Intracelluar Ligands of NCAM

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Introduction

Neural cell adhesion molecule (NCAM) (for review see: [1–3]) was first identified and characterized more than 30 years ago [4, 5]. For understanding how NCAM is implicated in its various functions such as neurite outgrowth, cell migration, stabilization of cell–cell contacts, synapse formation, learning and memory, it is necessary to analyze the adjacencies of the molecule outside and inside the cell. The identification of direct binding ligands or indirect associating molecules of NCAM can enlighten the signal transduction pathways of NCAM and give information on putative cross-talks with other signaling pathways. Furthermore, information on its interaction with the cytoskeleton helps to understand the specific functions of the different intracellular domains of NCAM.

Two of the three major isoforms of NCAM are transmembrane glycoproteins with large carboxy-terminal intracellular domains of different length resulting from alternative splicing of one single gene [6–8]. The intracellular domain of rat NCAM 140 has a length of 119 amino acids, whereas the intracellular part of NCAM 180 consists of an additional insert of 271 amino acids, resulting in a total intracellular length of 390 amino acids (Fig. 1). These large domains imply that they are implicated in the various functions of NCAM and can be the point of contacts for intracellular interactions. In particular, the presence of an additional insert in the intracellular domain of NCAM 180 suggests that the two transmembrane isoforms might have different functions.

In the following session, we will chronologically present the known intracellular binding proteins of NCAM.

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Fig. 1 Complete intracellular domain of rat-NCAM (*Black*: NCAM 140, *Red*: NCAM 180-specific insert). The sequence is divided into 4 constructs (*blue*), which were used for immunoprecipitation experiments. All potential phosphorylation sites (analyzed by NetPhos) are *underlined*

Spectrin, the First Identified Intracellular NCAM-Associated Protein

The first intracellular protein, demonstrated to bind to NCAM, was published by Pollerberg et al. [9]. Earlier studies already demonstrated that NCAM 180 appears later during development compared to NCAM 140, when mitosis and neuron migration has been completed and cells are morphologically differentiated [10, 11]. Furthermore, NCAM 180 was shown to have a reduced lateral mobility within the plasma membrane when compared with NCAM 140 [12]. These facts suggested a role of NCAM 180 in stabilizing of cell–cell contacts in differentiated cells by interacting with the cytoskeleton. By analyzing this in more detail by immunofluorescence using neuroblastoma and cerebellar cells, it was demonstrated that the NCAM 180 isoform is predominantly expressed at sites of cell–cell contacts of cell bodies and of growth cones contacting other cells [9, 12]. At these sites, cytoskeleton proteins such as brain spectrin and actin are also accumulated. In further experiments, brain spectrin and ankyrin but no other cytoskeleton proteins could be co-purified with NCAM 180 by immunoaffinity chromatography using adult mouse brain protein fractions. Furthermore, Pollerberg et al. [9] showed in a solid-phase radioligand-binding assaythat brain spectrin specifically binds to NCAM 180 but not to NCAM 140 or NCAM 120. The co-localization of brain spectrin and NCAM 180 and the association between these molecules indicate that spectrin directs the accumulation of NCAM 180 at specific sites of the cell membrane, but it is also possible that NCAM 180 direct the accumulation of spectrin in NCAM-rich areas. However, ankyrin could not be demonstrated to co-localize with NCAM 180. Therefore, it was speculated that only a very minor portion of ankyrin is associated with NCAM 180.

More than 10 years later, more recent studies revealed that not only NCAM 180 can bind spectrin but also NCAM 140, however, with lower efficiency than NCAM 180 [13,14]. In lipid rafts, even NCAM 120 is associated with spectrin [13].

Functional spectrin consists of two subunits building a heterodimer of one α and one β-subunit or a homodimer of two equal subunits [15]. Spectrin could be co-immunoprecipitated with NCAM from brain homogenate [13, 14] and vice versa also NCAM 120, NCAM 140 and NCAM 180 co-immunoprecipitated with spectrin from brain or NCAM-transfected CHO cells. ELISA-based protein ligand-binding assays with immobilized intracellular domains of NCAM 140 or NCAM 180 indicated that both isoforms directly bind the NH2-terminal region of isolated β1-spectrin, however only NCAM 180 interacts with the complete spectrin α1β1 [13]. The observation, that 13% of the total membrane-bound β1-spectrin is monomeric, suggests that also the NCAM-interaction with the isolated β1-subunit might play a role in vivo. The interaction of β1-spectrin with NCAM 120, which lacks an intracellular domain, is only possible within lipid rafts, where GPI-anchored NCAM 120 is mainly localized. NCAM 140- and NCAM 180-spectrin complexes can be found in both in rafts and raft-free membrane areas.

Spectrin's Function in Recruiting PKCβ

Activated PKCβ is known to interact with the pleckstrin homology domain of β1-2 spectrin [16] and plays a role in NCAM-mediated neurite outgrowth [17]. Therefore, spectrin was suggested to function as a molecular bridge between NCAM and PKCβ.

Extracellular activation of NCAM causes a recruitment of β1spectrin to NCAM 140/180 and a redistribution of NCAM–spectrin complexes into lipid rafts. The redistribution of NCAM–spectrin-complexes into lipid rafts correlates with an increased level of PKCβ within lipid rafts. Inhibition of the FGF-receptor, which is also involved in NCAM-mediated promotion of neurite outgrowth, inhibits the recruitment of the PKCβ to lipid rafts and abolishes its association with NCAM 140/180. Furthermore the formation of the NCAM-spectrin–PKCβ complex had also been shown to be important for NCAM-mediated neurite outgrowth, since the disruption of the complex abolishes NCAM-induced neurite outgrowth. These observations indicate an indirect association of NCAM 140 or NCAM 180 with activated PKCβ via spectrin in dependence of FGF receptor activation and are the basis of a novel model, where spectrin has the important role to form a scaffold to which signaling molecules such as the PKCβ become recruited.

Spectrin's Function in Recruiting NMDA Receptor and CaMKIIα

Recently, it was demonstrated that the NCAM–spectrin interaction is also involved in the formation of a postsynaptic signaling-complex [18]. After clustering of NCAM, not only spectrin but also the NMDA-receptor, CamKIIα, PSD95 or

α-actinin were accumulated in postsynaptic densities. Spectrin binds to the intracellular domain of the NMDA-receptor [19] and it could be demonstrated that the NMDA-receptor as well the CamKIIα and spectrin is co-immunoprecipitated together with NCAM using brain homogenate [18]. It was already speculated that the presence of NCAM is necessary in NMDA-receptor-dependent LTPs since in NCAM-deficient mice, LTPs were impaired [20, 21]. Also, the disruption of the spectrin-NCAM-scaffold in NCAM deficient mice prevents redistribution of the CaMKIIa and impairs NMDA-dependent LTP formation.

NCAM Interacts with the Tyrosine-Kinase Fyn

Besides binding and activating the FGF-receptor, NCAM 140, but not NCAM 180 activates the MAP-kinase pathway through Fyn and FAK. A small fraction of membrane-bound NCAM 140 (nearly 3%), but not NCAM 180, was shown to be constitutively associated with the lipid raft-associated nonreceptor tyrosine kinase Fyn [22]. Homophilic NCAM-binding results in the recruitment of the focal adhesion kinase FAK to the NCAM 140-fyn-complex, leading to activation of the MAP kinases ERK1/2 and phosphorylation of the transcription factor CREB [23]. The activation through Fyn and FAK and the resulting induction of neurite outgrowth are dependent on the localization of NCAM 140 within lipid rafts, whereas the activation of the FGF-receptor is mediated by NCAM molecules localized outside of lipid raft compartments.

A direct binding of Fyn to NCAM could not been proved yet. However, the activation of Fyn depends on its dephosphorylation by the receptor protein tyrosine phosphatase α (RPTPα) [24, 25]. RPTPα is present in high concentration in brain and recently a direct interaction of the RPTPα with NCAM 140 and with lower affinity to NCAM 180 was demonstrated [26]. In agreement with these data, in RPTPα-deficient mice, NCAM-Fyn interaction and NCAM-mediated neurite outgrowth is abolished [26].

NCAM Binds Not Only Spectrin, But Also Several Other Major Cytoskeletal Proteins

The observation that the over-expression of the intracellular domain of NCAM 180 in dominant-negative experiments leads to increased neurite outgrowth was explained by destabilization of NCAM-cytoskeleton interactions and indicated that especially NCAM 180 is associated with more cytoskeleton proteins and not only with spectrin [27]. Therefore, nearly 20 years after the identification of spectrin, we used ligand affinity chromatography of cytoskeleton-enriched fractions of rat brain and tried to identify novel binding proteins of NCAM 140 or NCAM 180.

We expressed the intracellular domains of rat NCAM 140 (NCAM cyt 140), NCAM 180 (NCAM cyt 180) and a control protein, purified them by immunoaffinity chromatography and immobilized them on CNBr-activated-Sepharose columns. Eluted proteins of the NCAM cyt 180-column were cut out of the gel, digested with trypsin and analyzed by peptide mass fingerprinting using MALDI-TOF MS. The following proteins could be identified: α- and β-tubulin, microtubule-associated protein MAP 1A, α-actinin 1, β-actin and surprisingly the rhoA-binding kinase α (ROKα), which might be associated to one of the cytoskeletal proteins [28]. All the eluates of the affinity chromatographies (empty columns, NCAM cyt 140- or NCAM cyt 180-columns and control protein-columns) were further analyzed by Western blot. We could identify all of the proteins identified by peptide mass fingerprinting in the eluates of the NCAM cyt 180 columns and reproduce the data obtained by MALDI-TOF MS. In addition, the eluates of the NCAM cyt 140 columns were also analyzed. Only α- and β-tubulin and α-actinin 1 were present in the eluate of the NCAM cyt 140 column, whereas microtubule-associated protein MAP 1A, β-actin and ROKα could not be detected [28]. None of these proteins was detected in eluates of the respective empty- or control columns [28]. Furthermore, we tested the presence of spectrin and tropomyosin by Western blot analysis. We could detect spectrin in the eluates of the NCAM cyt 140 and the NCAM cyt 180 columns, whereas tropomyosin was exclusively present in the eluate of the NCAM cyt 180-column [14]. In addition, spectrin could be co-immunoprecipitated together with NCAM from rat brain [14].

As mentioned before, spectrin is supposed to link NCAM to actin-filaments, which are composed of β-actin units. α-Actinin is an actin-binding protein, which mediates the bundling of actin-filaments, whereas tropomyosin is a small protein, which is responsible for the stabilization of the actin filaments. From this the question might arise, why β-actin and tropomyosin are present only in the NCAM 180-column eluate and not in the NCAM 140 column eluate, whereas the actin-binding proteins spectrin and α-actinin are present in both eluates. The following explanations are possible: First and most likely, NCAM 180, but not NCAM 140, interacts directly with β-actin and tropomyosin as well as via spectrin. Second, NCAM 140 interacts only with monomers of spectrin and α-actinin that are not associated with actin-filaments. Alternatively, NCAM 140-associated spectrin and α-actinin are in conformations that are not able to bind to actin-filaments. This explanation agrees with the observation by Leshchyns'ka et al. [13] that NCAM 140 only interacts with the spectrin β1-subunit and not with the complete spectrin, what might explain, that NCAM 140 does not associate with actin (filaments).

In a following study, it could be shown that α- and β-tubulin interact with the membrane-proximal domain of NCAM 140 and NCAM 180, whereas the ROKα interacts with the C-terminal part (amino acid residues 958–1,090) of the NCAM-180-specific insert [29]. The latter observation is in agreement with the identification of ROKα in the eluate of the NCAM 180 column and not in the eluate of the NCAM 140 column. ROKα was additionally demonstrated to co-immunoprecipitate with NCAM from mouse brain solubilisate, which supports the association also under more in vivo conditions [14]. The interaction with α- and β-tubulin reveals

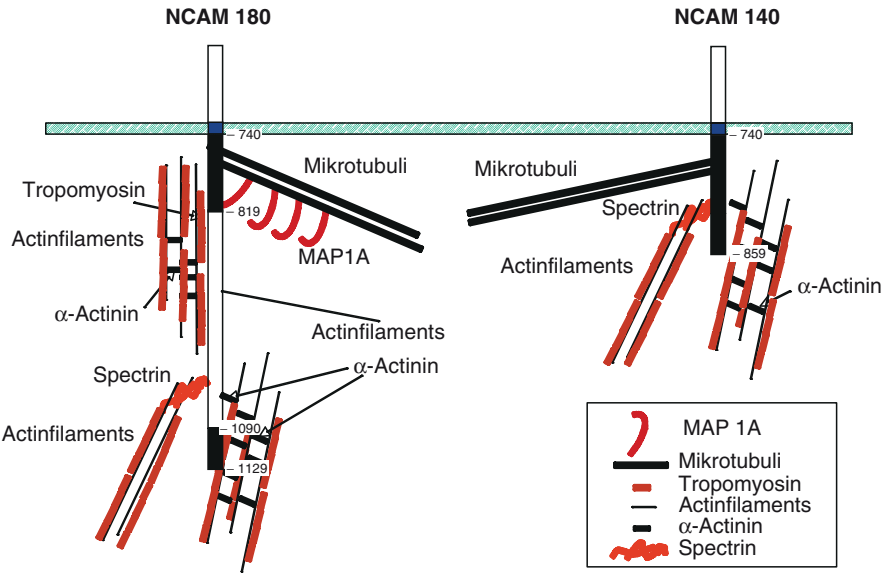

Fig. 2 Interaction of NCAM with cytoskeleton proteins. Note that only spectrin was shown to bind directly to NCAM

for the first time that NCAM is not only linked with the actin cytoskeleton but also to the microtubule-system. Figure 2 summarizes known interactions between NCAM and cytoskeletal proteins.

Interaction with Signaling Molecules

Using again the ligand affinity approach – now focusing more on cytosolic and not cytoskeleton proteins – we recently identified further novel proteins binding to the intracellular domains of NCAM [29]. This time, cytosolic-enriched protein fractions of rat brain were passed over the NCAM cyt 140 or NCAM cyt 180 columns. Proteins eluted from the NCAM cyt 180 column were again cut out of the gel, subjected to in-gel digestion with trypsin and analyzed by MALDI-TOF-MS. We were able to identify LANP (leucine-rich acidic nuclear protein), TOAD-64 (turned on after division-64), and syndapin in the NCAM cyt 180 column eluates by peptide mass finger printing and by Western blot analysis. Whereas LANP and syndapin could also be demonstrated to associate with NCAM 140, TOAD-64 interacts specifically with the NCAM 180 isoform [29].

Furthermore, we tested the presence of three other proteins in the eluates: phospholipase Cγ (PLCγ), which is implicated in NCAM-mediated signal transduction, and the serine/threonine phosphatases PP1 and PP2A since LANP is known to be a regulator of these phosphatases [30, 31]. We could detect all three proteins in the

eluate of the NCAM cyt 140 as well as of the NCAM cyt 180 column by Western blot analysis.

The interaction of PLCγ, LANP, TOAD-64, syndapin, PP1 or PP2A to NCAM was analyzed in more detail. Five fragments of the intracellular domain of NCAM, which comprise different parts of the complete intracellular domain of rat NCAM 140 and NCAM 180 were expressed and purified. All five NCAM fragments, representing together the complete sequence of both NCAM cyt 140 and 180, were further used to perform pull-down assays with cytosolic-enriched protein fractions of rat brain. PLCγ, TOAD-64, syndapin, PP1 and PP2A associate with NCAM cyt fragment 1, which represents the membrane close domain of 80 amino acids of both NCAM cyt 140 and NCAM cyt 180 (encoded by exon 16 and 17). LANP interacts with the N-terminal NCAM cyt fragment 5 (39 amino acids long), which represents the C-terminal part of NCAM 180 and NCAM 140 (encoded by exon 19) [29].

LANP has been described as a heat-stable inhibitor of the protein phosphatase PP2A [31] and positively regulates the activation of PP1 [30]. The interaction of PP1 and PP2A with the intracellular domains of NCAM indicates a possible dephosphorylation of serine and threonine residues of NCAM 140 and NCAM 180 by these phosphatases. The intracellular domain of NCAM 140 contains 5 putative serine and 6 putative threonine phosphorylation sites while NCAM 180 contains 28 putative serine and 21 putative threonine phosphorylation sites. First hints that NCAM could be dephosphorylated by protein phosphates came from experiments, where NCAM (phosphorylated by GSK-3) was dephosphorylated after incubation with PP2A [32]. We localized the binding of PP1 and PP2A to the first N-terminal 80 amino acids of the cytosolic domain of NCAM 140 and NCAM 180. This fragment contains two putative serine and three putative threonine phosphorylation sites, which could be dephosphorylated by PP1 or PP2A by regulating the phosphorylation of NCAM.

NCAM 180 might also be phosphorylated by the serine/threonine kinase ROK α, since the ROKα binding region within the NCAM 180-specific insert (NCAM cyt fragment 3) contains nine predicted serine and six predicted threonine phosphorylation sites.

Turned on after division-64 (TOAD-64) belongs to the TUC (TOAD/Ulip/CRMP)-family of proteins. TOAD-64 is highly up-regulated during early postnatal neuronal development [33] and is implicated in signaling events in growth cones of neurites. It is a cytoplasmic protein and can be associated with plasma membranes [34]. Surprisingly, TOAD-64 only associates with NCAM 180 although NCAM 140 is the active isoform for the stimulation of neurite outgrowth. The mapping studies suggested its association with a sequence of 80 amino acids close to membrane. However, this sequence is present in both NCAM-isoforms. An explanation could be that this membrane-proximal domain has another conformation in the NCAM 180- than in the NCAM 140-isoform due to the presence of the following additional insert of 267 amino acids, which might allow specifically an association with TOAD-64.

Syndapin, a protein highly enriched in brain [35], was shown to interact with the N-terminal 80 amino acids of the intracellular domain of NCAM 140 and NCAM 180. Qualman et al. showed that syndapin I associates with the GTPase dynamin I

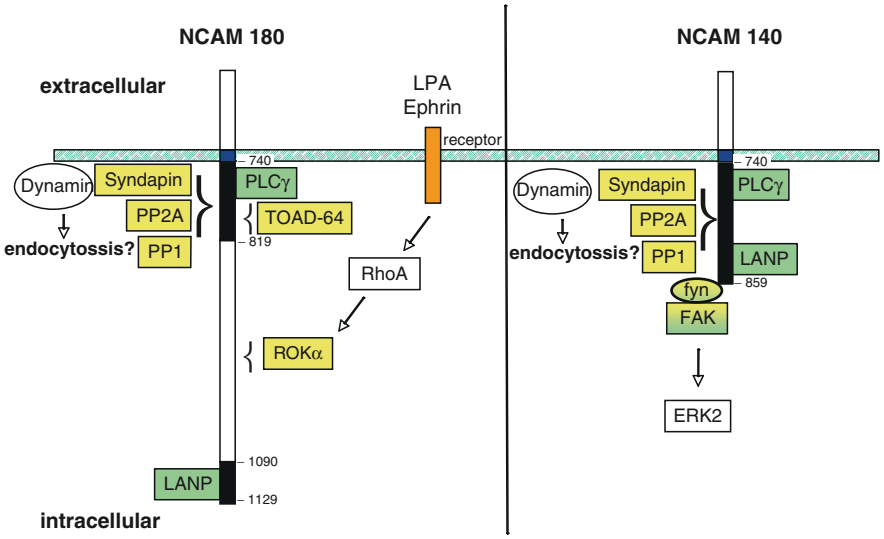

Fig. 3 Interaction of NCAM with signaling molecules. The intracellular domains of NCAM 140 and NCAM 180 are shown. Proteins, which bind direct to NCAM are indicated by *green* boxes. Proteins, which are not known to bind directly or indirectly to NCAM are indicated by *yellow* boxes. Numbers refer to the amino acids number of the NCAM sequence

and plays a role in clathrin-mediated endocytosis of synaptic vesicles [36]. Minana et al. (2001) demonstrated that internalized NCAM colocalizes with clathrin and α-adaptin of the adaptor complex AP-2, as well as with transferrin, which is used as a marker of early endosomes [37]. Very recently, it was demonstrated that NCAM is endocytosed via the clathrin-pathway [38]. The interaction of NCAM with syndapin indicates that syndapin is involved in the clathrin-mediated endocytosis of NCAM. Figure 3 summarizes the interactions between NCAM and signaling molecules described above.

Direct Binding of LANP and PLCγ to NCAM

LANP interacts directly with the intracellular domains of both NCAM 140 and NCAM 180. This was demonstrated by a GST-LANP pull-down assay with the purified recombinant intracellular domains of NCAM [29]. LANP, a member of the leucine rich protein family, also known as pp32 (phosphoprotein with a molecular weight of 32 kDa) was first identified in cerebellum from rat [39–41]. The N-terminal domain of the protein contains many leucine-rich repeats, which are supposed to mediate protein–protein interactions. LANP is upregulated in the central nervous system during the early stage of postnatal development. In undifferentiated neuronal cells, LANP is predominantly present in the nucleus, but during neuritogenesis

it translocates into cytoplasm [42]. There, it interacts with the light chain of free microtubule-associated protein 1B (MAP 1B), thereby inhibiting the binding rate of MAP 1B to microtubules, probably leading to improved flexibility of the neuronal cytoskeleton and facilitation of neurite outgrowth [42]. Since we identified MAP 1A as a binding partner of NCAM 180 and LANP has been demonstrated to interact not only with MAP 1B [42] but also with other members of the microtubule associated protein family such as *tau*, MAP 2 or MAP4 [43,44], it is possible that LANP might be a linker protein between NCAM 180 and MAP 1A.

We also investigated whether the interaction of the PLCγ with NCAM is directed or mediated by intermediate proteins and which domains of the PLCγ are implicated in direct interaction. For this, we performed GST-pull-down assays with GST-PLCγ fragments derived from human PLCγ comprising different structural and functional domains of PLCγ: the PH-domain, the N-terminal SH2-domain, the C-terminal SH2-domain and both SH2-domains in one construct. For the verification of direct interaction with some of the GST-PLCγ fragments, purified recombinant NCAM fusion proteins were used in the pull-down-experiments. As a negative control fusion protein of the intracellular domain of CEACAM, a transmembrane protein of the immunoglobulin superfamily was used. The intracellular domains of NCAM 140 or NCAM 180 directly interact with the PH-domain and the C-terminal SH2 domain of PLCγ. Both isoforms have a stronger affinity to the C-SH2 domain than to the PH-domain. A small amount of NCAM 180 also interacts with the N-terminal SH2 domain [29].

PLCγ could also be shown to co-immunoprecipitate with NCAM from mouse brain homogenates, which verifies also an interaction of PLCγ with NCAM under more in vivo conditions [29].

MyoNAP, a Novel NCAM-Binding Molecule in Avians

Very recently, Kim and Hirayama searched for highly expressed transcripts during myogenesis in quails [45]. One of the transcripts turned out to induce neurite-like processes after transfection in myoblasts. It could be demonstrated that the process formation depends on intact microtubules. A yeast-two-hybrid assay revealed that this protein directly binds the C-terminal domain of avian NCAM. Therefore, the yet unknown protein was named MyoNAP (Myogenesis-related and NCAM-associated protein). The MyoNAP-NCAM interaction could be shown to be necessary for the formation of MyoNAP-induced neurite like protrusions in quail myoblasts.

Conclusion

Taken together, there are up to now five proteins identified to directly bind to the intracellular domain of NCAM: spectrin, RPTPα, LANP, PLCγ and MyoNAP. The binding affinity and/or quantity to NCAM of these proteins partly depend on

the NCAM isoforms. Whether the other intracellular associated NCAM molecules presented here are directly or indirectly associated with NCAM has to be proven in the future.

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