimulated neurite outgrowth has been demonstrated in various cell systems as discussed in the introduction [20,44–46].

Our data indicate an increased activity of the FGF receptor 1 in NCAM180-Y734F cells in the absence of NCAM mediated stimulation leading to a significantly higher activity of the MAP kinase ERK1 and, concomitantly, to more extended neurites. Recently it has been shown that the FGF receptor 1 interacts directly with the extracellular region of NCAM [15]. Our results suggest that there is an additional intracellular direct or indirect interaction of the FGF receptor 1 with NCAM leading to activation of the FGF receptor and downstream signaling events when tyrosine 734 is substituted by a non-phosporylable residue. This hypothesis is consistent with the effect of the inhibitor PD 173074 which specifically inhibits the tyrosine kinase domain of the FGF receptor 1 [42], therefore not altering extracellular interactions. Although the MAP kinase pathway is activated in NCAM180-Y734F cells compared to NCAM180-wt cells, we see no downstream activation of the transcription factor CREB (not shown), which has been shown to be the result of NCAM140 crosslinking [18]. This observation indicates that neurite outgrowth stimulation of the NCAM180-Y734F expressing cells is probably mediated by other downstream effectors.

Neurite growth from wild type and mutant NCAM180 expressing cells appears to take place independent of trans interaction because it is roughly the same on pLL as substrate and also the same on COS cells that are or are not transfected with NCAM140. This result supports the view that NCAM180 is not primarily a promotor of neurite growth, in contrast to NCAM140 which is in agreement with the observation that NCAM140 expressing B35 cells exert significantly longer neurites on COS/140 cells compared to control COS cells (data not shown). As on pLL NCAM180-Y734F expressing cells have longer neurites on COS and COS/140 cells compared to NCAM180-wt cells. Treatment of cells with dbcAMP results in enhanced neurite length which under all experimental conditions analyzed increases to approximately twice the value of that for NCAM180-wt cells in the absence of dbcAMP ($\sim$40 μm compared to $\sim$80 μm).

The only difference between neurite growth on control COS cells and on NCAM140 expressing COS cells is that the FGF receptor dependent increase in neurite outgrowth of the NCAM180-Y734F expressing cells cannot be completely reduced to the level of NCAM180-wt expressing cells by incubating co-cultured cells with PD 173074. This result demonstrates that at least one other FGF receptor independent signal transduction pathway is activated in neurite outgrowth of NCAM180-Y734F transfected cells by NCAM–NCAM trans interaction.

It has been demonstrated that NCAM140 and NCAM180 can bind different intracellular molecules [8,9,17,41]. Since in general a spectrin-specific binding site has not been identified yet and since it is not known in which region of the cytoplasmic tail spectrin binds to NCAM180, it can be speculated that either the NCAM180 insert of 261 amino acids binds spectrin or interactions between amino acids of other cytoplasmic parts with NCAM180-specific amino acids take place thereby creating a tertiary structure providing a binding site for spectrin. On the other hand, NCAM140 specific binding partners as identified by Beggs et al. [17] and Büttner et al. [41] are prevented from interaction with NCAM180. It may be possible that an NCAM180-specific tertiary structure is stabilized by phosphorylation of tyrosine 734 and that the exchange of this tyrosine to phenylalanine leads to disturbances of putative interactions with amino acids in the NCAM180 insert resulting in another binding motif that leads to a direct/indirect activation of the FGF receptor 1. For the L1 subgroup of the Ig superfamily it has been shown that phosphorylation of the cytoplasmic tyrosine in the conserved motif FIGQY abolishes ankyrin binding and increases the lateral motility of the molecule [47]. Such a mechanism might also be true for NCAM180.

NCAM180-wt and NCAM180-Y734F are both located in detergent-resistant raft microdomains as well as in non-lipid-raft fractions indicating that a different localization of the mutated NCAM180 cannot be made responsible for the altered signal transduction and neurite outgrowth behavior (data not shown). Tyrosine 734 is located in the cysteine-rich region that is critical for raft association because of the palmitoylation of the four cysteine residues providing a second plasma membrane anchor [16,22]. Niethammer et al. [16] demonstrated that a mutant of NCAM140 with deletion of the four cysteine residues is located only in the non-lipid rafts and acts mainly via the FGF receptor whereas NCAM140-wt located in both subcellular fractions

signals through the nonreceptor tyrosine kinase p^{59fyn} as well as the FGF receptor. Taken together, from these results and our data it can be concluded that the increased neurite outgrowth and FGF receptor activity we observed are not due to different localization of NCAM180-Y734F in the plasma membrane but indeed due to differences in signaling.

Other studies and our data presented here lead to the hypothesis that NCAM180 possibly acts in tyrosine-phosphorylated form as a stabilizer of cell–cell contacts via interactions with cytoskeletal components, whereas non-tyrosine phosphorylated NCAM180 may have a less stabilizing effect but instead a neuritogenic function. The difference in phosphorylation may result from time- and/or spatial-dependent expression of a kinase responsible for the phosphorylation of tyrosine 734. It is tempting to speculate that dbcAMP treated B35 cells are comparable to more differentiated neurons in which the stabilizing effect is dominant over the neuritogenic effect which, on the other hand, may prevail during developmental stages of active neurite growth and which is mediated by enhanced direct or indirect interaction of non-tyrosine phosphorylated NCAM with the FGF receptor 1.

Acknowledgments

We thank P. Heiland for carrying out some of the phosphoamino acid analysis of NCAM, J. Kappler, Institute of Physiological Chemistry, University of Bonn, for helping us to isolate lipid rafts, M.T. Fergen for technical assistance, and the Deutsche Forschungsgemeinschaft for financial support (SFB 284, project A8).

References

[1] B.A. Cunningham, J.J. Hemperly, B.A. Murray, E.A. Prediger, R. Brackenbury, G.A. Edelman, Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell-surface modulation, and alternative RNA splicing, Science 236 (1987) 799–806.

[2] L.C. Rønn, B.P. Hartz, E. Bock, The neural cell adhesion molecule (NCAM) in development and plasticity of the nervous system, Exp. Gerontol. 33 (1998) 853–864.

[3] L.C. Rønn, V. Berezin, E. Bock, The neural cell adhesion molecule in synaptic plasticity and ageing, Int. J. Dev. Neurosci. 18 (2000) 193–199.

[4] V. Berezin, E. Bock, F.M. Poulsen, The neural cell adhesion molecule, Curr. Opin. Drug Disc. Dev. 3 (2000) 605–609.

[5] J.J. Hemperly, G.M. Edelman, B.A. Cunningham, cDNA clones of the neural cell adhesion molecule (N-CAM) lacking a membrane-spanning region consistent with evidence for membrane attachment via a phosphatidylinositol intermediate, Proc. Natl. Acad. Sci. 83 (1986) 9822–9826.

[6] K. Sadoul, A. Meyer, M.G. Low, M. Schachner, Release of the 120 kDa component of the mouse neural cell adhesion molecule N-CAM from cell surfaces by phosphatidylinositol-specific phospholipase C, Neurosci. Lett. 72 (1986) 341–346.

[7] A. Dityatev, G. Dityateva, M. Schachner, Synaptic strength as a function of post- versus presynaptic expression of the neural cell adhesion molecule NCAM, Neuron 26 (2000) 207–217.

[8] G.E. Pollerberg, M. Schachner, J. Davoust, Differentiation state-dependent surface mobilities of two forms of the neural cell adhesion molecule, Nature 324 (1986) 462–465.

[9] G.E. Pollerberg, K. Burridge, K.E. Krebs, S.R. Goodman, M. Schachner, The 180-kDa component of the neural cell adhesion molecule N-CAM is involved in a cell–cell-contacts and cytoskeleton-membrane interactions, Cell Tissue Res. 250 (1987) 227–236.

[10] V. Sytnyk, I. Leshchyns'ka, M. Delling, G. Dityateva, A. Dityatev, M. Schachner, Neural cell adhesion molecule promotes accumulation of TGN organelles at sites of neuron-to-neuron contacts, J. Cell Biol. 159 (2002) 649–661.

[11] S. Bhat, D.H. Silberberg, Developmental expression of neural cell adhesion molecules of oligodendrocytes in vivo and in culture, J. Neurochem. 50 (1988) 1830–1838.

[12] U. Rutishauser, Defining a role and mechanism for IgCAM function in vertebrate axon guidance, J. Cell Biol. 149 (2000) 757–760.

[13] B.C. Sorkin, S. Hoffman, G.M. Edelman, B.A. Cunningham, Sulfation and phosphorylation of the neural cell adhesion molecule, N-CAM, Science 225 (1984) 1476–1478.

[14] G.K. Povlsen, D.K. Ditlevsen, V. Berezin, E. Bock, Intracellular signaling by the neural cell adhesion molecule, Neurochem. Res. 28 (2003) 127–141.

[15] V.V. Kiselyov, G. Skladchikova, A.M. Hinsby, P.H. Jensen, N. Kulahin, V. Soroka, N. Pedersen, V. Tsetlin, F.M. Poulsen, V. Berezin, E. Bock, Structural basis for a direct interaction between FGFR1 and NCAM and evidence for a regulatory role of ATP, Structure (Camb) 11 (2003) 691–701.

[16] P. Niethammer, M. Delling, V. Sytnyk, A. Dityatev, K. Fukami, M. Schachner, Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neuritogenesis, J. Cell Biol. 157 (2002) 521–532.

[17] H.E. Beggs, S.C. Baragona, J.J. Hemperly, P.F. Maness, NCAM-140 interacts with the focal adhesion kinase p125fak and the src-related tyrosine kinase p59fyn, J. Biol. Chem. 272 (1997) 8310–8319.

[18] R.S. Schmid, R.D. Graff, M.D. Schaller, S. Chen, M. Schachner, J.J. Hemperly, P.F. Maness, NCAM stimulates the Ras-MAPK Pathway and CREB phosphorylation in neuronal cells, J. Neurobiol. 38 (1999) 542–558.

[19] D.K. Ditlevsen, L.B. Kohler, M.V. Pedersen, M. Risell, K. Kolkova, M. Meyer, V. Berezin, E. Bock, The role of phosphatidylinositol 3-kinase in neural cell adhesion molecule-mediated neuronal differentiation and survival, J. Neurochem. 84 (2003) 546–556.

[20] K. Kolkova, V. Novitskaya, N. Pedersen, V. Berezin, E. Bock, Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-Mitogen-activated protein kinase pathway, J. Neurosci. 20 (2000) 2238–2246.

[21] L.A. Krushel, B.A. Cunningham, G.M. Edelman, K.L. Crossin, NF-kappaB activity is induced by neural cell adhesion molecule binding to neurons and astrocytes, J. Biol. Chem. 274 (1999) 2432–2439.

[22] E.B. Little, G.M. Edelman, B.A. Cunningham, Palmitoylation of the cytoplasmic domain of the neural cell adhesion molecule N-CAM serves as an anchor to cellular membranes, Cell Adhes. Commun. 6 (1998) 415–430.

[23] W. van't Hof, M.D. Resh, Rapid plasma membrane anchoring of newly synthesized p59fyn: selective requirement for NH2-terminal myristoylation and palmitoylation at cysteine-3, J. Cell Biol. 136 (1997) 1023–1035.

[24] D. Schubert, S. Heinemann, W. Carlisle, H. Tarikas, B. Kimes, J. Patrick, J.H. Steinbach, W. Culp, B.L. Brandt, Clonal cell lines from the rat central nervous system, Nature 249 (1974) 224–227.
[25] O. Traub, J. Look, D. Paul, K. Willecke, Cyclic adenosine monophosphate stimulates biosynthesis and phosphorylation of the 26 kDa gap junction protein in cultured mouse hepatocytes, Eur. J. Cell Biol. 43 (1987) 48–54.
[26] E.Y. Shin, K.S. Shin, C.S. Lee, K.N. Woo, S.H. Quan, N.K. Soung, Y.G. Kim, C.I. Cha, S.R. Kim, D. Park, G.M. Bokoch, E.G. Kim, Phosphorylation of p85 beta PIX, a Rac/cdc42-specific guanine nucleotide exchange factor, via the Ras/ERK/PAK2 pathway is required for basic fibroblast growth factor-induced neurite outgrowth, J. Biol. Chem. 277 (2002) 44417–44430.
[27] P.H. Chuong, B.M. Seo, C.P. Chung, K.M. Yamada, J.H. Jang, Synergistic activity of fibronectin and fibroblast growth factor receptors on neuronal adhesion and neurite extension through extracellular signal-regulated kinase pathway, Biochem. Biophys. Res. Commun. 295 (2002) 898–902.
[28] D.W. Singleton, C.L. Lu, R. Colella, F.J. Roisen, Promotion of neurite outgrowth by protein kinase inhibitors and ganglioside GM1 in neuroblastoma cells involves MAP kinase ERK1/2, Int. J. Dev. Neurosci. 18 (2000) 797–805.
[29] J.C. Perron, J.L. Bixby, Distinct neurite outgrowth signaling pathways converge on ERK activation, Mol. Cell. Neurosci. 13 (1999) 362–378.
[30] J.K. Wang, G. Gao, M. Goldfarb, Fibroblast growth factor receptors have different signaling and mitogenic potentials, Mol. Cell. Biol. 14 (1994) 181–188.
[31] C. Creuzet, J. Loeb, G. Barbin, Fibroblast growth factors stimulate protein tyrosine phosphorylation and mitogen-activated protein kinase activity in primary cultures of hippocampal neurons, J. Neurochem. 64 (1995) 1541–1547.
[32] G. Paratcha, F. Ledda, C.F. Ibanez, The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands, Cell 113 (2003) 867–879.
[33] K. Mackie, B.C. Sorkin, A.C. Nairn, P. Greengard, G.M. Edelman, B.A. Cunningham, Identification of two protein kinases that phosphorylate the neural cell-adhesion molecule, N-CAM, J. Neurosci. 9 (1989) 1883–1896.
[34] B.G. Wallace, Regulation of the interaction of nicotinic acetylcholine receptors with the cytoskeleton by agrin-activated protein tyrosine kinase, J. Cell Biol. 128 (1995) 1121–1129.
[35] P.C. Heiland, B. Hertlein, O. Traub, L.S. Griffith, B. Schmitz, The neural cell adhesion molecule L1 is phosphorylated on tyrosine and serine residues, NeuroReport 7 (1996) 2675–2678.
[36] D. Barthels, G. Vopper, A. Boned, H. Cremer, W. Wille, High degree of NCAM diversity generated by alternative RNA splicing in brain and muscle, Eur. J. Neurosci. 4 (1992) 327–337.
[37] S.J. Small, G.E. Shull, M.J. Santoni, R. Akeson, Identification of a cDNA clone that contains the complete coding sequence for a 140-kD rat NCAM polypeptide, J. Cell Biol. 105 (1987) 2335–2345.
[38] P.C. Heiland, The role of the carbohydrate dependent association of neural cell adhesion molecules for neurite outgrowth and signal transduction, Ph.D. thesis, University of Bonn, 1998.
[39] R. Probstmeier, K. Kuhn, M. Schachner, Binding properties of the neural cell adhesion molecule to different components of the extracellular matrix, J. Neurochem. 53 (1989) 1794–1801.
[40] K. Takei, T.A. Chan, F.S. Wang, H. Deng, U. Rutishauser, D.J. Jay, The neural cell adhesion molecules L1 and NCAM-180 act in different steps of neurite outgrowth, J. Neurosci. 19 (1999) 9469–9479.
[41] B. Büttner, C. Kannicht, W. Reutter, R. Horstkorte, The neural cell adhesion molecule is associated with major components of the cytoskeleton, Biochem. Biophys. Res. Commun. 310 (2003) 967–971.
[42] S.D. Skaper, W.J. Kee, L. Facci, G. Macdonald, P. Doherty, F.S. Walsh, The FGFR1 inhibitor PD 173074 selectively and potently antagonizes FGF-2 neurotrophic and neurotropic effects, J. Neurochem. 75 (2000) 1520–1527.
[43] P.J. Green, F.S. Walsh, P. Doherty, Promiscuity of fibroblast growth factor receptors, Bioessay 18 (1996) 639–646.
[44] J.L. Saffell, E.J. Williams, I.J. Mason, F.S. Walsh, P. Doherty, Expression of a dominant FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs, Neuron 18 (1997) 231–242.
[45] L.C. Rønn, P. Doherty, A. Holm, V. Berezin, E. Bock, Neurite outgrowth induced by a synthetic peptide ligand of neural cell adhesion molecule requires fibroblast growth factor receptor activation, J. Neurochem. 7 (2000) 665–671.
[46] D. Kiryushko, T. Kofoed, G. Skladchikova, A. Holm, V. Berezin, E. Bock, A synthetic peptide ligand of neural cell adhesion molecule (NCAM), C3d, promotes neuritogenesis and synaptogenesis and modulates presynaptic function in primary cultures of rat hippocampal neurons, J. Biol. Chem. 278 (2003) 12325–12334.
[47] T.D. Garver, Q. Ren, S. Tuvia, V. Bennett, Tyrosine phosphorylation at a site highly conserved in the L1 family of cell adhesion molecules abolishes ankyrin binding and increases lateral mobility of neurofascin, J. Cell Biol. 137 (1997) 703–714.