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A peptide from the first fibronectin domain of NCAM acts as an inverse agonist and stimulates FGF receptor activation, neurite outgrowth and survival

Alexandra A. Anderson,* Claire E. Kendal,* Mitla Garcia-Maya,* Anna V. Kenny,* Samantha A. Morris-Triggs,* Teresa Wu,† Richard Reynolds,† Erhard Hohenester* and Jane L. Saffell*

**Division of Cell and Molecular Biology, Faculty of Life Sciences and †Department of Cellular and Molecular Neuroscience, Imperial College London, London, UK*

Abstract

Neural cell adhesion molecule (NCAM) contributes to axon growth and guidance during development and learning and memory in adulthood. Although the Ig domains mediate homophilic binding, outgrowth activity localizes to two membrane proximal fibronectin-like domains. The first of these contains a site identified as a potential FGF receptor (FGFR) activation motif (FRM) important for NCAM stimulation of neurite outgrowth, but its activity has hitherto remained hypothetical. Here, we have tested the effects of a domain-specific antibody and peptides corresponding to the FRM in cellular assays *in vitro*. The first fibronectin domain antibody inhibited NCAM-stimulated outgrowth, indicating the importance of the domain for NCAM function. Monomeric FRM peptide behaved as an inverse agonist; low concentrations specifically inhibited neurite outgrowth stimulated by NCAM and cellular responses to FGF2, while saturating concentrations stimulated FGFR-dependent neurite outgrowth equivalent to NCAM itself. Dendrimeric FRM peptide was 125-fold more active and stimulated FGFR activation, FGFR-dependent and FGF-mimetic neurite outgrowth and cell survival (but not proliferation). We conclude that the FRM peptide contains NCAM-mimetic bioactivity accounted for by stimulation of FGF signalling pathways at the level of or upstream from FGF receptors, and discuss the possibility that FRM comprises part of an FGFR activation site on NCAM.

Keywords: bioactive peptide, cell survival, fibroblast growth factor receptor, fibronectin type III domain; neural cell adhesion molecule, neurite outgrowth.

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Neural cell adhesion molecule (NCAM) expressed on the surface of neurons, oligodendrocytes and astrocytes is the prototypic immunoglobulin-like (Ig) member of a family of cell adhesion molecules (CAMs) that induce cell adhesion by homophilic binding (Crossin and Krushel 2000). It mediates neuronal migration, axon growth and guidance (Walsh and Doherty 1997; Bruses and Rutishauser 2001) and synaptic plasticity associated with learning and memory (Cremer *et al*. 1994; Luthl *et al*. 1994; Muller *et al*. 1996; Schachner 1997). NCAM stimulation of neurite outgrowth depends on intracellular signalling (Schuch *et al*. 1989; Doherty *et al*. 1991) involving Fyn (Beggs *et al*. 1994) and the fibroblast growth factor receptor (FGFR) (Williams *et al*. 1994a; Saffell *et al*. 1997). FGFs and their receptors (FGFR1-4) contribute to a range of physiological processes (Bikfalvi *et al*. 1997), including survival and differentiation of neurons (Walicke *et al*. 1986). FGFRs are required for NCAM-

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Address correspondence and reprint requests to Dr Jane L. Saffell, Division of Cell and Molecular Biology, Biochemistry Building, Imperial College London, South Kensington Campus, London SW7 2AZ, UK. E-mail: j.saffell@imperial.ac.uk

Abbreviations used: APP, amyloid precursor protein; BrdU, bromodeoxyuridine; CAM, cell adhesion molecule; CHD, cell adhesion molecule homology domain; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FRM, fibroblast growth factor receptor activating motif; FnIII, fibronectin type III; HRP, horseradish peroxidase; MBP, myelin basic protein; MTS, tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCAM, neural cell adhesion molecule; OD, optical density; OLP, oligodendrocyte progenitor medium; PBS, phosphate-buffered saline; PND, post-natal day; PSA, polysialic acid; SDS, sodium dodecyl sulfate.

stimulated neurite outgrowth (Saffell *et al*. 1997; Niethammer *et al*. 2002), can co-precipitate with (Cavallaro *et al*. 2001) and be activated by NCAM (Saffell *et al*. 1997). It has been hypothesized that NCAM *trans* homophilic binding co-clusters and activates FGFRs in *cis*, perhaps by direct binding (Williams *et al*. 1994a; Doherty and Walsh 1996).

The extracellular domain of NCAM comprises five Ig and two fibronectin type III-like domains (FnIII) (Fig. 1a) and, while the Ig domains mediate homophilic adhesion (Ranheim *et al*. 1996; Soroka *et al*. 2003; Johnson *et al*. 2004), the FnIII domains contain the neurite outgrowth stimulating activity (Frei *et al*. 1992). Kiselyov *et al*. (2003) have identified a site (FGL) in the second FnIII domain (Fig. 1a) that mediates binding to the third Ig domain of FGFR1 (shown by NMR), and stimulated neurite outgrowth (Neiiendam *et al*. 2004), FGFR signalling (Kiselyov *et al*. 2003) and enhanced learning *in vivo* (Cambon *et al*. 2004). Interestingly, however, when binding of the FnIII domains to FGFR1 was assessed by surface plasmon resonance, a fragment containing both FnIII domains bound, but not the second domain alone, indicating that the first FnIII domain also contributes to optimal FGFR binding (Kiselyov *et al*. 2003). Contained within the first FnIII domain is a motif (putative FGF receptor activation motif, FRM) that has been hypothesized to contribute to NCAM activation of FGFRs leading to neurite outgrowth (Fig. 1a) (Doherty and Walsh 1996), although this has not been tested experimentally. Doherty and Walsh (1996) have identified a similar motif in L1 and N-cadherin and have hypothesized that these CAMs activate FGFRs by binding via their FRM to the FGFR CAM homology domain (CHD) site in the second receptor Ig domain, also important for FGF and heparan sulfate binding (Plotnikov *et al*. 1999; Pellegrini *et al*. 2000) (Fig. 1a). Peptide and antibody competition assays have established that N-cadherin FRM is an important motility motif (Williams *et al*. 2001) but the functional importance of the NCAM FRM remains theoretical and is the subject of this study.

The NCAM FRM (^{504}SIDRVEPYSSTAQ^{516} in rodent) is highly conserved between species (Fig. 1b), in particular the SI and VEPYSSTA residues. Our model of the first FnIII domain (rodent), based on the experimental structure of the D3 domain of gp130 (Chow *et al*. 2001), shows that the FRM maps to a descending loop, almost entirely surface-exposed, comprising β-strand A, the A–B turn and half of β-strand B (Fig. 1c). We have investigated the biological activity of this FRM by testing a domain-specific antibody and FRM peptides for their ability to modulate cell behaviour in cellular assays using primary neurons, oligodendrocytes and cell lines *in vitro*. The results indicate that the first FnIII domain is important for NCAM stimulation of neurite outgrowth, and that the FRM peptide contains NCAM and selective FGF mimetic bioactivity, stimulating FGFR activation, FGFR-dependent neurite outgrowth and cell survival but not proliferation.

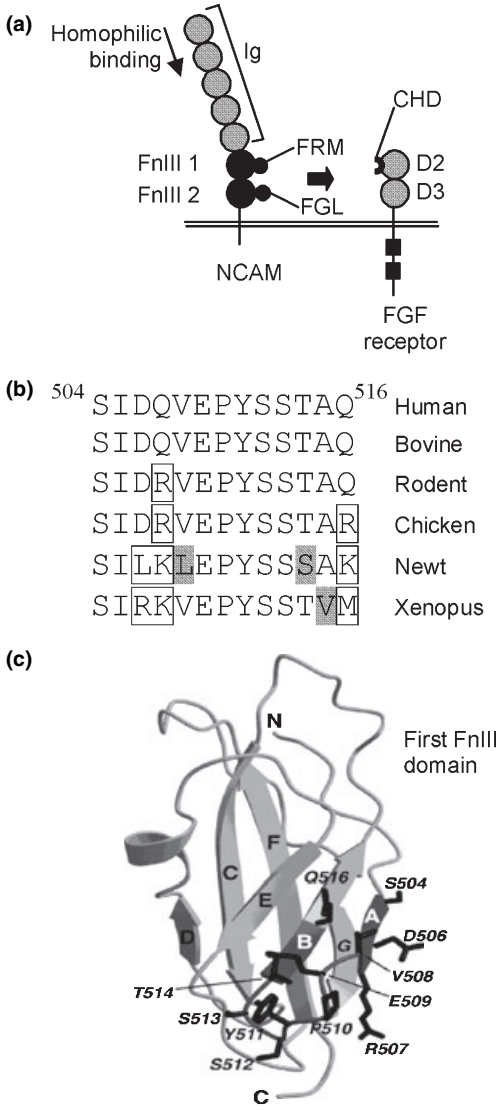


Fig. 1 The NCAM FRM is highly conserved and maps to a surface loop in the first FnIII domain. (a) Diagram depicting the interaction hypothesized between the FRM site on NCAM and the CHD site on the FGFR following homophilic binding of NCAM. The two-Ig domain form of FGFR1 is shown. The CHD site in the second Ig-like domain of FGFR1 (D2) and the FRM in the first FnIII domain of NCAM are at a similar distance from the membrane. An FGL site in the second FnIII domain of NCAM identified by Kiselyov *et al.* (2003) binds to FGFR D3 at an unidentified site. The flexibly hinged depiction of NCAM is taken from Johnson *et al.* (2004). (b) Sequence alignment of the NCAM FRM across species. Conservative substitutions are shaded and amino acids differing from human/bovine are boxed. (c) Model of the first FnIII domain of NCAM based on the experimental structure of the 30% identical D3 domain of gp130 (Chow *et al.* 2001). Polypeptide chain termini and β-strands A-G are labelled. The FRM is in black; selected side chains are shown as stick models and are labelled.

Materials and methods

Neurite outgrowth

For cerebellar neurons, post-natal day 4 (PND4) rat cerebella were trypsinised, dissociated and plated at 2500 per well in Sato's medium in the absence or presence of peptides, NCAM antibody or FGF2, on monolayers of parental, NCAM- (140 kDa) or amyloid precursor protein (APP695)-expressing 3T3 cells established by plating 80 000 3T3 cells overnight in eight-chamber LabTek slides sequentially coated with poly lysine and fibronectin. For hippocampal neurons, embryonic day 18 rat hippocampi were isolated, trypsinised, dissociated and plated as above, but in Ham's F12 medium supplemented with B27 supplement. After 16 h, co-cultures were fixed with 4% paraformaldehyde and neurons visualised using anti-GAP 43 antibody for determination of neurite length using fluorescence microscopy and Zeiss KS300 image analysis software. Slides were systematically scanned and the mean length of the longest neurite per neuron (cerebellar neurons) or the mean total neurite length per neuron (hippocampal neurons) was determined for 150–250 neurons in each well.

Survival/proliferation

For 3T3, L6 or L6/FGFR1cβ survival, 6000 quiescent cells were plated per well of a 96-well plate in DMEM (Dulbecco's modified Eagle's medium) containing 0.5% FCS (foetal calf serum) (survival) or DMEM containing 10% FCS (proliferation). After 1 day, an index of cell number at the start of the experiment was determined by adding CellTiter 96 [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution (Promega, Madison, WI, USA), incubating for 4 h, then reading optical density (OD) at 490 nm. In the rest of the wells, medium was removed and replaced with DMEM (survival assay) or DMEM containing 0.5% FCS (proliferation assay) alone or supplemented with FGF2, FRM peptides, insulin, or FCS, four wells per condition. Relative cell number after 2 days (proliferation) or 3 days (survival) was determined using MTS as above. 3T3 cell survival was also assessed morphologically by fixing cells with 4% paraformaldehyde, then staining the actin cytoskeleton with rhodamine-conjugated phalloidin (Sigma, St Louis, MO, USA). For neuronal survival, 40 000 PND4 cerebellar neurons were plated per well in a 96-well plate overnight in Sato's medium containing 2% FCS. The following day the medium was removed and replaced with DMEM alone or supplemented with 400 nM FRM-10d peptide (four wells per condition). The neurons were cultured for a further 24 h, after which relative cell number remaining was determined using MTS as above. The presence of contaminating glial cells was monitored in parallel by immunocytochemistry and cultures were confirmed to be > 95% neurons throughout.

Oligodendrocyte culture

Oligodendrocyte progenitors were purified (~98% pure) from trypsinised cell suspensions of PND4 rat cortices by immunopanning using the O4 monoclonal antibody (Barres *et al*. 1992). Specifically bound cells were washed off and plated at 5×10^4 cells/cm^2 on poly ornithine-coated glass coverslips in DMEM/10% FCS before being switched to defined oligodendrocyte progenitor medium (OLP) (Hardy and Reynolds 1993). After 3 days, FRM-10d peptide, insulin, or both, was added and the cells incubated for a further 3 days before cell survival was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The percentage of oligodendrocyte survival is MTT-positive cells expressed as percentage total cells. Surviving cells express both O4 and myelin basic protein (MBP) and represent pre-myelinating oligodendrocytes. In the absence of supplements, cell viability at 6 days was only 8%. For O4/MBP, co-immunolabelling cells were incubated with O4 monoclonal antibody diluted 1 : 50 in phosphate-buffered saline (PBS) containing 10% goat serum, followed by rhodamine-conjugated goat anti-mouse IgM. After fixing, cells were permeablised in 0.1% TritonX100, incubated with anti-MBP antibody diluted 1 : 200 in PBS containing 10% goat serum, then biotin-conjugated goat anti-rabbit antibodies and Alexa 488-conjugated streptavidin.

BrdU incorporation

Quiescent 3T3 cells were plated in 96-well plates at 30 000 cells/well in DMEM/0.5% FCS and cultured for 3 days before addition of FGF2 or FRM-10d and 12 μM BrdU, in the same medium (four wells each). Cells were fixed with ice-cold methanol, treated with 20% HCl, neutralized with 100 mM Tris pH 7.5. Non-specific binding was blocked with 10% goat serum, then cells were incubated with anti-BrdU antibody (DAKO) and horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Pharmacia, Little Chalfont, UK). Relative antibody binding per well was determined by reading the OD at 650 nm following incubation with K-blue substrate (Neogen, Europe Ltd, Ayr, UK) for 30 min. Identical sister plates were incubated with CellTiter 96 MTS reagent (Promega) instead of being fixed, to confirm that cell number did not change over the 7-h treatment period.

Immunoprecipitation and western blotting

Overnight cultures of 2×10^6 3T3 cells or 1.5×10^5 L6/FGFR1cβ were serum starved for 5 h, then incubated with FGF2 or FRM peptide for 10 min at 37°C. Cells were lysed in ice-cold RIPA buffer (50 mM Tris pH 7.4, 1% NP40, 0.2% deoxycholate, 150 mM NaCl, 1 mM EGTA) containing protease inhibitors, NaF and phosphatase inhibitor bpV (Calbiochem, San Diego, CA, USA). After removal of insoluble material, lysates were incubated overnight at 4°C with either Flg antibody (C-15 Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 4G10-agarose (UBI) -recognizing FGFR1 or tyrosine phosphorylated proteins, respectively. Flg immunoprecipitations were subsequently incubated with Protein A sepharose (Amersham Pharmacia) for 2 h at 4°C. Washed beads were boiled in sodium dodecyl sulfate (SDS) sample buffer, proteins separated by SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed for tyrosine phosphorylated proteins with 4G10 monoclonal antibody (UBI) or with anti-FRS-2 polyclonal antibody (H-91, Santa Cruz Biotechnology) and HRP-conjugated anti-mouse or anti-rabbit secondary antibodies, respectively (Bio-Rad Laboratories, Hercules, CA, USA). Antibody binding was detected using ECL (Amersham Pharmacia).

FGF2, peptides and NCAM antibody

Recombinant human FGF2 (157 aa) was purchased from R & D Systems Ltd (Minneapolis, MN, USA), and peptides from Mimotopes Ltd. (Melbourne, Vic, Australia) Monomeric peptides were synthesized to > 95% purity with acetyl and amide blocked N- and

C-termini, respectively. The FRM-10d dendrimeric peptide was synthesized with blocked N- and C-termini attached to backbone and side chain amino groups of two lysine residues. These were in turn attached to the backbone and side chain amino groups of a third lysine with a free C-terminus to result in a peptide comprising four copies of the FRM-10 sequence attached to a triple lysine backbone. The monoclonal antibody 123C3 was purchased from Abcam Ltd (Cambridge, UK).

Results

A first FnIII domain antibody inhibits NCAM-stimulated neurite outgrowth

Cerebellar neurons plated on monolayers of 3T3 cells stably expressing NCAM transgenes extend neurites over a 16-h period that are approximately twice as long as neurites extended on untransfected control 3T3 monolayers (Doherty *et al*. 1991). The advantage of this very well-characterized assay system is that it relies on endogenous neuronal NCAM isoforms, and NCAM (140 kDa) is presented to neurons in the context of a cellular substratum approximating the physiological *in vivo* presentation of the CAM. Using this co-culture assay, we first tested the functional importance of the first FnIII domain for neurite outgrowth stimulated by NCAM using monoclonal antibody 123C3 that recognizes the domain in intact conformation (Moolenaar *et al*. 1990). Increasing concentrations of 123C3 (supplied as tissue culture supernatant) dose-dependently inhibited NCAM-stimulated neurite outgrowth back to baseline lengths (Fig. 2a). The effect was specific for outgrowth stimulated by NCAM, as even the highest concentration of 123C3 had no effect on basal outgrowth over control monolayers (Figs 2a and b), or outgrowth stimulated by monolayers expressing amyloid precursor protein (Fig. 2b). The ability of this first FnIII domain antibody to completely inhibit NCAM-stimulated outgrowth indicates that the domain may contain a functionally important site.

FRM peptide displays dual NCAM-mimetic and NCAM/FGF inhibitory activity

To investigate the significance of the FRM site within the first FnIII domain, we synthesized a peptide corresponding to the 13-amino acid FRM (FRM-13, Table 1) and tested its effect on neurite outgrowth in the presence or absence of substratum NCAM in co-culture experiments. The results in Fig. 3(a) show that at low concentrations FRM-13 dose-dependently decreased the NCAM response back to baseline outgrowth on control 3T3 monolayers, with an IC_{50} of ~6 μM. However, beyond 25 μM the peptide actually stimulated neurite outgrowth on both control 3T3 and NCAM-expressing monolayers. The effect was dose-dependent and considerable, outgrowth over control 3T3 monolayers in the presence of 100 μM being equivalent to that stimulated by NCAM itself

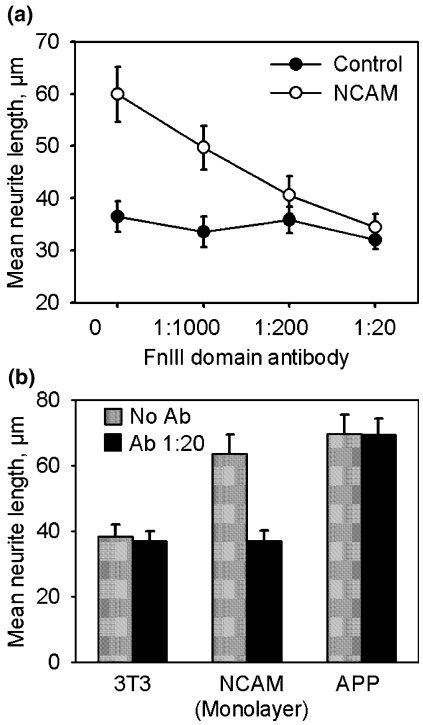


Fig. 2 A first FnIII domain antibody inhibits specifically NCAM-stimulated neurite outgrowth. (a) Effect of increasing concentrations of 123C3 monoclonal antibody recognizing the intact first FnIII domain of NCAM on neurite outgrowth over control or NCAM-expressing 3T3 monolayers. (b) Effect of 123C3 antibody (diluted 1 : 20) on neurite outgrowth stimulated by either 3T3-expressed amyloid precursor protein or NCAM. For each data point mean neurite length is the mean length of the longest neurite per cell measured for 150–250 PND4 rat cerebellar neurons.

Table 1 FRM and control peptides

Type	Sequence	Name
Monomeric	SIDRVEPYSSTAQ	FRM-13
	DRVEPYSSTA	FRM-10
Substituted	ARVEPYSSTA	
	DRSEPYSSTA	
	ARSEPYSSTA	
Truncated	DRVE	
	PYSSTA	
	SIDRV	
Cyclic	–CGDRVEPYSSTAGC–	FRM-10cyclic
Dendrimeric	$[(DRVEPYSSTA)_2K]_2K$	FRM-10d
	$[(ARSEPYSSTA)_2K]_2K$	ARSE-10d
	$[(ADTRSVSEYP)_2K]_2K$	Scrambled-10d

Underlining denotes amino acids substituting for residues in the wild-type peptide sequence.

(Fig. 3a). The stimulatory activity of the peptide was robust, reproducible, and retained in a shorter FRM-10 peptide (Table 1) that stimulated outgrowth with a sigmoidal dose–

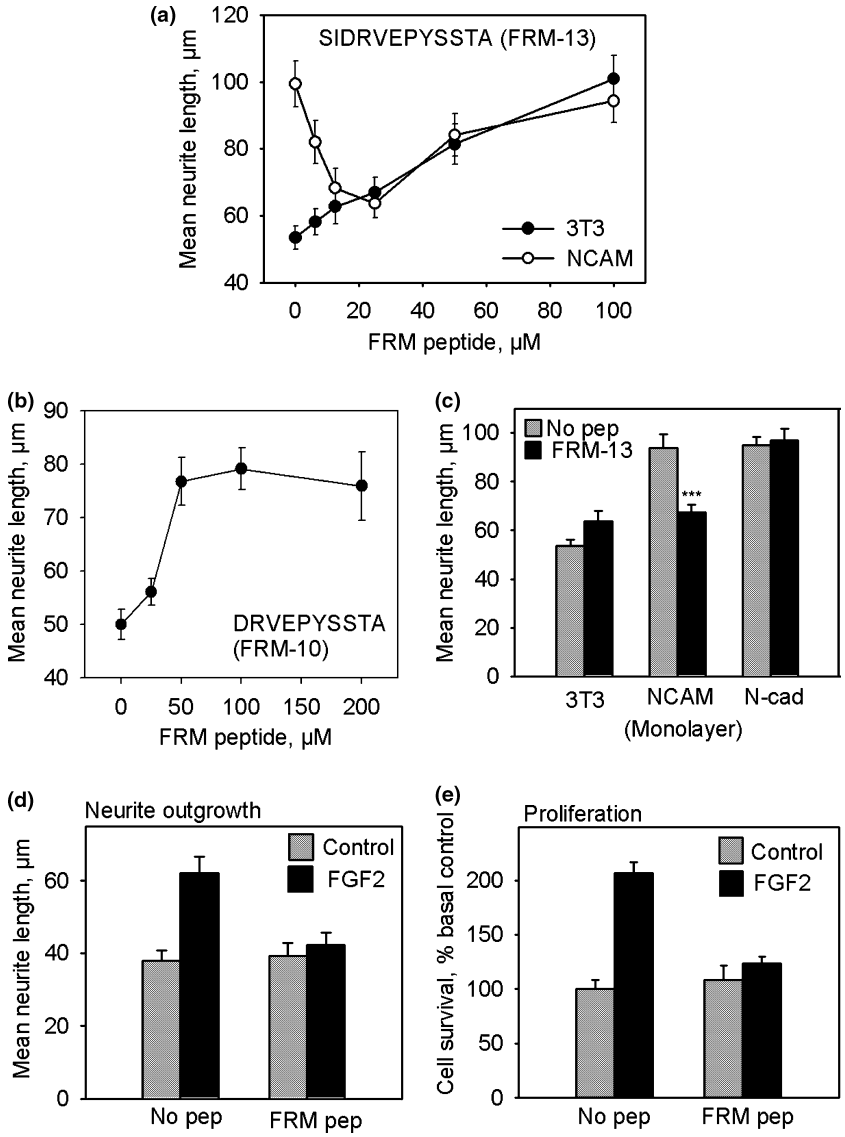


Fig. 3 FRM peptide displays dual NCAM-mimetic and NCAM/FGF inhibitory activity. (a) Neurite outgrowth over control or NCAM-expressing 3T3 monolayers in the presence of 0–100 µM FRM-13 peptide. (b) Stimulation of neurite outgrowth over control 3T3 monolayers by FRM-10 peptide (pooled from eight independent experiments). (c) Neurite outgrowth from cerebellar neurons cultured on control 3T3 monolayers or monolayers expressing NCAM or N-cadherin, in the absence (grey bar) or presence (black bar) of NCAM FRM-13 peptide (12.5 µM) pooled from four independent experiments. ***$p < 0.025$. (d) The increase in neurite outgrowth on monolayers of 3T3 cells in medium containing FGF2 (1 ng/mL) (black bars) compared with control medium (grey bars) is inhibited by the additional presence of FRM-10 (25 µM). (e) Increased 3T3 cell proliferation after 2-day culture in DMEM/0.5% FCS alone (grey bars), or supplemented with FGF2 (10 ng/mL) (black bars) is inhibited by the additional presence of FRM-10 (50 µM). The increase in cell number for each data point was determined using an MTS colorimetric assay, each value being the mean ± SEM from four identical wells. Cell proliferation is expressed as percentage of the basal value in the absence of FGF2 supplement. (a–d) PND4 rat cerebellar neurons were used and neurite outgrowth measured as for Fig. 2.

response curve and was maximally active at 50 µM (Fig. 3b, pooled from eight independent experiments). Thus, the FRM peptide displays dual NCAM-mimetic and NCAM inhibitory activity at high and low concentrations, respectively, both of which are relevant for understanding the activity of the FRM site on NCAM. The inhibitory effect was specific as 12.5 µM FRM-13 did not inhibit basal outgrowth over control monolayers (Figs 3a and c) or outgrowth stimulated by N-cadherin (Fig. 3c), but specifically inhibited the NCAM response back to basal levels. In addition, subthreshold concentrations of FRM-10 (25 µM) fully inhibited neurite outgrowth stimulated by FGF2 (Fig. 3d) and also FGF2-stimulated 3T3 fibroblast proliferation (Fig. 3e). Thus, monomeric FRM specifically inhibits NCAM and FGF responses at low concentrations, but at saturating concentrations itself stimulates neurite outgrowth equivalent to NCAM, the behaviour of an inverse agonist.

Residues within DRVE are important for FRM stimulatory activity

The ability of the NCAM FRM peptide to itself stimulate neurite outgrowth was initially surprising, particularly as a peptide corresponding to a related site in N-cadherin showed no such bioactivity (Williams *et al*. 2001). We therefore set out to determine the amino acids within the NCAM FRM that were important for the stimulatory effect, beginning with the activity of peptides corresponding to the N- and C-termini, DRVE and PYSSTA, respectively (Table 1). Whilst the PYSSTA peptide retained the ability to fully inhibit the NCAM response, albeit with a higher IC_{50} of 60 µM, it contained no stimulatory activity even at 300 µM (Fig. 4a). However, the DRVE peptide showed a profile of inhibition/stimulation (Fig. 4b) that was very similar to that of FRM-13 (Fig. 3a) and FRM-10 (Fig. 4d) despite being less active, inhibiting the NCAM response with an IC_{50} of

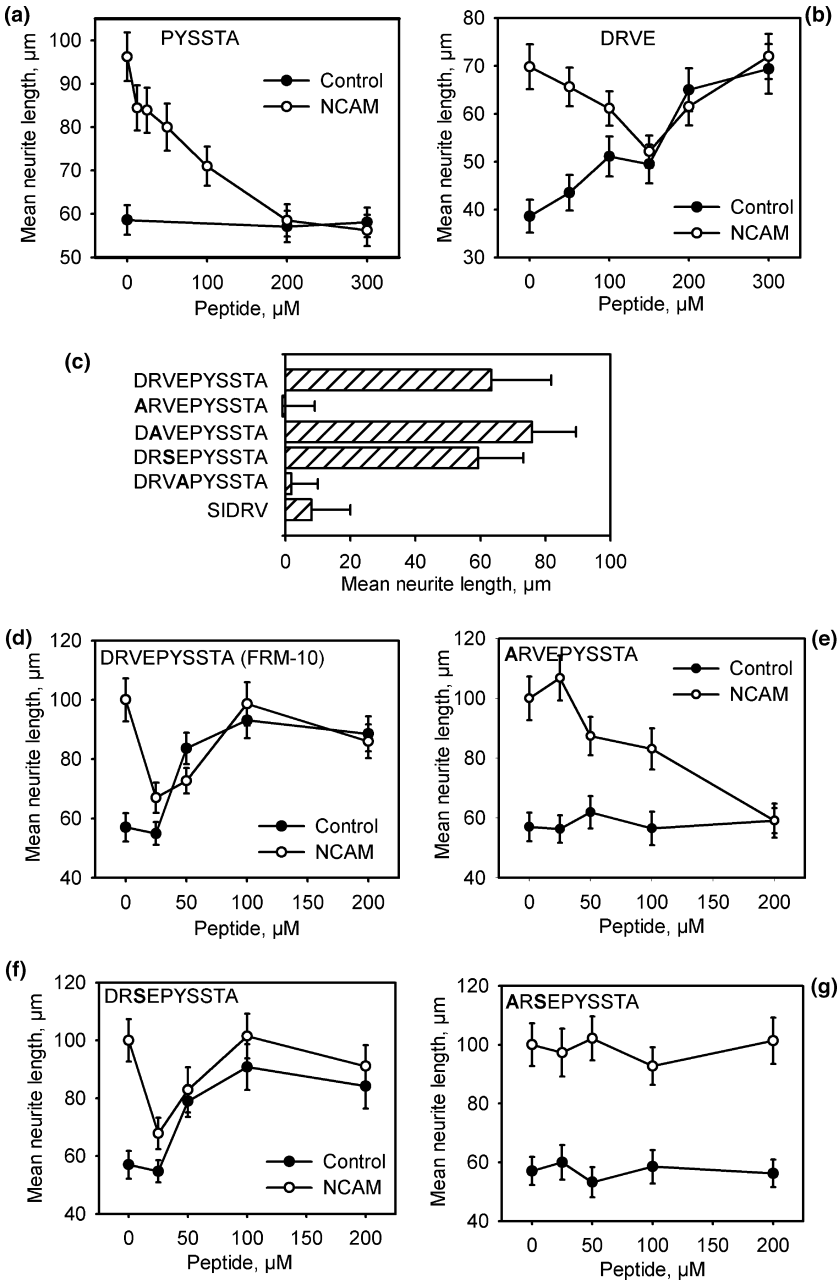


Fig. 4 Mapping activity within the FRM. Neurite outgrowth of PND4 rat cerebellar neurons over control or NCAM-expressing 3T3 monolayers in the presence of (a), PYSSTA peptide (0–300 μM) or (b) DRVE peptide (0–300 μM). (c) Neurite outgrowth stimulation by FRM-10 wild-type, singly and doubly substituted peptides shown and of truncated peptide SIDRV N-terminal sequence of FRM-13, all at 100 μM. (d–g) Concentration-response inhibitory and stimulatory effects of wild-type and substituted peptides on control and NCAM-expressing monolayers. (d) Wild-type FRM-10, (e) effect of D/A substitution, (f) Effect of V/S substitution and (g) Effect of double substitution D/A and S/V. Bold residues denote amino acid substitutions.

~75 μM and stimulating neurite outgrowth equivalent to NCAM at 300 μM. Interestingly, the model of NCAM's first FnIII domain (Fig. 1c) indicated that DRVEPY within the FRM motif was particularly solvent exposed (> 50% surface area accessible to a 1.4 A probe).

We next determined the effect of specific amino acid substitutions within the DRVE region on the stimulatory activity of the FRM-10 peptide at 100 μM (Table 1). Alanine (A) was used to substitute for aspartate (D), arginine (R) and glutamate (E) whilst serine (S) rather than similarly hydrophobic alanine was used to substitute for valine (V). The results in Fig. 4(c) show that D/A and E/A substitutions abolished the stimulatory activity of FRM-10 while peptides with R/A and V/S substitutions retained activity. A peptide SIDRV from the N-terminal of FRM-13 contained no stimulatory activity. The concentration–response inhibitory/stimulatory effect of wild-type FRM-10 (Fig. 4d) was compared with that of the D/A substituted peptide (ARVEPYSSTA) (Fig. 4e) and the V/S substituted peptide (DRSEPYSSTA) (Fig. 4f). Although the D/A substituted peptide remained non-stimulatory even up to 200 μM, it retained the ability to inhibit neurite outgrowth stimulated by NCAM, albeit with a higher IC_{50} of 100 μM (Fig. 4e). In contrast, substitution V/S did not alter the activity profile of the peptide, which was identical to wild-type FRM-10 (Fig. 4f). Surprisingly, however, substitution of V/S and

D/A together resulted in a peptide (ARSEPYSSTA) which lacked both inhibitory and stimulatory activity (Fig. 4g), showing that the V does contribute to peptide-target binding, although this is only unmasked when the dominant aspartate is also absent. Comparison of the relative ability of these peptides to inhibit or mimic NCAM stimulated outgrowth indicates that residues across the whole FRM contribute to inhibition (PYSSTA and DRVE), while the stimulatory activity appears to specifically depend upon amino acids within the DRVE region.

FRM neurite outgrowth activity is FGFR-dependent and enhanced by cyclization or multimerisation

Because FRM fully mimics the outgrowth response of NCAM itself (Fig. 3a) and outgrowth stimulated by NCAM is FGF-receptor dependent (Saffell *et al*. 1997), we investigated the FGF receptor dependence of the peptide effect using neurons lacking functional FGF receptors. Cerebellar neurons from transgenic mice in which FGF receptor function was disabled by expression of dominant-negative FGF receptors have previously been shown to be unable to extend neurites in response to NCAM (Saffell *et al*. 1997). We tested the response of these neurons to FRM-10 and found that they were likewise unable to extend longer neurites in response to the peptide, although the neurons from wild-type mice approximately doubled in neurite length (Fig. 5a), indicating that the neurite outgrowth response stimulated by FRM is dependent on FGF receptor function in the responding neurons.

FRM on our model of the first FnIII domain (Fig. 1c) maps to a surface exposed loop, a conformation unlikely to be retained in the linear FRM peptide. Cyclizing enhanced the neurite outgrowth stimulating activity of FRM-10 50-fold, FRM-10cyclic giving maximal stimulation of neurite outgrowth from cerebellar neurons at 1 μM compared with 50 μM for the linear peptide (Fig. 5b). The FRM-10cyclic peptide similarly stimulated neurite outgrowth from E18 hippocampal neurons, with maximal increase in length being induced by 1–10 μM peptide, equivalent to neurite outgrowth stimulated by NCAM itself (Fig. 5c). In an alternative modification to multimerize the peptide, we synthesized a dendrimeric FRM peptide (Table 1) consisting of four FRM-10 linked at the

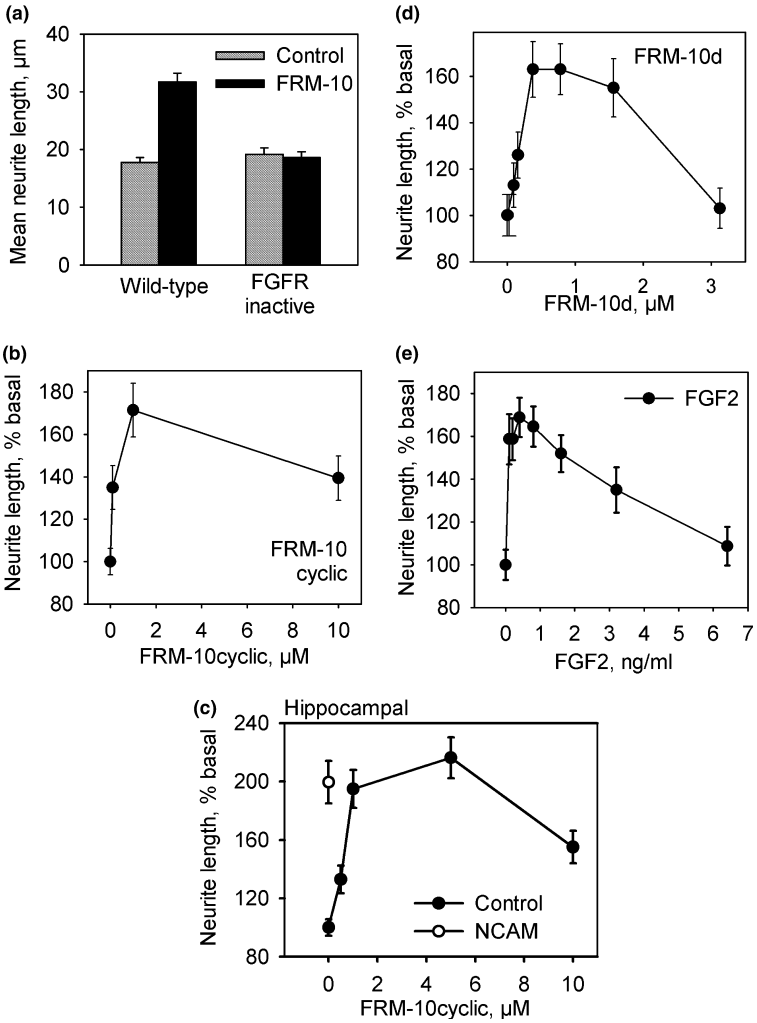


Fig. 5 FRM neurite outgrowth activity is FGFR-dependent and enhanced by structural modification. (a) Neurite outgrowth of PND4 cerebellar neurons from wild-type mice or mice expressing dominant-negative FGFRs in neurons (FGFR inactive) on 3T3 monolayers in the absence (grey bars) or presence (black bars) of FRM-10 (100 μM). (b, c) Dose-dependent stimulation by cyclic peptide FRM-10cyclic of neurite outgrowth over 3T3 monolayers from (b) PND4 rat cerebellar neurons and (c) E18 rat hippocampal neurons. Open circle in (c) shows neurite length of hippocampal neurons cultured on NCAM-expressing 3T3 monolayer for comparison. (d, e) Dose-dependent stimulation of neurite outgrowth from cerebellar neurons by (d), dendrimeric peptide FRM-10d, and (e) FGF2. (b–d) Neurite outgrowth is expressed as percentage basal mean neurite length on 3T3 monolayers to allow pooling of three experiments with differing basal values.

C-terminus to a triple-lysine backbone (FRM-10d). In neurite outgrowth assays, this peptide was 125 times more active than linear monomeric FRM-10, stimulating neurite outgrowth optimally at 0.4 μM (Fig. 5d) while control peptide ARSE-10d (Table 1) contained no activity (not shown). Notably, the most active peptides, FRM-10cyclic and FRM-10d, showed reduced neurite outgrowth when peptide exceeded the optimal stimulatory concentration (Figs 5b–d), a biphasic dose–response curve similar to the response of these neurons to FGF2 (Fig. 5e; Williams *et al*. 1994b).

FRM promotes cell survival but not proliferation

Intrigued by the possibility that these small synthetic FRM peptides were able to activate neuronal FGF receptors to stimulate neurite outgrowth, we tested whether they could mimic effects of FGF2 on non-neuronal cells that express FGF receptors but not NCAM, namely survival and proliferation of NIH3T3 cells. To investigate specifically the survival effect of FGF2 and FRM, 3T3 cells were plated at low density in low serum-containing medium, then switched after a day to serum-free medium alone or supplemented with test factors. Cells were cultured for 3 days, whereupon the relative cell number remaining per well was quantitated using MTS reagent. Figure 6(a) shows the comparative ability of FGF2, FRM-10d, FRM-10cylic and a scrambled FRM-10d peptide to stimulate 3T3 cell survival. While FRM-10d and FRM-10cylic stimulated cell survival equivalent to FGF2, the FRM-10d scrambled peptide was inactive, unable to stimulate cell survival even at 200 μM. The morphology of 3T3 cells cultured for 3 days in DMEM alone, or supplemented with insulin, FGF2 or FRM-10d (Fig. 6b) shows that the peptide is as effective as insulin and FGF2 at preventing cell death induced by serum deprivation. The dose-dependent effects of FGF2 and FRM are shown in Figs 6(c and d). FGF2 stimulated 3T3 cell survival dose dependently to reach a maximum, in the presence of 0.1 ng/mL FGF2, of 171 ± 5% over basal survival in the absence of supplement (Control). Dose-dependent increases in cell survival were similarly stimulated by FRM-10d and FRM-10cyclic (Fig. 6d), which at 50 or 70 μM, respectively, increased cell survival to 225 ± 11 and 210 ± 7% of basal survival in the absence of supplement. We also tested the ability of FRM-10d to stimulate survival of other cell types. The peptide increased the survival of cerebellar neurons by 95 ± 38% above baseline survival in the absence of supplements (pooled from four independent experiments) at 400 nM, the same concentration that stimulated maximal neurite outgrowth from these cells (Fig. 4b). In addition, FRM-10d was as effective as insulin at stimulating survival of pre-myelinating oligodendrocytes purified from rat cortex (Fig. 6e). In the absence of supplement, fewer than 10% of oligodendrocytes survived over 6 days in culture compared with 29 ± 5.9% in the presence of FRM-10d (12.5 μM) and 24 ± 3.7% in the presence of 10 μg/mL insulin (pooled from three independent experiments). Whereas, in the absence of supplement, most pre-myelinating oligodendrocytes underwent degenerative changes, those surviving following supplementation with FRM-10d (6 μM) grew an extensive process network and expressed the myelin protein MBP (Fig. 6e).

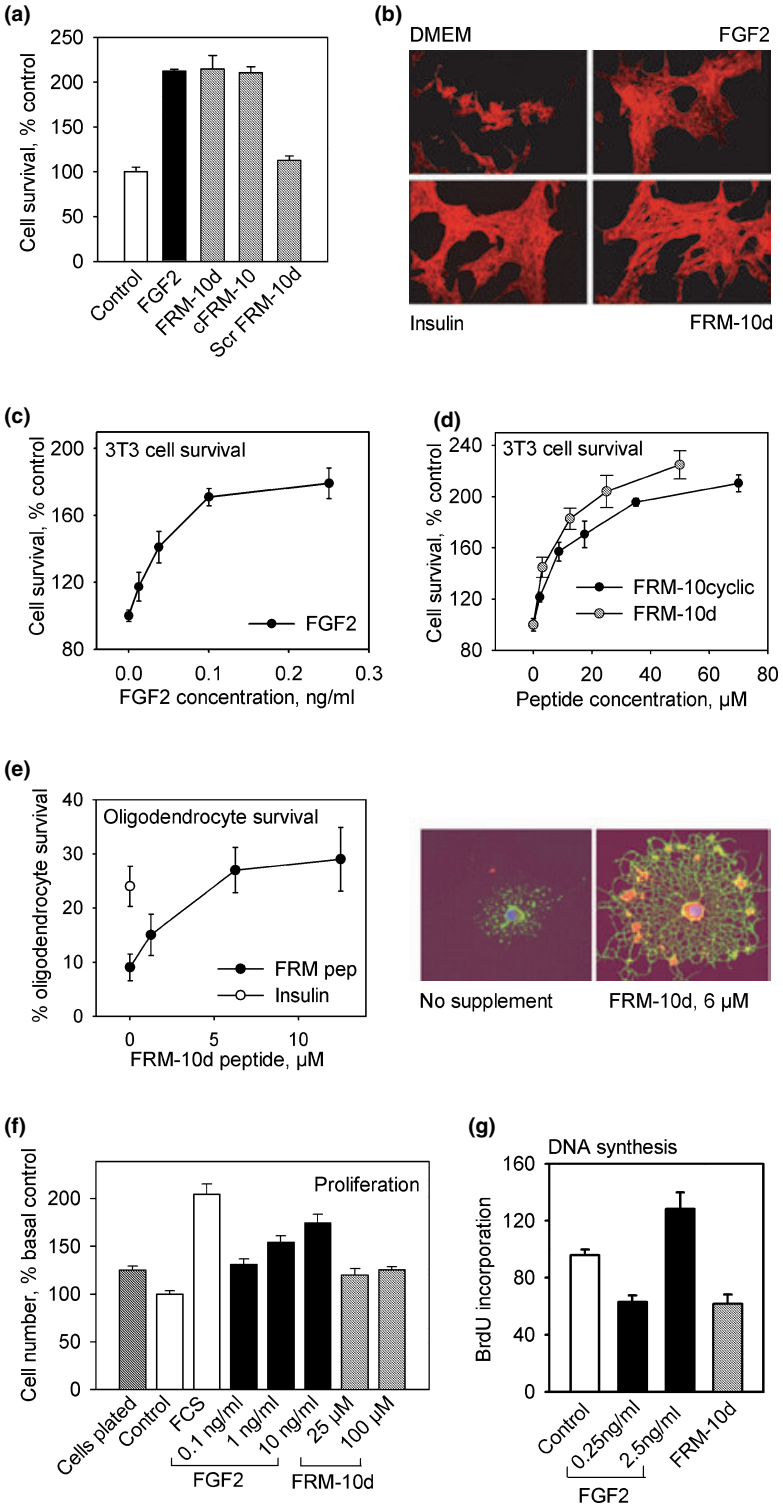
As FGF2 is also a potent mitogen for fibroblasts, we tested the mitogenicity of FRM-10d in MTS proliferation and DNA synthesis assays. 3T3 cells were plated in DMEM/10% FCS, then switched after 1 day to DMEM/0.5% FCS alone, or supplemented with test agents (FGF2, FRM-10d) or FCS for 2 days, after which relative cell number per well was determined using the MTS assay. The results in Fig. 6(f) show that cell number increased substantially in the presence of FCS, dropped slightly in basal medium (Control), and 1 and 10 ng/mL FGF2 stimulated proliferation dose-dependently. In contrast, 0.1 ng/mL FGF2 and 25–100 μM FRM-10d prevented the drop in cell number observed in basal medium (100% survival), but did not stimulate any increase in cell number above the plating value that would signify proliferation. Furthermore, FRM-10d and 0.1 ng/mL FGF2 decreased BrdU incorporation into 3T3 cells in a DNA synthesis assay in which 2.5 ng/mL FGF2 stimulated an increase in BrdU incorporation (Fig. 6g). These data indicate that FRM peptides mimic the cellular effects of low concentrations of FGF2, which also stimulate survival and differentiation but inhibit proliferation (Garcia-Maya *et al*. 2005).

FRM activates FGF receptors and proximal substrate FRS-2 and its survival activity is FGFR-dependent

To test the FGF receptor dependency of survival stimulated by FRM peptide, we used the L6 cell line that does not express FGF receptors in place of 3T3 cells in the MTS survival assay. L6 cells were plated at low density in DMEM/0.5% FCS, and switched after 1 day to DMEM alone or supplemented with 1% FCS, 1 μg/mL insulin, 0.1–10 ng/mL FGF2, or 25–100 μM FRM-10d peptide. Cells were cultured for 3 days, before relative cell number remaining was determined by MTS assay. Figure 7(a) shows that L6 cell-survival was supported by insulin and FCS but not by FGF2 or the FRM peptide. However, L6 cells engineered to express a FGFR1cβ transgene acquired the ability to survive in response to FRM peptide and FGF2, both of which stimulated a doubling in cell survival compared with basal survival in the absence of supplement (Fig. 7b). These data indicate that FRM-10d not only mimics the survival-stimulating effects of FGF2, but also that its effects are dependent on functional FGF receptor expression in the responding cells.

Our functional data show that FRM stimulates neurite outgrowth and cell survival that are FGF mimetic and FGF-receptor dependent. If these cellular effects result from direct or indirect peptide activation of FGFR signalling pathways, this should be detectable by western blotting for tyrosine phosphorylated FGF receptors and specific downstream receptor substrates. We first tested the ability of

FRM-10d to activate FGFR1, both in 3T3 cells expressing physiological levels of endogenous receptor and in L6 cells expressing the FGFR1cb transgene. Following 10-min incubation of cells with no supplement, FGF2 or FRM-10d, FGFR1 was immunoprecipitated using a specific antibody and activated receptor detected on western blots using anti-phosphotyrosine antibody 4G10 (UBI). The results in Fig. 7(c) show that FRM-10d (30 μM) stimulated activation of FGFR1 in 3T3 cells and L6/FGFR1b comparable with that stimulated by 10 ng/mL FGF2, but control peptide ARSE-10d was inactive (Figs 7c and d). One of the most proximal FGFR substrates is the 90-kDa



adapter protein FRS-2, which is rapidly tyrosine phosphorylated by activated FGFR (Kouhara *et al*. 1997). We compared tyrosine phosphorylation of FRS-2 immunoprecipitated from 3T3 cells treated with different concentrations of FGF2 or FRM-10d. Figure 7(e) shows dose-dependent activation of FRS-2 by 1 and 10 ng/mL FGF2 (but not 0.1 ng/mL) and activation by FRM-10d comparable with that of 10 ng/mL FGF2. The pooled results of three independent experiments show that the peptide stimulated a 8.3-fold increase in FRS-2 activation, compared with FGF2 at 0.1 ng/mL (1.6-fold), 1 ng/mL (5.5-fold) and 10 ng/mL (12.2-fold). These data show that the FRM peptide activates FGFR1 and the downstream substrate FRS-2.

Discussion

The penultimate membrane proximal domain of the cell adhesion molecules N-cadherin, NCAM and L1 contains a motif postulated to contribute to FGFR activation by these CAMs leading to neurite outgrowth (Doherty and Walsh 1996). Here, we show for the first time that a peptide corresponding to this motif (FRM) in the first FnIII domain of NCAM stimulates FGFR activation and FGFR-dependent neurite outgrowth and cell survival. At low concentrations, however, monomeric FRM acts as an inverse agonist, specifically inhibiting NCAM and FGF-dependent cellular responses. An antibody recognizing the FRM-containing first FnIII domain blocks NCAM-stimulated neurite outgrowth, indicating the importance of the domain for NCAM function. These findings provide the first evidence that the FRM of NCAM contains bioactivity consistent with its postulated involvement in FGFR activation leading to neurite outgrowth.

NCAM is an abundant molecule and its concentration on the neuronal surface has been calculated as 50 μM (Kiselyov *et al*. 2003). Thus, the concentrations of FRM peptide required to stimulate neurite outgrowth in this study (0.4–50 μM) are within the physiological range of FRM-containing NCAM on the neuronal surface.

The stimulatory effects of the FRM peptide on cells are substantial. Mean neurite lengths in the presence of the peptide are at least as high as in the presence of NCAM itself or FGF2, and 3T3 and oligodendrocyte survival stimulated by FGF2 and insulin is matched by FRM. Our finding that FRM activates FGFRs, even in cells that do not express NCAM, and stimulates outgrowth and survival that is FGFR dependent, indicates that the cellular responses result from FRM peptide stimulation of FGF receptors either upstream of or at the level of the receptor itself. Interestingly, peptide activity differs between cell types in the order neurons > oligodendrocytes >> fibroblasts, perhaps indicating that the receptor microenvironment and/or cofactor expression on the cell surface is optimal in cells that normally express NCAM.

While our results show that a peptide corresponding to the FRM in the first FnIII domain of NCAM stimulates FGFR activation and FGFR-dependent cellular responses, Kiselyov *et al*. (2003) have shown in a separate study that a peptide (FGL) from the second FnIII domain also stimulates FGFR activation and neurite outgrowth. What is the significance of the finding that two completely different NCAM peptides from neighbouring domains can independently stimulate FGFR activation and NCAM-mimetic neurite outgrowth? The FGL site comprises an ascending loop at the N-terminal end of FnIII domain 2 (Kiselyov *et al*. 2003), and the FRM maps to a descending loop at the C-terminal end of FnIII domain 1 in our model (Figs 1c and 8). There are very few rotations of the two domains that would not place the loops in close apposition. We hypothesize that these loops together comprise the FGFR binding site of NCAM, and this is depicted in the model diagram shown in Fig. 8. This is consistent with the suggestion of Kiselyov *et al*. (2003) that both FnIII domains contribute to the FGFR binding site, based on their finding that, while recombinant FnIII domains 1 and 2 together bound to FGFR1 by surface

Fig. 6 FRM stimulates FGFR-dependent cell survival but not proliferation. (a) Comparison of 3T3 cell survival over 3 days in the presence of DMEM alone (Control), or supplemented with FGF2 (0.1 ng/mL), FRB-10d (25 μM), cyclic FRM-10 (40 μM), and scrambled FRM-10d (200 μM). (b) Morphology of 3T3 cells after 3-day culture in DMEM alone or supplemented with either insulin (10 μg/mL), FGF2 (0.1 ng/mL) or FRM-10d peptide (25 μM), visualized by TRITC-phalloidin staining of the actin cytoskeleton. (c) Dose-dependent enhancement of 3T3 cell survival after 3-day culture in DMEM alone, or supplemented with FGF2, or (d) FRM-10d or FRM-10cyclic peptides. Survival is expressed as a percentage relative to survival in control medium (DMEM alone). (e) Effect of FRM-10d peptide (0–12.5 μM) or insulin (10 μg/mL) on survival of pre-myelinating oligodendrocyte precursors over 6 days in culture. The graph shows the pooled results of three independent MTT survival assays, representing the mean survival ± SEM. The image shows that in the absence of peptide cells underwent degenerative changes (No supplement, left) but in the presence of 6 μM FRM-10d peptide cells grew an extensive process network and expressed the myelin protein MBP (red) (right image). O4 mAb staining is shown in green. (f) Proliferation response of 3T3 cells plated in DMEM/10% FCS after 2-day culture in DMEM/0.5% FCS alone (Control) or supplemented with 1% FCS, FGF2 (0.1–10 ng/mL), or FRM-10d peptide (25–100 μM). The effect of FGF2, insulin and FRM-10d is compared with basal cell number in the absence of supplement (Control) and the starting cell number on the day the cells were switched to DMEM/0.5% FCS (Cells plated). Cell numbers were quantitated from quadruplicate wells for each data point using MTS colorimetric reagent, and are expressed as percentage cell number in control wells. (g) Effect of FRM-10d peptide (200 μM) on DNA synthesis in 3T3 cells, compared with 0.25 or 2.5 ng/mL FGF2. DNA synthesis during 7-h incubation with FGF2 or peptide in the presence of BrdU was assessed using a specific antibody to detect BrdU incorporation followed by ELISA quantitation, measured in arbitrary OD units.

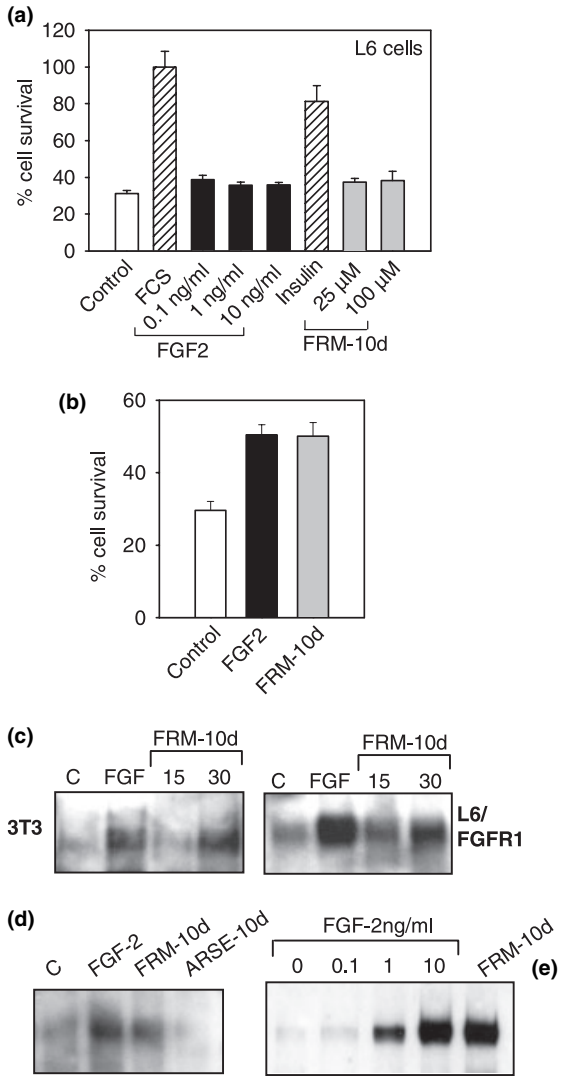


Fig. 7 FRM activates FGFR and FRS-2 and its survival activity is FGFR dependent. (a) Survival of FGFR-deficient L6 cells over 3-day culture in the presence of DMEM alone (Control), or supplemented with 1% FCS, FGF2 (0.1–10 ng/mL), insulin (1 μg/mL), and FRM-10d peptide (25–100 μM). (b) Survival of FGFR1β-expressing L6 cells (L6/FGFR1β) in the presence of DMEM alone, or supplemented with 1 ng/mL FGF2 or 125 μM FRM-10d peptide. (a, b) Cell survival is expressed as percentage survival relative to starting cell number, and was determined from quadruplicate wells using MTS reagent. (c) Tyrosine phosphorylation of FGFR1 immunoprecipitated from 2 million 3T3 cells (left) or 150 000 L6/FGFR1β cells (right) incubated for 10 min with either no supplement (c), 10 ng/mL FGF2, 15 or 30 μM FRM-10d peptide. Activated FGFR1 was detected with 4G10 anti-phosphotyrosine antibody. (d) Effect of 10 ng/mL FGF2, 50 μM FRM-10d and 50 μM control peptide ARSE-10d on tyrosine phosphorylation of FGFR1 in 3T3 cells, detected by anti-phosphotyrosine antibody 4G10. (e) Tyrosine phosphorylation of FGFR substrate FRS-2 in 3T3 cells stimulated with 0, 0.1, 1 and 10 ng/mL FGF2 or 50 μM FRM-10d. Immunoprecipitated from 2 million 3T3 cells with 4G10 phosphotyrosine antibody-conjugated agarose, blotted with FRS-2 antibody. (c–e) Representative blots of experiments repeated at least three times.

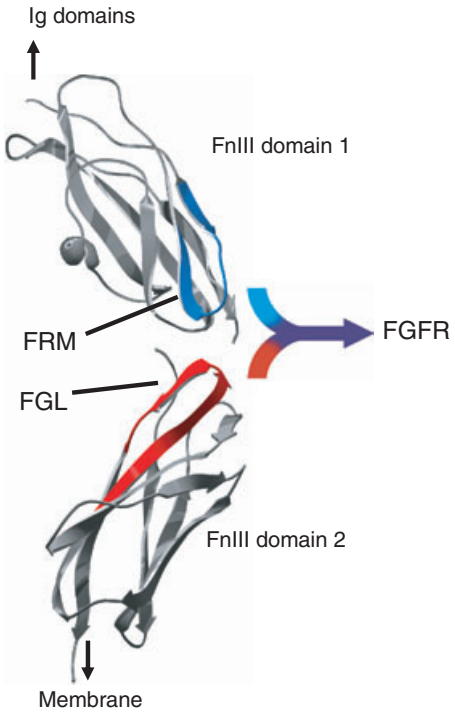


Fig. 8 Hypothetical scheme for FGFR activation by NCAM FnIII domains 1 and 2. We hypothesize that the FRM and FGL loops together comprise an FGFR-activating site on NCAM. Our model of the first domain suggests that FRM is a descending loop (Fig. 1c), and NMR structural analysis shows FGL to be an ascending loop in the second domain (Kiselyov *et al.* 2003). Peptides corresponding to the FRM loop in the first FnIII domain and the FGL loop in the second FnIII domain each activate FGFR1 and stimulate neurite outgrowth (this study; (Kiselyov *et al.* 2003; Cambon *et al.* 2004).

plasmon resonance with a K_d of 10 μM, removal of the first FnIII domain abolished all binding. It is not uncommon to find adjacent domains contributing target protein binding loops; indeed, the D2 and D3 FnIII domains of gp130 (structurally related to NCAM) provide a binding site for IL6 at the interface between the two domains (Chow *et al.* 2001). Kiselyov *et al.* (2003) were able to show using NMR that NCAM FnIII domain 2 bound via FGL to D3 of FGFR1; FRM is hypothesized to interact with FGFR D2 at the CAM homology domain (CHD) site (Doherty and Walsh 1996), which overlaps with a major FGF binding site on the receptor (Plotnikov *et al.* 1999; Pellegrini *et al.* 2000). Our finding that FRM peptide at low concentrations inhibits FGF as well as NCAM responses is consistent with this (Fig. 3). The data we describe will inform the structural and biochemical protein binding studies required to test the hypothesis that FRM and FGL together comprise an FGFR activation site on NCAM.

The finding that monomeric FRM peptide inhibits NCAM-stimulated outgrowth at low concentrations, but itself stimulates the response at saturating concentrations, is reminiscent of the behaviour of protean agonists of G-protein

coupled receptors (Milligan *et al*. 1995; Kenakin 2001). These are receptor agonists that produce a receptor-active state of lower intrinsic efficacy than does the natural ligand. Thus, they act as inverse agonists in the presence of ligand, but produce the full response themselves at saturating concentrations. For example, low concentrations of FRM monomer could compete with NCAM for receptor binding though, being less efficacious at activating the receptor than NCAM itself, have the effect of inhibiting NCAM-stimulated neurite outgrowth. However, the same peptide stimulates the full neurite outgrowth response equivalent to NCAM itself, but only at high (saturating) concentration as it has lower efficacy than NCAM. It would not be surprising for FRM peptides to be less efficient than NCAM at stimulating neurite outgrowth as NCAM itself contains both the FRM and FGL sites. Peptide multimerization increases efficacy, as the dendrimeric peptide FRM-10d is 125 times more active than the monomer, stimulating the full neurite outgrowth response equivalent to NCAM or FGF2 at 0.4 μM. Cyclization also increases FRM-10 activity 50-fold.

The predominant model for activation of growth factor receptors such as the FGFR is ligand-induced receptor dimerization (Spivak-Kroizman *et al*. 1994). It has been hypothesized that NCAM similarly dimerizes FGFRs by co-clustering following homophilic binding (Doherty and Walsh 1996). It is interesting therefore that the FGFR can be activated by small peptide fragments from NCAM (this study and Kiselyov *et al*. 2003). NCAM–FGF receptor interactions are subject to spatial restriction not applicable to soluble peptides, so it is possible that the receptor activation mechanism for NCAM differs from that of the FGFR activating peptides FRM (this study) and FGL (Kiselyov *et al*. 2003). The dendrimeric FRM-10d and FGL peptides could theoretically dimerize FGFRs, although without the extensive receptor contacts used by FGF (Plotnikov *et al*. 1999; Pellegrini *et al*. 2000). However, these peptides and the small FRM monomer that stimulates FGFR-dependent neurite outgrowth and survival at saturating concentrations may activate FGFRs by an alternative mechanism such as dimer stabilization and/or allosteric activation. It is becoming clear that many tyrosine kinase receptors, including the closely related epidermal growth factor receptor (Gadella and Jovin 1995), can be activated by an allosteric mechanism, exemplified by erythropoietin (Remy *et al*. 1999), as well as by ligand-induced dimerization. In this context, two different receptor dimer conformations have been observed in FGF : FGFR crystal structures, perhaps reflecting different modes of receptor activation (Plotnikov *et al*. 1999; Pellegrini *et al*. 2000), and FGFR signalling stimulated by FGF and NCAM has been shown to be somewhat divergent (Hinsby *et al*. 2004). We have shown in a separate study that even different concentrations of FGF2 can stimulate divergent responses in the same cell type, low and high concentrations stimulating survival and differentiation but inhibiting proliferation, and intermediate concentrations stimulating proliferation (Garcia-Maya *et al*. 2005). It is notable that, for cells such as fibroblasts that can proliferate in response to FGF2, FRM-10d, which activates FGF receptors in these cells and stimulates their survival, actually inhibits cell proliferation. This may provide a possible mechanism for the inhibition by NCAM of neural progenitor cell proliferation (Amoureux *et al*. 2000).

The bioactivity profile of the FRM peptide indicates that it may have useful therapeutic application, stimulating as it does cell survival and neurite outgrowth, but not proliferation *in vitro.* We have recently discovered that FRM-10d peptide enhances spatial learning and memory *in vivo* (Seymour, Regan and Saffell, unpublished observations). For multiple sclerosis, the development of novel therapeutic strategies designed to stimulate re-myelination of lesions requires an understanding of axonal signals for oligodendrocyte survival (Levine *et al*. 2001). NCAM, being expressed by both oligodendrocytes and axons, is a candidate for such a signal and indeed there is evidence that NCAM can support oligodendrocyte survival (Gard *et al*. 1996). Our finding that FRM stimulates survival of pre-myelinating oligodendrocytes indicates that the survival effects of NCAM may result from FRM-mediated activation of downstream survival pathways in oligodendrocytes following homophilic binding to axonal NCAM.

The results of the present study and of Kiselyov *et al*. (2003) establish the importance of the FnIII-like domains for NCAM function. The domains seem to be rich in molecular interaction sites as prion protein also binds to the FnIII domain I (Schmitt-Ulms *et al*. 2001), and it will be interesting to determine the significance of this binding for NCAM and prion protein function. In addition, the long chains of electronegative polysialic acid (PSA) that enhance the axon outgrowth, migration and plasticity-stimulating properties of NCAM (Doherty *et al*. 1992; Hu *et al*. 1996; Bruses and Rutishauser 2001) are added to the fifth Ig domain which lies adjacent to FnIII domain 1 containing the FRM site (Nelson *et al*. 1995). It is tempting to speculate that the steric effects of PSA (Yang *et al*. 1992) enhance NCAM activity by influencing the FRM/FGL site conformation to maximize binding and activation of FGFRs by NCAM. Similarly, the finding that homophilically bound NCAM contains a hinge, perhaps between the fifth Ig-like domain and the first FnIII domain (Johnson *et al*. 2004), may indicate a mechanism for coupling homophilic binding of NCAM to its activation of the FGFR, if the FGL/FRM binding site is exposed only after homophilic binding. Furthermore, alternative splicing of 'muscle specific domain' exon A introduces a putative hinge between the two FnIII domains, which may serve to modulate interactions in this region. Many other cell surface receptors of the Ig superfamily contain membrane proximal FnIII domains, and it will be interesting to see whether enrichment in protein binding sites that influence cellular responses is a general feature of these domains.

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