Triple Effect of Mimetic Peptides Interfering with Neural Cell Adhesion Molecule Homophilic Cis Interactions†

Shizhong Li,§ Kateryna Kolkova,§ Olga Rudenko,§ Vladislav Soroka,§ Flemming M. Poulsen,‡ Elisabeth Bock,§ and Vladimir Berezin*,§

Protein Laboratory, Institute of Molecular Pathology, Panum Institute Bld. 6.2., University of Copenhagen, Blegdamsvej 3C, DK-2200, Copenhagen N, Denmark, and the Department of Protein Chemistry, Institute of Molecular Biology, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen, Denmark

Received September 15, 2004; Revised Manuscript Received December 15, 2004

ABSTRACT: The neural cell adhesion molecule (NCAM) is pivotal in neural development, regeneration, and learning. Here we characterize two peptides, termed P1-B and P2, derived from the homophilic binding sites in the first two N-terminal immunoglobulin (Ig) modules of NCAM, with regard to their effects on neurite extension and adhesion. To evaluate how interference of these mimetic peptides with NCAM homophilic interactions in cis influences NCAM binding in trans, we employed a coculture system in which PC12-E2 cells were grown on monolayers of fibroblasts with or without NCAM expression and the rate of neurite outgrowth subsequently was analyzed. P2, but not P1-B, induced neurite outgrowth in the absence of NCAM binding in trans. When PC12-E2 cells were grown on monolayers of NCAM-expressing fibroblasts, the effect of both P1-B and P2 on neurite outgrowth was dependent on peptide concentrations. P1-B and P2 acted as conventional antagonists, agonists, and reverse agonists of NCAM at low, intermediate, and high peptide concentrations, respectively. The demonstrated in vitro triple pharmacological effect of mimetic peptides interfering with the NCAM homophilic cis binding will be valuable for the understanding of the actions of these mimetics in vivo.

The neural cell adhesion molecule, NCAM,[1] is a member of the immunoglobulin (Ig) superfamily, and it plays multiple roles in nervous system development and maintenance (*1*−*5*). The extracellular domain of NCAM is composed of five Ig modules (IgI−V) and two fibronectin type III homology modules (F3,I and F3,II). Three major isoforms, NCAM-120, NCAM-140, and NCAM-180, resulting from alternative splicing of a single gene are found in the nervous system as well as in muscle, heart, kidney, and gonads. One important feature of NCAM is that it can induce neuronal differentiation as reflected by neurite outgrowth through homophilic (NCAM−NCAM) as well as heterophilic interactions, which activate various signal transduction pathways (*6*−*9*). All five Ig modules have been suggested to be involved in homophilic binding (*10*−*16*). Recently a novel model has been proposed in which the IgI and IgII modules mediate dimerization of NCAM molecules positioned on the same cell surface (cis interaction), and the NCAM−IgIII module on one cell binds the IgI and IgII modules of NCAM molecules expressed on the surface of opposing cells (trans interactions). These cis-assisted trans interactions result in the formation of a double-zipper-like NCAM adhesion complex (*17*).

The P1-B and P2 peptides are conserved sequence fragments of NCAM derived from the IgI and IgII modules, respectively. On the basis of structural studies, these peptides have been suggested to represent the IgI−IgII contact area. P1-B corresponds to residues 10−21 (GEISVGESKFFL) of human, rat, mouse, and chicken NCAM and covers the C-terminal end of the A and B β-strands of IgI including Phe[19], the key residue of the IgI−II binding interface (*17*). The P2 sequence corresponds to residues 172−183 (GRILARGEINFK) of human, rat, mouse, and chicken NCAM, covering the C-terminal part of the F β-strand, the F−G turn, and the N-terminal part of the G β-strand of IgII. The tetrameric dendrimer of P2 (P2d) has been shown to bind to recombinant NCAM IgI with an apparent K_d of $(4.7 \pm 0.9) \times 10^{-6}$ M. At the cellular level P2 exhibits a dual effect. It acts as an NCAM antagonist, inhibiting cell aggregation, but it promotes neurite outgrowth and protects against neuronal cell death, indicating that P2 is an agonist of NCAM (*18*, *19*).

In the present study we employed NMR titration analysis to demonstrate that P1-B binds to IgII and induces chemical shifts of a number of amino acid residues representing the P2 sequence. We also investigated the dual function of the peptides representing the IgI−IgII contact area in NCAM, P1-B, and P2, employing a coculture of PC12-E2 cells grown on monolayers of fibroblasts with and without NCAM expression. We here demonstrate that in the absence of NCAM homophilic trans binding P2, but not P1-B, induces

† This work was supported by the Lundbeck Foundation and the Danish Medical Research Council.

* To whom correspondence should be addressed: Tel 45-35-32-73-35; fax 45-35-36-01-16; e-mail berezin@plab.ku.dk.

§ Institute of Molecular Pathology.

‡ Institute of Molecular Biology.

[1] Abbreviations: NCAM, neural cell adhesion molecule; PC12-E2, pheochromocytoma 12, subline E2; P1-Bd, P1-B dendrimer; P2d, P2 dendrimer; P1-Bm, monomeric version of the P1-B peptide; P2m, monomeric version of the P2 peptide; scrP1-Bd, scrambled version of the P1-B peptide; scrP2d, scrambled version of the P2 peptide; FGF, fibroblast growth factor; ppm, parts per million.

10.1021/bi0480133 CCC: $30.25
Published on Web 03/09/2005

neurite outgrowth from PC12-E2 cells. We show that physiological NCAM homophilic trans interactions result in an induction of neurite outgrowth, which is inhibited by treatment with either P1-B or P2 in low and high, but not intermediate, peptide concentrations. These observations exemplify a complex functional relationship between NCAM homophilic cis and trans interactions, making it challenging to design pharmacological agents mimicking NCAM function.

MATERIALS AND METHODS

Antibodies. The mouse monoclonal antibody against rat CD90 (Thy-1) was purchased from Caltag. The secondary Alexa Fluor488 goat antibodies against mouse immunoglobulins were from Molecular Probes Inc.

Preparation of Peptides. All peptides were purchased from Loke Diagnistics (Aarhus, Denmark). The P1-B (GEISVGESKFFL) and P2 (GRILARGEINFK) peptides were synthesized as monomers (P1-Bm and P2m, respectively) and tetrameric dendrimers (P1-Bd and P2d, respectively) composed of four monomers coupled to a lysine backbone. The scrambled versions of the peptides, scrP1-Bd (LGSFEVKIFSEG) and scrP2d (NLFEKGRRAIGI), were synthesized as dendrimers.

Recombinant Proteins. The recombinant Ig module 2 of NCAM was expressed in a *Pichia pastoris* expression system and purified by gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden) followed by ion-exchange chromatography on a HiTrap SP 5 mL column (Pharmacia) as previously described (*14*). Two samples of NCAM module 2 were produced: unlabeled and ^{15}N-labeled IgII.

NMR Titration Analysis. ^{1}H$-^{15}$N HSQC spectra of uniformly ^{15}N-labeled IgII (0.1 mM) alone and in the presence of the P1-B peptide (1.0 mM) were recorded at 298 K on a Varian Unity Inova 750 MHz spectrometer. The transformation and analysis of spectra were performed with MNMR and PRONTO computer programs (*20*). All samples were prepared in 90% H_2O/10% D_2O, 150 mM NaCl, 5 mM sodium phosphate, and 0.02% sodium azide, pH 7.34.

Cell Culture. PC12-E2 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS), 10% (v/v) horse serum (HS), 2 mM GlutaMAX, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

The fibroblastoid mouse cell line L929 was from the European Cell Culture Collection. L929 cells (L-cells) were transfected with eukaryotic expression vector PHβ-Apr-1-neo containing a full-length cDNA encoding human 140 kDa NCAM (*7*). The cells were routinely grown at 37 °C and 5% CO_2 in DMEM supplemented with 10% (v/v) FCS, 2 mM GlutaMAX, 100 units/mL penicillin, and 100 μg/mL streptomycin.

For coculture experiments, transfected L-cells were seeded in eight-well LabTek Permanox plastic chamber slides at a density of 7×10^4 cells/well and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h, 5000 PC12-E2 cells were seeded on top of confluent monolayers of L-cells with or without NCAM expression in DMEM containing 1% HS, and grown for 24 h.

Immunostaining and Image Analysis.The cocultures were fixed in 4% paraformaldehyde, followed by blocking with 10% FCS, and then incubated with anti-rat (Thy-1) CD90 mouse monoclonal antibody (1:200 dilution with 10% FCS) followed by incubation with secondary Alexa Fluor488 goat anti-mouse antibodies (1:1000 dilution with 10% FCS).

Recording was done by computer-assisted microscopy on a Nikon Diaphot inverted microscope (Tokyo, Japan) equipped with an epifluorescence attachment and a Nikon Plan 20× objective. Images were grabbed with a CCD video camera (Grundig Electronics, Germany) by use of the software package "Prima" developed at the Protein Laboratory (University of Copenhagen, Denmark) and stored as 768 × 576 pixels, eight-bit, gray scale GIF images. The length of neuronal processes per cell was estimated by a stereological approach with a software package "Process length" developed at the Protein Laboratory (*21*).

Dynamic Light Scattering Analysis. DLS was performed at 37 °C on a DynaPro DLS instrument (Protein Solutions Inc.) DLS provides direct measurement of diffusion coefficient and hydrodynamic radius, allowing an approximate estimate of molecular mass. The peptides were dissolved in PBS. It appeared that both P1-Bd, at a concentration of 3.4 mg/mL, and P2d, at a concentration of 3.8 mg/mL, exist as monomers with molecular masses of 4−5 kDa, which is in reasonable agreement with their calculated masses, 5.6 and 5.9 kDa, respectively.

Statistics. Statistical evaluation was performed by the paired *t*-test employing the commercially available software package Fig-P, version 2.98 (Biosoft, Cambridge, U.K.). The results are given as mean values ± SEM from at least four independent experiments performed on different days. For estimation of neurite outgrowth at least 200 ± 50 PC12-E2 cells were processed in each individual experiment.

RESULTS

P1-B Peptide Binds to the Second Ig Module of NCAM. The P1-B and P2 peptide sequences represent the IgI−IgII contact area (Figure 1) imperative for the formation of NCAM cis dimers (*14*, *15*, *17*). In agreement with this, a segment of IgII, the P2 peptide, has by surface plasmon resonance analysis been shown to bind to the first Ig module of NCAM (*18*). We therefore tested whether a segment of IgI, the P1-B peptide, can bind IgII. In the ^{15}N-HSQC spectrum of ^{15}N-labeled protein, a signal for each amino acid residue with both an amide proton and nitrogen can be observed, and the changes in chemical shifts of the signals provide information on the sites involved in molecular interactions (*14*). The differences in chemical shifts between ^{15}N-labeled IgII and a mixture of ^{15}N-labeled IgII and P1-B are shown in Figure 2A. The recorded changes in ^{1}H and ^{15}N chemical shifts for each residue were mapped onto the ribbon structure of IgII, with cutoffs at 0.1 and 0.02 ppm for the ^{15}N and ^{1}H chemical shifts, respectively (Figure 2B). The changes in spectra observed during the titration are consistent with the formation of a specific low-affinity complex with IgII. Moreover, the amino acid residues of IgII displaying chemical shifts upon binding of P1-B were largely identical to the residues affected by binding to the natural ligand, the IgI module (*14*). The residues within the P2 sequence of IgII that exhibit pronounced chemical shifts are

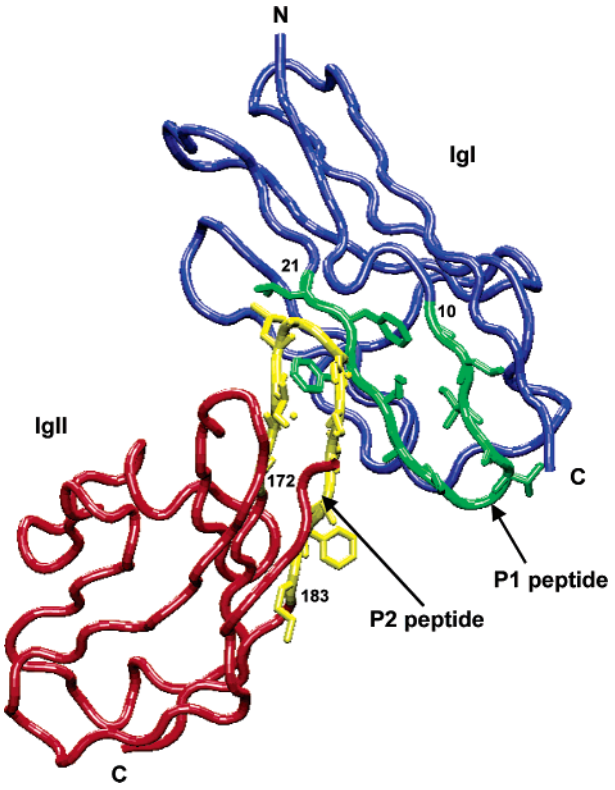

FIGURE 1: Localization of the P1-B peptide and P2 peptide sequences in the double NCAM IgI−IgII module. The IgI and IgII modules are marked in blue and red, respectively. The P1-B and P2 sequences are marked in green (10-GEISVGESKFFL-21) and yellow (172-GRILARGEINFK-183), respectively. The coordinates of the NCAM IgI−IgII module (residues 1−190) are derived from the X-ray structure of the IgI−IgIII fragment of rat NCAM (Protein Data Bank accession number 1QZ1). The figure was prepared with the program Insight II (Accelrys, San Diego, CA).

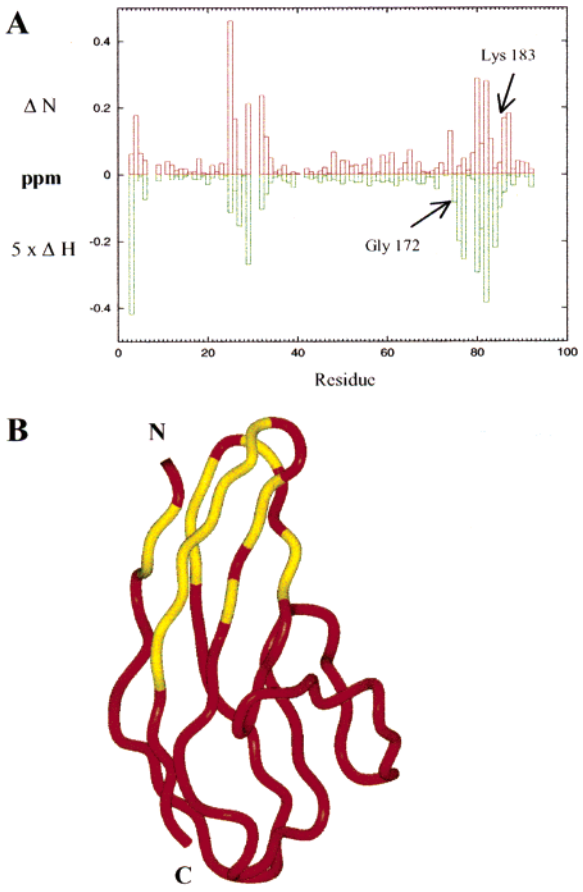

FIGURE 2: Identification of residues in the binding site of P1-B in NCAM-IgII by use of chemical shift changes for ^{15}N and ^{1}H resonance lines of the H−N groups. (A) Difference in chemical shift by residue between a sample of ^{15}N-labeled IgII (0.1 mM) and a mixture of ^{15}N-labeled IgII (0.1 mM) and unlabeled monomeric P1-B (1.0 mM). Bars above $y = 0$ are differences in ^{15}N chemical shift, and bars below are differences in ^{1}H chemical shift (5-fold increased). (B) Mapping (yellow) of the backbone positions on a ribbon presentation of NCAM-IgII that have chemical changes >0.1 ppm for ^{15}N and >0.02 ppm for ^{1}H.

Arg 173, Ile 174, Arg 177, Gly 178, Glu 179, Ile 180, Asn 181, and Lys 183. This strongly suggests that the P1 peptide binds to the NCAM IgII module and that the binding site includes at least eight amino acid residues from the P2 sequence that undergo fast exchange between the bound and unbound states (*14*).

P2 Peptide, But Not P1-B, Induces Neuronal Differentiation of PC12-E2 Cells. We also tested whether P1-B and P2 were capable of inducing differentiation of PC12-E2 cells when grown on monolayers of NCAM-negative fibroblasts. The PC12-E2 cells were seeded sparsely in order to avoid NCAM homophilic trans interactions. The peptides were prepared as tetrameric dendrimers (see Materials and Methods) in order to increase their potency (*18*). From the top panel of Figure 3, it appears that treatment of cocultures with various concentrations of P1-Bd or its scrambled version (scrP1-Bd) did not affect neurite outgrowth from PC12-E2 cells, whereas treatment with P2d, but not with scrP2d, resulted in an induction of neurite outgrowth at relatively high peptide concentrations (10 and 30 μg/mL) (Figure 3, bottom panel). These data together with previously published results (*18*) indicate that, in the absence of NCAM homophilic trans interactions, interference with the NCAM homophilic cis interactions (by P2) can result in stimulation of neurite outgrowth.

P1-B and P2 Both, in a Dose-Dependent Manner, Inhibit Neuronal Differentiation of PC12-E2 Cells Induced by NCAM Homophilic Trans Interactions. NCAM mediates cell adhesion through a mechanism in which a complex zipperlike structure is formed by the trans interactions of NCAM cis dimers (*17*). We therefore tested whether P1-B and P2 affect differentiation of PC12-E2 cells when they grown on monolayers of fibroblasts expressing NCAM, the condition known to mimic physiological NCAM homophilic trans interactions. Under these circumstances NCAM is known to induce neurite extension from a variety of neuronal cell types (reviewed in ref *5*). In Figure 4 it can be seen that PC12-E2 cells grown in coculture with NCAM expressing fibroblasts extend longer neurites than PC12-E2 cells grown on monolayers of fibroblasts without NCAM expression (compare to Figure 3). This confirms that NCAM homophilic trans interactions have the potential to induce neurite outgrowth. Treatment of PC12-E2 cells grown on monolayers of fibroblasts expressing NCAM with either P1-Bd or P2d in low concentrations (1 and 3 μg/mL) resulted in an inhibition of NCAM-mediated neurite outgrowth (Figure 4), probably due to an inhibitory effect on cell adhesion. P2d had a stronger effect than P1-Bd, as reflected by the fact that P2d totally abrogated the NCAM-specific neurite outgrowth response, whereas P1-B inhibited it only partly (ca. 40%). In a higher concentration (10 μg/mL), P1-Bd did not affect NCAM-induced neurite outgrowth, whereas P2d

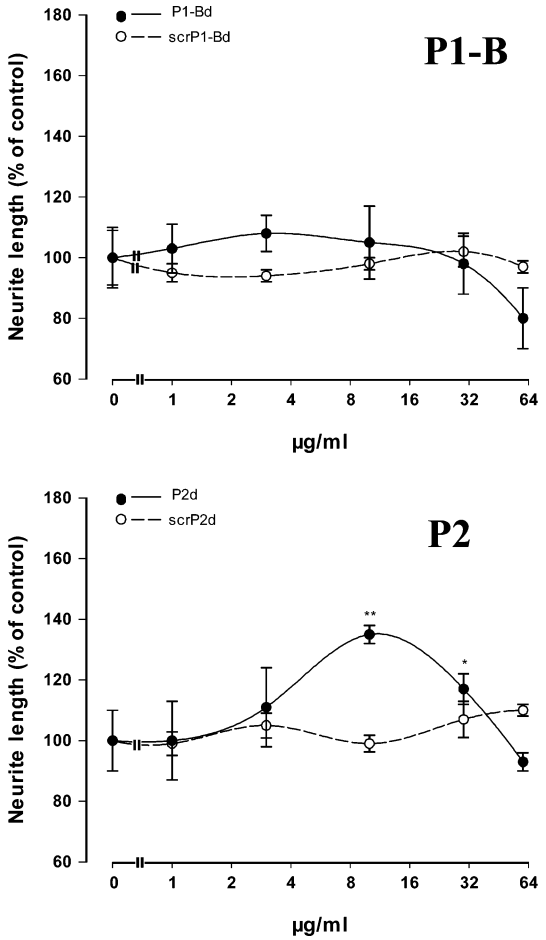

FIGURE 3: Effect of the dendrimeric form of the P1-B and P2 peptides on neurite outgrowth from PC12-E2 cells grown in coculture with NCAM-negative fibroblasts. Results from at least four independent experiments are in all cases expressed as a percentage ± SEM, with untreated controls set as 100%, corresponding to an average neurite length of 43 ± 2.1 μm. Cultures were treated with P1-Bd or scrambled P1-Bd (top panel) and with P2d or scrambled P2d (bottom panel) for 24 h. *$p < 0.05$, **$p < 0.01$ compared with control.

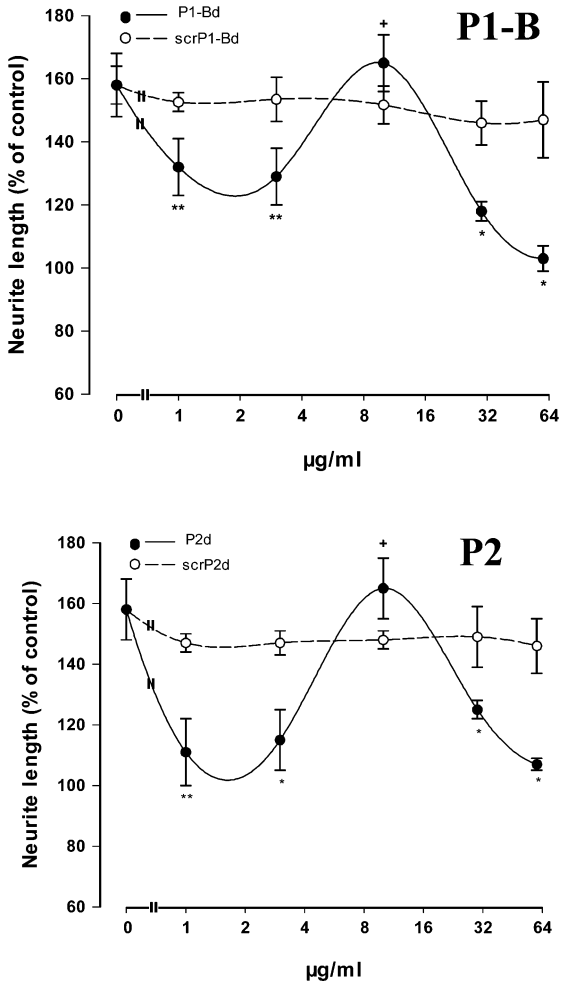

FIGURE 4: Effect of the dendrimeric form of the P1-B and P2 peptides on neurite outgrowth from PC12-E2 cells grown in coculture with NCAM-expressing fibroblasts. Results from at least four independent experiments are in all cases expressed as a percentage ± SEM, with untreated control cultures growth on monolayers of NCAM-negative fibroblasts (Figure 3) set as 100%. Cultures were treated with P1-Bd or scrambled P1-Bd (top panel) and with P2d or scrambled P2d (bottom panel) for 24 h. *$p < 0.05$, **$p < 0.01$, compared with untreated cultures grown on monolayers of fibroblasts expressing NCAM; +, $p < 0.05$ compared with cultures grown on monolayers of NCAM-expressing fibroblasts treated with peptide at a concentration of 3 μg/mL.

stimulated it further (statistically significantly when compared to the effect of scrP2d, $p < 0.05$; Figure 4, bottom panel). An additional increase in concentration of both peptides (30 and 60 μg/mL) led to an inhibition of neurite extension. This dose-dependent effect of the two peptides on NCAM-mediated neurite outgrowth points to a complex functional relationship between NCAM homophilic cis and trans interactions.

Simultaneous Treatment of Cultures with P1-Bd and P2d Abrogates the Effects Observed with Separately Applied Peptides. As described above, the P1-B and P2 peptides represent the IgI−IgII contact area in a NCAM cis dimer, indicating that the P1-B segment in IgI probably interacts with the P2 segment in IgII. We therefore tested whether application of these peptides together can block the effects of separately incubated peptides on neurite outgrowth. Treatment of PC12-E2 cells grown on monolayers of control fibroblasts with P1-Bd in concentrations of 1, 3, and 10 μg/mL had no effect on neurite outgrowth (Figures 3 and 5A), whereas P2d induced neurite outgrowth in a concentration of 10 μg/mL. The effect of P2d was abrogated if cultures were treated with P2d and P1-Bd together, each at a concentration of 10 μg/mL. Treatment of PC12-E2 cells grown on monolayers of fibroblasts expressing NCAM with P1-Bd or P2d separately at concentrations of 1 and 3 μg/mL inhibited NCAM-induced neurite outgrowth (Figure 4), whereas combined treatment with both peptides in the same concentrations had no effect (Figure 5B). These data suggest that P1-Bd and P2d may interact with each other in solution, thereby reciprocally blocking the ability of peptides to interact with the IgI and IgII modules of NCAM.

Identification of Important Amino Acid Residues of P1-B and P2 Sequences Involved in Inhibition of Cell Adhesion. As shown above, P1-Bd and P2d both inhibited NCAM-mediated neurite outgrowth at low peptide concentrations. To analyze the contribution of individual amino acid residues to the inhibiting effect of the peptides on cell adhesion, single amino acid residues in monomeric forms of P1-B (P1-Bm) and P2 (P2m) were replaced with alanine residues. Peptides were tested at a concentration of 200 μg/mL. At this concentration, neither P1-Bm nor P2m stimulated neurite outgrowth from PC12-E2 cells grown in coculture with

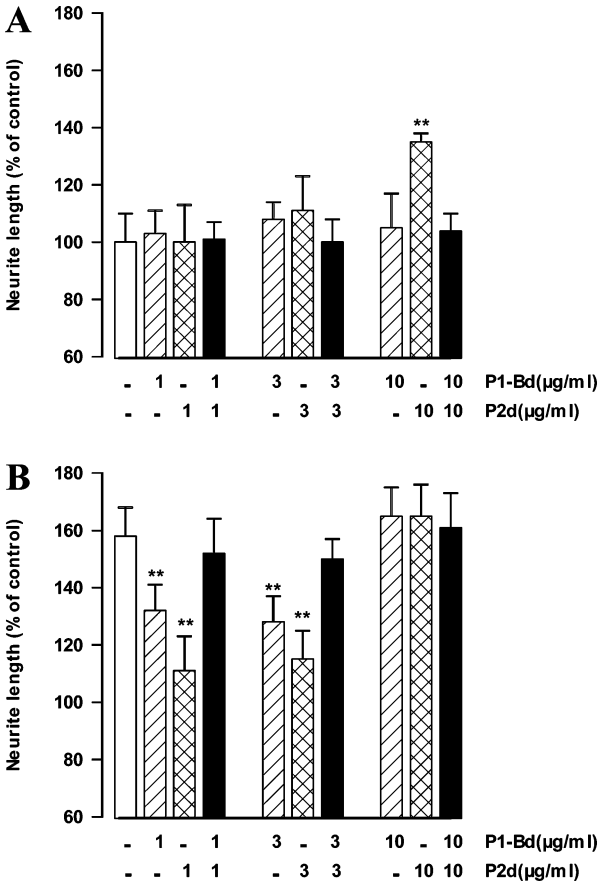

FIGURE 5: Effect of simultaneous treatment of cultures with P1-Bd and P2d on neurite outgrowth from PC12-E2 cells grown in coculture of fibroblasts without (A) or with (B) NCAM expression. Results from four independent experiments are in all cases expressed as a percentage ± SEM, with neurite outgrowth from untreated control cultures grown on monolayers of NCAM-negative fibroblasts set as 100%. $^{**}p < 0.01$, compared with untreated cultures grown on monolayers of control fibroblasts (A) and fibroblasts expressing NCAM (B).

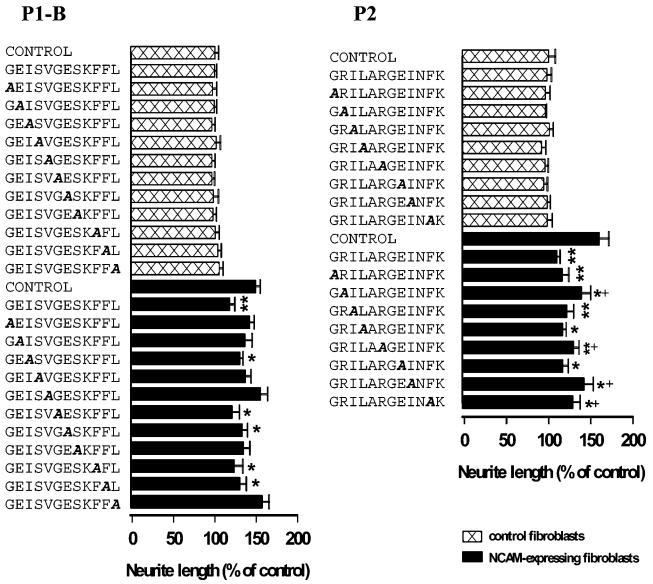

FIGURE 6: Effect of monomeric P1-B and P2 peptide derivatives containing single alanine substitutions on neurite outgrowth from PC12-E2 cells grown on monolayers of fibroblasts with or without NCAM expression. Results from at least four independent experiments are in all cases expressed as a percentage ± SEM, with untreated control cultures grown on monolayers of NCAM-negative fibroblasts set as 100%. Alanine substitutions are shown in boldface type. All peptides were tested at a concentration of 200 μg/mL. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with untreated cultures grown on monolayers of NCAM-expressing fibroblasts; +, $p < 0.05$ compared with cultures grown on monolayers of NCAM-expressing fibroblasts and treated with nonmutated P2.

NCAM-negative fibroblasts, but they clearly inhibited cell adhesion as reflected by a statistically significant inhibition of the NCAM-mediated neurite outgrowth response (Figure 6). From Figure 6 (left panel) it appears that single substitutions of Gly[1], Glu[2], Ser[4], Val[5], Ser[9], and Leu[12] abrogated the inhibitory effect of P1-Bm on NCAM-mediated neurite outgrowth, whereas none of the single amino acid substitutions resulted in a complete inactivation of P2m (Figure 6, right panel). However, single substitutions of Arg[2], Arg[6], Ile[9], and Phe[11] did partly decrease the effect of P2m, pointing to an important contribution of basic amino acid residues in the N-terminal peptide segment and hydrophobic residues in the C-terminal peptide segment in NCAM-mediated cell adhesion.

DISCUSSION

Because of the complex multimodular structure of cell adhesion molecules (CAMs), studies of the biological effects of CAM stimulation are impeded by the difficulties in presenting the molecules to appropriate ligands mimicking or specifically inhibiting CAM-mediated adhesion and signal transduction. Recent studies of the structural mechanisms of NCAM-mediated cell adhesion have revealed a substantial complexity of NCAM homophilic interactions at the atomic level (*14*, *15*, *17*). At the same time, these studies have promoted the identification and development of NCAM-derived peptides mimicking NCAM homophilic binding and heterophilic interactions with the fibroblast growth factor (FGF) receptor (reviewed in ref *22*). Two of the identified peptide mimetics, P1-B and P2 peptides, represent the contact area between IgI and IgII of NCAM, respectively. P2 has by surface plasmon resonance been demonstrated to bind IgI (*18*). Likewise, we have here shown that P1-B bound to IgII and caused chemical shifts of eight out of 12 residues constituting the P2 sequence in IgII. The IgI and IgII modules are involved in a double-reciprocal interaction, presumably leading to the binding of two NCAM molecules expressed on the surface of the same cell (dimerization). The formation of NCAM dimers in cis is presumably a requirement for NCAM-mediated cell−cell adhesion, involving interaction of NCAM dimers in trans. In other words, NCAM mediates cell adhesion by cis-assisted trans interactions. Interference with the formation of dimers in cis may therefore be expected to inhibit cell adhesion, abstructing binding in trans. Indeed, P1-B and P2 have been shown to reduce NCAM-dependent adhesion (*18*, *17*).

Cell−cell adhesion by NCAM results in an induction of a number of intracellular signal-transducing cascades, ultimately leading to a series of biological responses including neurite outgrowth (reviewed in ref *5*). By employing a coculture system, in which neuronal cells are grown on monolayers of fibroblasts with and without NCAM expression, it is possible to test the effect of compounds interfering with NCAM-mediated adhesion by measuring the rate of neurite growth. This model cell system closely mimics physiological conditions, and it is widely used to study

NCAM function and to test NCAM mimetic compounds (*23*, *7*, *8*). We here demonstrate that the peptides P1-B and P2, belonging to the class of NCAM mimetics affecting homophilic binding in cis, influence NCAM-mediated neurite outgrowth with a complex dose−effect relationship.

At low peptide concentrations, P1-B and P2 inhibited NCAM-dependent neurite extension, probably by competing with their structural counterparts IgI and IgII in relation to the physiological NCAM binding mechanism, indicating that interference with NCAM dimers in cis inhibits cell adhesion and thereby reduces neurite outgrowth. Therefore, P1-B and P2 can be regarded as conventional antagonists of NCAM when used in low concentrations. It should be noted that this definition is valid only for the effect of the peptides on NCAM-mediated cell adhesion.

At intermediate peptide concentrations, P1-B did not affect NCAM-mediated neurite outgrowth, whereas P2 enhanced it. We suggest that the actual mechanism of this apparently anomalous activity of the peptides may be as follows: NCAM homophilic interactions in trans are absent, since the concentration of the peptides already is higher than those abrogating NCAM-mediated cell adhesion. Therefore, we may disregard the influence of NCAM expressed on the surface of the fibroblast monolayer on neurite outgrowth. Under these circumstances the peptides interact with the NCAM cis dimers expressed on the surface of PC12-E2 (the situation also observed in control cultures) and, in the absence of NCAM homophilic trans interactions, interference with NCAM homophilic cis interactions results in stimulation of neurite outgrowth. P2 clearly has a stronger potential to stimulate neurite outgrowth than P1-B does. Therefore, the effect of P2 appears as an increase in NCAM-mediated neurite outgrowth, whereas P1-B seems to have no effect (Figure 4; compare to Figure 3B). However, at an intermediate peptide concentration, P1-B actually is capable of stimulating neurite outgrowth statistically significantly if this effect is compared to that observed at low concentrations of P1-B (Figure 4, top panel). Accordingly, both P2 and P1-B may be regarded as conventional agonists as regards their effect on neurite outgrowth in the absence of NCAM homophilic interactions in trans. The P2 peptide has previously been shown to induce neurite outgrowth from hippocampal neurons under conditions excluding NCAM homophilic interactions in trans (*18*).

At high peptide concentrations, both P1-B and P2 inhibited NCAM-mediated neurite outgrowth (Figure 4). As discussed above, at intermediate and high peptide concentrations, the NCAM homophilic interactions in trans are probably abrogated. Therefore, P1-B and P2 may be regarded as inverse agonists (see IUPAC Recommendations, 1998; http://www.chem.qmul.ac.uk/iupac/medchem/) when used in high concentrations, because they act at the same receptor (NCAM) in the absence of its interaction with a natural ligand (i.e., NCAM expressed on an opposing cell). The molecular mechanism of the inhibitory effect of the peptides in high concentrations in the absence of NCAM homophilic interactions in trans might be due to a growth factor-like effect of P1-B and P2. Since neurite outgrowth mediated by NCAM is known to involve the activation of the FGF receptor (*24*, *25*), it is possible that high concentrations of the peptides hyperactivate the FGF receptor, resulting in its desensitization, for example, due to endocytosis.

In conclusion, the structural complexity of the NCAM homophilic binding mechanism based on the interplay between NCAM homophilic cis and trans interactions makes it difficult to design pharmacological compounds unambiguously mimicking the functional role of this cell adhesion molecule. We have here shown that peptides interfering with NCAM cis binding, depending on their concentrations, may act as conventional antagonists, agonists, or reverse agonists.

ACKNOWLEDGMENT

We thank Dr. Klaus Seedorf (Hagedorn Research Institute, Gentofte, Denmark) for his gift of the PC12-E2 cell line.

REFERENCES

1. Rønn, L. C., Hartz, B. P., and Bock, E. (1998) The neural cell adhesion molecule (NCAM) in development and plasticity of the nervous system, *Exp. Gerontol. 33*, 853−864.
2. Rønn, L. C., Doherty, P., Holm, A., Berezin, V., and Bock, E. (2000) Neurite outgrowth induced by a synthetic peptide ligand of NCAM requires fibroblast growth factor receptor activation, *J. Neurochem. 75*, 665−671.
3. Berezin, V., Bock, E., and Poulsen, F. M. (2000) The neural cell adhesion molecule, *Curr. Opin. Drug. Discovery Dev. 3*, 605−609.
4. Povlsen, G. K., Ditlevsen, D. K., Berezin, V., and Bock, E. (2003) Intracellular signaling by the neural cell adhesion molecule, *Neurochem. Res. 28*, 127−141.
5. Hinsby, A. M., Berezin, V., and Bock, E. (2004) Molecular mechanisms of NCAM function, *Frontiers Biosci. 9*, 2227−2244.
6. Schmid, R. S., Graff, R. D., Schaller, M. D., Chen, S., Schachner, M., Hemperly, J. J., and Maness, P. F. (1999) NCAM stimulates the Ras-MAPK pathway and CREB phosphorylation in neuronal cells, *J. Neurobiol. 38*, 542−558.
7. Kolkova, K., Novitskaya, V., Pedersen, N., Berezin, V., and Bock, E. (2000) 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*, 2238−2246.
8. Jessen, U., Novitskaya, V., Serup, P., Berezin, V., and Bock, E. (2001) The transcription factors CREB and c-Fos play key roles in NCAM-mediated neuritogenesis in PC12-E2 cells, *J. Neurochem. 79*, 1149−1160.
9. Ditlevsen, D. K., Køhler, L. B., Pedersen, M. V., Rissel, M., Kolkova, K., Meyer, M., Berezin, V., and Bock, E. (2003) The role of phosphatidylinositol 3-kinase in neural cell adhesion molecule-mediated neuronal differentiation and survival, *J. Neurochem. 84*, 546−556.
10. Rao, Y., Wu, X. F., Yip, P., Gariepy, J., and Siu, C. H. (1993) Structural characterization of a homophilic binding site in the neural cell adhesion molecule, *J. Cell Biol. 268*, 20630−20638.
11. Sandig, M., Rao, Y., and Siu, C. H. (1994) The homophilic binding site of the neural cell adhesion molecule NCAM is directly involved in promoting neurite outgrowth from cultured neural retinal cells, *J. Biol. Chem. 20*, 14841−14848.
12. Ranheim, T. S., Edelman, G. M., and Cunningham, B. A. (1996) Homophilic adhesion mediated by the neural cell adhesion molecule involves multiple immunoglobulin domains, *Proc. Natl. Acad. Sci. U.S.A. 93*, 4071−4075.
13. Kiselyov, V. V., Berezin, V., Maar, T. E., Soroka, V., Edvardsen, K., Schousboe, A., and Bock, E. (1997) The first immunoglobulin-like neural cell adhesion molecule (NCAM) domain is involved in double-reciprocal interaction with the second immunoglobulin-like NCAM domain and in heparin binding, *J. Biol Chem. 272*, 10125−10134.
14. Jensen, P. H., Soroka, V., Thomsen, N. K., Ralets, I., Berezin, V., Bock, E., and Poulsen, F. M. (1999) Structure and interactions of NCAM modules 1 and 2—basic elements in neural cell adhesion, *Nat. Struct. Biol. 6*, 486−493.
15. Kasper, C., Rasmussen, H., Kastrup, J. S., Ikemizu, S., Jones, E. Y., Berezin, V., Bock, E., and Larsen, I. K. (2000) Structural basis of cell−cell adhesion by NCAM, *Nat. Struct. Biol. 7*, 389−393.
16. Atkins, A. R., Chung, J., Deechongkit, S., Little, E. B., Edelman, G. M., Wright, P. E., Cunningham, B. A., and Dyson, H. J. (2001) Solution structure of the third immunoglobulin domain of the

neural cell adhesion molecule N-CAM: can solution studies define the mechanism of homophilic binding? *J. Mol. Biol. 311*, 161−172.

17. Soroka, V., Kolkova, K., Jette, S. Kastrup, Diederichs, K., Breed, J., Kiselyov, V. V., Poulsen, F. M., Larsen, I. K., Welte, W., Berezin, V., Bock, E., and Kasper, C. (2003) Structure and interaction of NCAM IgI-2−3 suggest a novel zipper mechanism for homophilic adhesion, *Structure 10*, 1291−1301.
18. Soroka, V., Kiryushko, D., Novitskaya, V., Ronn, L. C., Poulsen, F. M., Holm, A., Bock, E., and Berezin, V. (2002) Induction of neuronal differentiation by a peptide corresponding to the homophilic binding site of the second Ig module of NCAM, *J. Biol. Chem. 277*, 24676−24683.
19. Pedersen, M. V., Køhler, L. B., Ditlevsen, D. K., Li, S., Berezin, V., and Bock, E. (2004) Neuritogenic and survival-promoting effects of the P2-peptide derived from a homophilic binding site in NCAM, *J. Neurosci. Res. 75*, 55−65.
20. Kjær, M., Andersen, M. K., and Poulsen, F. M. (1994) Automated and semiautomated analysis of homo- and heteronuclear multidimensional nuclear magnetic resonance spectra of proteins; the program Pronto, *Methods Enzymol. 239*, 288−307.
21. Rønn, L. C., Ralets, I., Hartz, B. P., Morten, B., Berezin, A., Berezin, V., Møller, A., and Bock, E. (2000) A simple procedure for quantification of neurite outgrowth based on stereological principles, *J. Neurosci. Methods 100*, 25−32.
22. Berezin, V., and Bock, E. (2003) NCAM mimetic peptides: Pharmacological and therapeutic potential, *J. Mol. Neurosci. 22*, 33−40.
23. Rønn, L. C., Olsen, M., Østergaard, S., Kiselyov, V., Berezin, V., Mortensen, M. T., Lerche, M. H., Jensen, P. H., Soroka, V., Saffels, J. S., Doherty, P., Poulsen, F. M., Bock, E., and Holm, A. (1999) Identification of a neuritogenic ligand of the neural cell adhesion molecule using a combinatorial library of synthetic peptides, *Nat. Biotechnol. 17*, 1000−1005.
24. Doherty, P., and Walsh, F. S. (1996) CAM−FGF receptor interactions: a model for axonal growth, *Mol. Cell. Neurosci. 8*, 99−111.
25. Kiselyov, V. V., Skladchikova, G., Hinsby, A. M., Jensen, P. H., Kulahin, N., Soroka, V., Pedersen, N., Tsetlin, V., Poulsen, F. M., Berezin, V., and Bock, E. (2003) Structural basis for a direct interaction between FGFR1 and NCAM and evidence for a regulatory role of ATP, *Structure 11*, 691−701.

BI0480133