NCAM and the FGF-Receptor

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Structural Biology of NCAM

In mammals, there are two related neural cell adhesion molecules, termed NCAM1 [1, 2] and NCAM2 [3], which are encoded by two different genes. The two molecules belong to the immunoglobulin (Ig) superfamily and have similar domain structure: they consist extracellularly of five Ig-like modules, two fibronectin type III (F3) modules and intracellularly of a cytoplasmic part of varying length. Not much is known about the NCAM2 function, structure and signalling and in the following, only NCAM1 will be considered, which for simplicity is termed NCAM.

NCAM is expressed as three major isoforms, which arise due to alternative splicing of a single gene consisting of at least 26 exons [4–9]. Two of the isoforms are transmembrane proteins, NCAM-A and NCAM-B, with apparent molecular weights of 180 kD and 140 kD, respectively (as determined by SDS-PAGE) while the third isoform, NCAM-C (with an apparent molecular weight of 120 kD), is glycosylphosphatidylinositol (GPI)-anchored. The structures of the first three Ig modules and the two F3 modules have been determined by X-ray crystallography and nuclear magnetic resonance (NMR) analysis [10–16] while the structures of the fourth and fifth Ig modules, as well as of the cytoplasmic part are still unknown. The Ig fold of the first three N-terminal fold is of the intermediate type and the other two Ig modules are also predicted to be of this type [10]. The extracellular part of NCAM is encoded by exons 1–14 (two exons per module) and is similar for the three major isoforms. The two transmembrane isoforms are in addition encoded by exons 16–19 for NCAM-A and exons 16, 17, 19 for NCAM-B. The NCAM-C isoform, which is GPI-anchored, additionally contains only exon 15. The extracellular part of NCAM may also contain additional short sequences such as the 30-basepair exon VASE (Variable Alternatively Spliced Exon), which is inserted in

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the middle of the fourth Ig module between exons 7 and 8, and exons a, b, c, AAG, inserted between the first and second F3 modules of NCAM. NCAM molecules are post-translationally modified by attachment of long chains of polysialic acid (PSA) to the fifth Ig module [17, 18] and the first F3 module [16].

NCAM Functions

NCAM is involved in the regulation of cell adhesion and neurite outgrowth [19–22] and plays a major role during development of the nervous system. NCAM also regulates synaptic plasticity, learning and memory consolidation [23–27]. During embryonic development, NCAM is heavily polysialilated and is widely expressed in the whole organism. However, in the adult it is mainly found in tissues of neural origin; the amount of NCAM polysialylation is markedly decreased [28, 29], whereas expression of exon VASE is increased [30], and the polysialilated form of NCAM (without exon VASE) is only expressed in areas which retain a high degree of plasticity such as the hippocampus and the olfactory bulb [31], indicating that a decrease in polysialylation together with an increase in expression of the VASE exon changes NCAM from a plasticity promoting molecule to a molecule that generates stability [20, 25].

NCAM regulates cell adhesion and neurite outgrowth by means of homophilic binding (NCAM self binding) and subsequent activation of intracellular signaling. The major signaling partner of NCAM is the fibroblast growth factor (FGF) receptor [15, 32]. NCAM is also known to activate non-receptor tyrosine kinases Fyn and FAK [33]. The NCAM mediated signaling results in activation of the mitogen-activated protein kinase (MAPK) pathway [34, 35]. At present, it is unclear whether the MAPK pathway is activated via the FGF-receptor or the Fyn/FAK complex (or maybe both).

NCAM is also involved in heterophilic interactions with other molecules, such as heparin, heparan/chondroitin sulfates [36–40], various types of collagen [40, 41], L1 [42], TAG-1/axonin-1 [43], neurocan [44], phosphacan [45], agrin [46], glial cell line derived neurotrophic factor (GDNF), GDNF family receptor α [47], ATP [15, 48, 49], glucocorticoid receptor [50], cellular prion protein [51], brain-derived neurotrophic factor (BDNF) [52], platelet-derived growth factor (PDGF) [53], rabies virus [54], spectrin [55] and various cytoskeletal components [56].

This review focuses on the structural basis for the FGF-receptor activation by NCAM. For review of the signaling pathways activated by NCAM, see [57–60].

Mechanism of the NCAM Homophilic Binding

The mechanism of the NCAM mediated homophilic binding has been the subject of much controversy. Several alternative models have been proposed, which involve binding between multiple NCAM modules in various configurations (see Fig. 1).

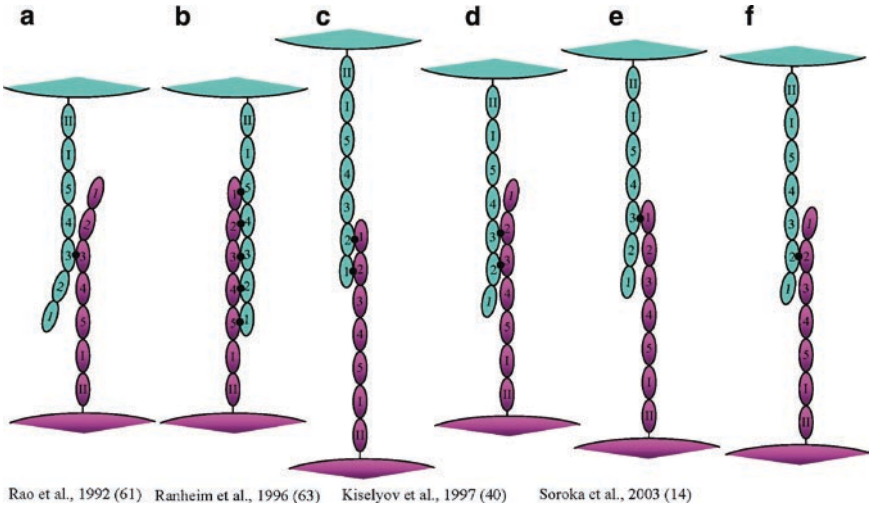

Fig. 1 Models for the NCAM homophilic binding proposed by different researchers. The contact site between the interacting modules is indicated by a *black dot*

In aggregation experiments with various truncated forms of NCAM, it was established that the third Ig module of NCAM was important for the NCAM homophilic binding, and the model in which the third Ig module binds to itself (shown in Fig. 1a) was suggested [61, 62]. In experiments with micro-beads coated with the various modules of NCAM, the binding of the third Ig module to itself was confirmed, and it was also found that the first module bound to the fifth, and the second – to the fourth [63], which allowed to modify the above model (see Fig. 1b). However, neither of these interactions could be detected by means of surface plasmon resonance (SPR) analysis [40]. Furthermore, the self-binding of the third Ig module could not be detected by one of the most sensitive methods currently available – NMR [13]. It should be noted that the third Ig module used in the NMR study [13] was properly folded (as judged by the NMR structure determination of the module) meaning that the absence of binding was not due to the module mis-folding. Using SPR experiments, binding between the first and second Ig modules of NCAM was detected and a model shown in Fig. 1c was suggested [40]. This model was later confirmed in multiple NMR and crystallography experiments [11, 12, 14, 64]. Recently, the crystal structure of the first three Ig modules of NCAM was determined [14]. This structure confirmed interaction between the first and second Ig modules of NCAM (Fig. 1c) and also demonstrated the importance of the third Ig module for the homophilic binding. However, the third Ig module was found to make a contact with the second Ig module in an anti-parallel way (Fig. 1d) and with the first Ig module (Fig. 1e). A contact between two second Ig modules of NCAM was also shown (see Fig. 1f). Combination of the interactions shown in Fig. 1c with those in Fig. 1d leads to a one-dimensional "zipper" formation of NCAM molecules [14]. Another one-dimensional "zipper" can be made if one combines contacts

shown in Fig. 1e, f [14]. These two types of "zippers" are not mutually exclusive and their combination can lead to a two-dimensional "zipper" array of NCAM molecules. For review of these interactions, see [65]. As shown in Fig. 1, the membrane separations spanned by the adhesive complexes are different for the various models. Using surface force measurements, the distance dependence of the attractive and repulsive forces for the membrane-bound NCAM extracellular modules was determined [66]. The NCAM fragment consisting of all five Ig modules of NCAM exhibited adhesion at two different distances: 29 nm and 18 nm. The first distance corresponds to the anti-parallel interaction of the first and second Ig modules (Fig. 1C), whereas the second – to the models in which an overlap of approximately all five Ig modules occurs (see Fig. 1a, b and d). In order to discriminate between the models involving self-binding of the third module (Fig. 1a, b) and the anti-parallel binding of the second and third module (Fig. 1d), the first and the second Ig modules were deleted from the extracellular NCAM domain, and this construct appeared to adhere at the membrane separation corresponding to the full overlap of the Ig modules [66]. This seems to support the models involving self-binding of the third module (Fig. 1a, b). However when deleting the first and second Ig modules from the extracellular NCAM domain, amino acids 28–219 were also deleted [66], and thus the whole A β-strand (amino acids 209–215) and the loop region between the A and A' β-strands (residues 216–218) were deleted, according to the NMR and crystal structures of the module [13, 14]. It was demonstrated by NMR [13] that the third Ig module, when lacking only a part of the A β-strand (residues 209–213), was unfolded. This means that the deletion construct used in the surface force measurements [66] most likely contained an unfolded third Ig module, and thus the self-binding of this construct cannot be used as evidence in support of the models shown in Fig. 1a, b.

Thus, it appears that multiple inter-module interactions are involved in the NCAM homophilic binding. Despite numerous studies, only the anti-parallel binding of the first and second Ig modules can be considered firmly established (Fig. 1c). This interaction has been detected in many experimental set-ups employing SPR, NMR, X-ray diffraction and surface force measurements. There is a lot of evidence showing that all of the other contacts shown in Fig. 1 may also be relevant for the NCAM homophilic binding, but the direct evidence (such as binding between the individual modules with estimation of the interaction's affinity) is still lacking.

Interaction of NCAM with the FGF-Receptor

The FGF-receptor family consists of four closely related receptor tyrosine kinases (FGF-receptor 1–4), which regulate a multitude of cellular process via interactions with FGFs (FGF1–FGF23) [67, 93]. The prototypical FGF-receptor consists of three Ig modules, a transmembrane domain and a cytoplasmic tyrosine kinase domain. The Ig1 and Ig2 modules are separated by a very long linker (20 to 30 amino acids, depending on the isoform), which contains a stretch of acidic residues, termed the

acid box. FGF binding to the FGF-receptor results in the receptor dimerization [68, 69] leading to auto-phosphorylation of the receptor tyrosine kinase domains. Regulation of the ligand binding to the FGF-receptor is primarily achieved by alternative splicing of the receptor, leading to the receptor isoforms lacking the first Ig module [67, 94]. The triple-Ig module FGF-receptor isoforms have lower affinity for FGFs when compared with the double-Ig module receptor [70, 71]. The structure of the first Ig module has recently been determined by NMR [72] and the module's inhibitory effect appears to be due to the intra-molecular binding of the module to the receptor's second Ig module in the area of the second module's binding sites for FGF [73]. For review of the FGF-receptor activation by FGFs, see [74].

The FGF-receptor can also be activated by cell adhesion molecules such as NCAM, L1 and N-cadherin [32, 58, 59, 75]. It is believed that these cell adhesion molecules interact directly with the FGF-receptor. However, a direct binding to the FGF-receptor has only been demonstrated for NCAM [15, 76]. Using SPR, it was shown that a recombinant protein consisting of the combined first and second F3 modules of NCAM bound to a recombinant protein consisting of the combined second and third Ig modules of the FGF-receptor1 [15] and FGF-receptor2 [76] with a Kd value of approximately 10 μM. However, binding of the individual F3 modules of NCAM to the FGF-receptor1 fragment could not be detected by SPR indicating that both F3 modules of NCAM are required for an efficient binding, and a model shown in Fig. 2a was suggested [15]. When using a more sensitive method such as NMR, a weak binding between amino acids located in the FG loop region of the second F3 module of NCAM and the FGF-receptor1 could be shown [15]. A peptide corresponding to the FG loop region was shown to stimulate the FGF-receptor1 phosphorylation [15], induce neurite outgrowth and neuronal survival in primary rat neurons [77], protect hippocampal neurons from ischemic insult [78], promote early postnatal sensorimotor development and enhance social memory

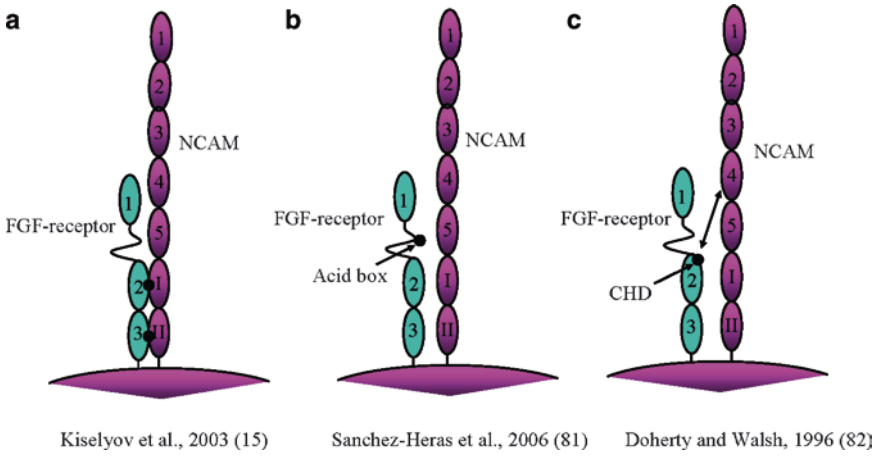

Fig. 2 Models for the interaction between NCAM and the FGF-receptor proposed by different researchers. The contact site between the interacting modules is indicated by a *black dot*

retention [79], and reduce neuropathological signs and cognitive impairment induced by beta-amyloid 25–35 peptide [80], supporting the notion that the FG loop region of the second F3 module of NCAM is important for activation of NCAM signaling. Affinity of the NCAM binding to the FGF-receptor (with a Kd value of 10 μM) may seem rather low in comparison with that of the FGF binding to the FGF-receptor (with a Kd value of 10–100 μM). However, in view of a very high concentration of NCAM in the membrane, approximately 85% of the FGF-receptor molecules are estimated to be involved in a transient binding to NCAM [15]. Recently, it has been shown by immunoprecipitation that the acid box region of the FGF-receptor is necessary for binding to NCAM, N-cadherin and L1 (see Fig. 2b) while the FGF-receptor's Ig modules appear not to be required for the binding [81], which seems to contradict the SPR and NMR experiments showing importance of the second and third Ig modules of the FGF-receptor for binding to NCAM [15, 76]. It should be noted that this immunoprecipitation experiment [81] employed a detergent triton X-100 (at a concentration of 1%) for cell lysis and subsequent washing of the complex between NCAM and the FGF-receptor. Therefore, the reported absence of binding of the second and third Ig modules of the FGF-receptor to NCAM could be due to the denaturation effect of the employed detergent. At present, it is not clear whether or not the acid box of the FGF-receptor is involved in a direct binding to NCAM. Another area of the FGF-receptor previously suggested to be involved in binding to NCAM, L1 and N-cadherin is the CHD (Cell adhesion molecule Homology Domain) region located in the second Ig module of the FGF-receptor [82]. CHD shares sequence homology with functional motifs present in NCAM (exon VASE in the fourth Ig module) and N-cadherin (HAV motif), which formed the basis for the model shown in Fig. 2c [82]. However, recent data [81] indicate that the CHD region is not necessary for the interaction with NCAM.

Mechanism of the FGF-Receptor Activation by NCAM

As mentioned above, approximately 85% of the FGF-receptor molecules are estimated to be bound to NCAM if the mechanism of interaction between NCAM and the FGF-receptor is according to the model shown in Fig. 2a [15]. It should be noted that the FGF-receptor binding to NCAM (according to this model) is not expected to influence significantly the receptor activation by FGF due to the much higher affinity (approximately 1000-fold) of the FGF binding compared to that of NCAM. In order to activate the FGF-receptor, the FGF-receptor binding to NCAM should also lead to the receptor dimerization. Furthermore, the FGF-receptor is believed to be activated by NCAM only when NCAM is involved in the homophilic binding (mediating cell–cell adhesion). If the mechanism of the NCAM homophilic binding is according to any of the models shown in Fig. 1, then it is not clear how the FGF-receptor binding to NCAM could lead to the receptor dimerization. However, combination of the models shown in Fig. 1c,d and in Fig. 1e, f leads to two kinds of one-dimensional "zipper" formations. The two one-dimensional "zippers"

can be combined to form a tightly packed two-dimensional array of NCAM molecules, as seen in the crystal structure of the first three Ig modules of NCAM [14, 65]. The peptides corresponding to the interactions shown in Fig. 1d–f inhibit the NCAM homophilic binding and also inhibit the NCAM-mediated neurite outgrowth [14]. This suggests that NCAM clustering achieved by the formation of one-dimensional and/or two-dimensional "zipper" formations of NCAM molecules may be a possible mechanism for the FGF-receptor activation by the NCAM homophilic binding [65]. NCAM clustering is expected to cluster the FGF-receptor molecules and therefore increase the local concentration of the receptor. It has previously been suggested that the FGF-receptor could also be dimerized through a direct receptor-receptor binding in the absence of FGF [83, 84], explaining the background level of the FGF-receptor activation in the absence of FGF. This notion is supported by the fact that in the crystal structure of the FGF-receptor1 in complex with FGF2 [68], the receptor molecules are found to be involved in a direct interaction. The increase in the local concentration of the FGF-receptor is expected to shift the equilibrium between the monomeric and dimeric receptors toward the receptor dimer, which is supported by the fact that over expression of the FGF-receptor1 results in a considerable increase in the background level of the receptor activation [85]. Thus, the "zipper" model of the NCAM homophilic binding allows explaining the FGF-receptor activation by NCAM. Furthermore, this model also provides a possible explanation for the effect of PSA on NCAM signaling. During development (and in the adult brain in areas retaining high degree of plasticity), NCAM is heavily polysialilated. Removal of PSA from NCAM inhibits the NCAM stimulated neurite outgrowth [86] and increases the adhesive properties of NCAM [87, 88]. Since the hydrated volume of the PSA-moiety is approximately three times larger than the size of the NCAM molecule [89], it is obvious that the tightly packed two-dimensional "zipper" cannot be formed when NCAM is heavily polysialilated because there is very little room between NCAM molecules in this "zipper." However, it is reasonable to speculate that the two types of one-dimensional "zippers" may still be formed when NCAM is polysialilated because in this case there is sufficient room for the flexible PSA chains to the side of the "zippers." Since these two "zippers" contain smaller number of stabilizing contacts per NCAM molecule than the tight two-deminsional "zipper,", the latter is expected to produce stronger adhesion than the other two types of one-dimensional "zippers." On the other hand, NCAM molecules in the tight "zipper" probably cannot interact with the FGF-receptor due to the lack of room between the tightly packed NCAM molecules, whereas it is reasonable to assume that the polysialilated NCAM molecules in one-dimensional "zippers" are accessible for interaction with the receptor because the receptor molecules can now get to the "NCAM-zipper" from the side. One can conclude therefore that expression of non-polysialilated NCAM is expected to result in a stronger homophilic binding, but weaker (if any) activation of the FGF-receptor; while polysialilated NCAM molecules probably have a reduced capacity for the homophilic binding, but can interact and activate the FGF-receptor. Thus, expression of PSA by NCAM may function as a "switch" regulating whether NCAM is involved in adhesion or signaling. Another molecule which may

function as a "switch" in the regulation of the FGF-receptor activation by NCAM is adenosine triphosphate (ATP), which is one of the most abundant neurotransmitters in the nervous system [90, 91]. NCAM has been shown extracellularly to bind and hydrolyze ATP [48, 49]. The role of this ATPase activity is little understood. However, ATP has been shown to inhibit interaction between NCAM and the FGF-receptor1 [15] and to inhibit the NCAM mediated neurite outgrowth [15, 92]. Using NMR, it was demonstrated that ATP binds to the nucleotide binding motif of NCAM (A^{686}ENQQGKS^{693}) located in the FG loop of the second F3 module, which is the same area shown to be involved in binding to the FGF-receptor1; and by SPR, it was shown that ATP could inhibit the binding of NCAM to the FGF-receptor1 with an inhibition constant of 0.3 mM [15]. In the extracellular environment in the brain, ATP is found in sufficiently high concentrations (in the mM range) for inhibition of the NCAM binding to the FGF-receptor only in the vicinity of synapses. One may therefore hypothesize that in the absence of ATP, the FGF-receptor activation by NCAM provides a stimulating environment for growth cone extension. However, when the growth cone reaches its target and a new synaptic contact is formed, release of ATP may switch NCAM from the signaling mode to a mode promoting adhesion.

Conclusion

Despite numerous studies, the mechanisms of both the NCAM homophilic binding and the NCAM binding to the FGF-receptor are still controversial. It appears that the multiple inter-module contacts are involved in both interactions. The affinities corresponding to these contacts are probably rather low, which is probably the reason why some of these interactions can be detected in one experimental set-up (favoring these interactions), but not in another. Recent structural data [14, 15] allow making a speculative model [65] of the FGF-receptor activation by NCAM which, as shown above, is the only one that can explain from the structural point of view how interaction between NCAM and the FGF-receptor could lead to the receptor dimerization; why the interaction between NCAM and the FGF-receptor activates the receptor *only* when NCAM is involved in the homophilic binding; and how PSA could regulate the strength of the NCAM homophilic binding and the signaling properties of NCAM. Further study is required to come to the conclusion whether this model is true or not.

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