Novel Cytosolic Binding Partners of the Neural Cell Adhesion Molecule: Mapping the Binding Domains of PLCγ, LANP, TOAD-64, Syndapin, PP1, and PP2A[†]

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ABSTRACT: The neural cell adhesion molecule (NCAM) is implicated in important functions during development and maintenance of the nervous system. Two of the three major isoforms, NCAM 140 and NCAM 180, are transmembrane glycoproteins with large cytoplasmic domains of different length. The purpose of this study was to identify novel intracellular binding partners of NCAM 140 and NCAM 180. We expressed both cytoplasmic domains, as well as cytoplasmic fragments of NCAM, as fusion proteins in *Escherichia coli* and used them for ligand affinity chromatography or glutathione S-transferase (GST) pull-down assays. By peptide mass fingerprinting Western blot analysis, or both, we identified PLCγ, LANP, syndapin, PP1, and PP2A as binding partners for both NCAM 140 and NCAM 180, whereas TOAD-64 was identified as a NCAM 180-specific interacting protein. Furthermore, we were able to show that binding of these novel binding proteins, as well as the previously described interaction partners ROK α (rho A binding kinase α) and α- and β-tubulin, bind to specific cytosolic sequences of NCAM. For this purpose, we performed GST pull-down experiments using cytosolic fragments of NCAM as GST-fusion proteins and cytosolic- or cytoskeleton-enriched protein fractions of rat brain.

The neural cell adhesion molecule (NCAM) was identified as one of the first members of the immunoglobulin superfamily in 1977 (*1*). NCAM is implicated in numerous cellular functions. Thus, during embryonic development, it controls tissue formation (e.g., neurulation) and is involved in many processes of neuronal development such as migration of neuroblasts, regulated outgrowth, and fasciculation of neurites and the formation of communication contacts, for example, nerve−muscle cell interactions (*2*). Furthermore, NCAM is involved in synaptogenesis and synaptic plasticity (*3*), learning and memory processes (*4*), and the regeneration of neurites (*5*).

NCAM is a Ca^{2+}-independent homophilic cell adhesion molecule existing in three major isoforms, which are generated by alternative splicing of a single gene (*6*). With reference to their apparent molecular weights, they are termed NCAM 120, NCAM 140, and NCAM 180 (*7*). NCAM 120 is anchored to the membrane via glycosyl-phosphatidylinositol, whereas NCAM 140 and NCAM 180 are transmembrane glycoproteins with large cytoplasmic domains of different lengths. The cytoplasmic domain of NCAM 180 consists of an additional insert of 267 amino acids, encoded by the alternatively spliced exon 18, whereas the flanking cytoplasmic regions are identical to those of NCAM 140 (119 amino acids). The three isoforms show different temporal and spatial expression patterns and functions. NCAM 140 is predominantly expressed in neural growth cones (pre- and postsynaptically) and mediates neurite outgrowth by activating signal transduction pathways. It is expressed on neurons and muscle cells. NCAM 180 is exclusively expressed in the nervous system and seems to appear later in development than NCAM 140, when neural cell migration is completed. In neurons, NCAM 180 is expressed on postsynaptic membranes, and it stabilizes cell−cell contacts by association with the cytoskeleton linker protein spectrin (*8*). Glia cells mainly express NCAM 120.

All three isoforms are present in specialized membrane subcompartments, the lipid rafts. Four cysteine residues in the cytoplasmic domains of NCAM 140 and NCAM 180 adjacent to the transmembrane domain represent palmitoylation sites (*9*), which have been demonstrated to be necessary for the presence of NCAM within lipid rafts (*10*).

In the past decade, many studies have addressed the NCAM-mediated signal transduction initiated through homophilic NCAM−NCAM interaction or by more recently investigated by heterophilic interactions.

NCAM directly interacts with the FGF-receptor and thereby activates FGF-receptor-specific signaling pathways, leading to the activation of the PLCγ pathway, which results in a Ca^{2+} influx into neurons and an activation of protein kinase C (PKC) or the activation of the MAP kinases ERK1 and ERK2 (*11*).

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Recently Kiselyov et al. demonstrated a direct interaction between the two extracellular fibronectin-like domains of NCAM and the second and third immunoglobulin-like domain of the FGF receptor (*12*).

Both NCAM 140 and NCAM 180 are able to activate the FGF-receptor, and CHO cells transfected with one or the other of the isoforms show increased activation of the MAP kinases ERK1 and ERK2 after external NCAM stimulation (*10*). However, only NCAM 140 promotes neurite outgrowth, so it was concluded that FGF-receptor stimulation alone is not sufficient for NCAM-induced neuritogenesis.

Besides activating the FGF-receptor pathway, 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 (*13*). 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 and -2 and phosphorylation of the transcription factor CREB (*14*). 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 (*10*).

Both described signaling mechanisms, the FGF-receptor−PLCγ-pathway and the NCAM 140-mediated activation of the MAP kinases ERK1 and ERK2 through Fyn and FAK, are required for maximal NCAM-mediated neurite outgrowth.

Recent evidence indicates that the cAMP/protein kinase A (PKA) pathway and the phosphatidylinositol-3-kinase (PI3-kinase)/Akt-kinase (protein kinase B (PKB)) pathway are involved in NCAM-mediated signaling and neurite outgrowth (*15*, *16*). In addition to palmitoylation of the cysteine residues, the phosphorylation of cytoplasmic serine or threonine residues of NCAM may also control the function of NCAM (*17*).

Diestel et al. demonstrated that the only cytoplasmic tyrosine residue of NCAM, present in the sequence DITCYFL located proximal to the transmembrane domain, could be phosphorylated in NCAM 180 by a yet unknown kinase (*18*). They suggest that the tyrosine phosphorylated form of NCAM 180 acts as a stabilizer of cell−cell contacts, whereas non-tyrosine-phosphorylated NCAM 180 has a less stabilizing activity.

Although NCAM 140 and NCAM 180 have large cytoplasmic domains with many potential serine and threonine phosphorylation sites, not many cytosolic interaction partners have been identified during the last three decades.

Recently we identified the cytoskeleton proteins α- and β-tubulin and α-actinin as binding partners of both NCAM 140 and NCAM 180. Furthermore, we showed that the microtubuli-associated protein MAP 1A, β-actin, tropomyosin, and the rhoA-binding kinase α are NCAM 180 interacting partners (*19*). In addition, we demonstrated that overexpression of the cytoplasmic domain of NCAM 180 in PC12 cells (dominant-negative experiments) leads to increased neurite outgrowth due to destabilization of NCAM−cytoskeleton interactions (*20*).

In the present study, we identified PLCγ, LANP, syndapin, PP1, and PP2A as novel intracellular binding partners of NCAM 140 and NCAM 180, while TOAD-64 was shown to be a binding partner of NCAM 180. Some of these proteins may be implicated in novel, as yet undescribed signal transduction pathways of NCAM. We also identified those parts of the NCAM molecule responsible for binding the interacting molecules.

MATERIALS AND METHODS

Antibodies. The following antibodies were used: monoclonal anti-NCAM-antibody 5B8 (Hybridoma Bank), monoclonal anti-mouse NCAM antibody H28 (Cerdarlane), monoclonal anti-PLCγ (BD Transduction Laboratories), polyclonal anti-TOAD-64 (BD PharMingen), monoclonal anti-syndapin (BD Transduction Laboratories), monoclonal anti-PP1 (BD Transduction Laboratories), monoclonal anti-PP2A (BD Transduction Laboratories), polyclonal anti-LANP (kindly provided by Dr. K. Danker), monoclonal anti-rhoA binding kinase α (BD Transduction Laboratories), monoclonal anti-α-tubulin (Sigma), monoclonal anti-β-tubulin (Sigma), mouse-antirabbit antibody (Dianova), and monoclonal anti-CEACAM (mAb Be 9.2) (*21*).

Plasmids. The pGEX-2T plasmids containing the cDNA of the PH-domain of human PLCγ, the N-terminal SH2-domain, and the C-terminal SH2-domain of PLCγ or both SH2-domains together were kindly provided by Dr. M. Falasca (Bone and Mineral Centre, University College London, England; see ref *22*). The plasmid encoding for human LANP (pp32 "phosphoprotein with a molecular mass of 32 kDa"; pGEX-5X-2/pp32) was kindly provided by J. A. Steitz and C. M. Brennan (Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut).

Cloning of Rat NCAM cyt 140 and NCAM cyt 180. cDNA encoding the whole cytoplasmic domain of rat NCAM 140 and NCAM 180 was cloned as described in Büttner et al. (*19*). The pRSET C plasmids (Invitrogen) containing NCAM cyt 140 cDNA or NCAM cyt 180 cDNA were transformed in the *Escherichia coli* strain BL21.

Expression and Purification of His-tag NCAM cyt. Expression of His-tag NCAM cyt in BL21 or the empty vector-encoded control protein (His-tag fusion part) was induced by 1 mM IPTG. The protein expression proceeds for 4 h at 37 °C. Harvested cells of 1 L of BL21 culture were resuspended in 12 mL of PBS containing 1% Triton X-100, protease inhibitor cocktail, PMSF (Sigma), DNAse (10 μg/mL), and RNAse (10 μg/mL). Cells were lysed by five freezing/thawing cycles with liquid nitrogen. NCAM cyt 140 and NCAM cyt 180 were purified from bacterial lysate by immunoaffinity chromatography using the monoclonal anti-NCAM antibody 5B8 as described elsewhere. Empty vector-encoded control protein, which represents the his-tag-fusion part (vector pRSET C) was purified by Ni−NTA affinity chromatography according to the manufacturer's instructions (Qiagen).

Cloning of the Cytosolic Fragments of Rat NCAM. The cDNA of the cytosolic fragments of NCAM was generated by PCR, using the plasmid encoding the entire cytoplasmic domain of rat NCAM 180 (pcDNA3.1 (+)/NCAM-TM-cyt 180) (*20*) as a template, together with following primer pairs:

NCAM cyt fragment (number)	NCAM cyt 180 amino acid residues	NCAM 180-specific	Sense Primer (s) Antisense Primer (as) (5′-3′-sense)
1	740 - 819	no	s: GAATTCGAATTCGACATCACCTGCTACTTCC as: CTCGAGCTCGAGCTCGGGCTCTGTCAGTGGTG
2	820 - 957	yes	s: GAATTCGAATTCCTGCCTGC CGACACCACAGC as: CTCGAGCTCGAGGGAGGGCTTACTGGTTGC
3	958 – 1090	yes	s: GAATTCGAATTCCCTACTCCCACCCCCACTC as: CTCGAGCTCGAGCTCGGTCT TTG CTGGTGCGG
4	910 - 1011	yes	s: GAATTCGAATTCGACCTGAG TGATACCCCA ACC as: CTCGAGCTCGAGGGCTGCCT CAGTTGGGTTC
5	1091 - 1129	no	s: GAATTCGAATTCGAGAAGGGTCCTGTAGAAACA AAG as: CTCGAGCTCGAGTCATGCTT TGCTCTCATTCTC

The sense primers consist of two 5′-terminal *Eco*R1-recognition sites and the antisense primers of two *Xho*I-sites, which are underlined in each sequence. The resulting PCR products representing the different cytosolic fragments of NCAM were digested with *Eco*R1 and *Xho*I and ligated in the prokaryotic expression vector pGEX-4T-1 (Amersham Pharmacia Biotech). Plasmids were transformed in the *E. coli* strain BL21 (Invitrogen) for protein expression.

Expression of Recombinant GST Fusion Proteins and Cell Lysis. Expression of the recombinant NCAM cyt fragments with a GST-tag or the GST-tag alone was induced by adding IPTG to a final concentration of 0.1 mM (GST, NCAM cyt fragments 2, 3, and 5) or 0.5 mM (NCAM cyt fragments 1 and 4). During expression, cells were cultured at 30 °C for 2−3 h (NCAM cyt fragment 1) or 3−5 h (GST, NCAM cyt fragments 2−5). The expression of GST-LANP (pp32) and the different GST-PLCγ-fragments was induced by 0.1 mM IPTG at 30 °C for 3−5 h. BL21 cells were harvested by centrifugation (3000*g*, 10 min, 4 °C). BL21-GST-NCAM cyt cell pellets were resuspended in PBS containing protease inhibitor cocktail, as well as PMSF (Sigma), DNAase (10 μg/mL), and RNAse (10 μg/μL). GST-PLCγ-BL21 cells were resuspended in lysis buffer containing 10 mM TRIS (pH 8.0), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, protease inhibitor cocktail, PMSF (Sigma), DNAse (10 μg/mL) and RNAse (10 μg/mL), and 0.1% Triton X-100. GST-LANP-BL21 cell pellets were resuspendend in a lysis buffer containing 50 mM Tris (pH 8.0), 200 mM NaCl, 1 mM EDTA, 5 mM DTT, PMSF (Sigma), DNAse (10 μg/mL), and RNAse (10 μg/mL). Cells were lysed by five freezing/thawing cycles with liquid nitrogen.

Ligand Affinity Chromatography with His-tag NCAM cyt 140 and NCAM cyt 180. Empty vector-encoded control protein, purified NCAM cyt 140, and purified NCAM cyt 180 were immobilized to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Amersham). Cytosolic- or cytoskeletal-enriched protein fractions of rat brain were prepared and passed first over an empty column (ethanolamine-blocked precolumn) and then over the NCAM cyt 140 or NCAM cyt 180 column (4 °C, overnight). As a control, a portion of each protein fraction was also passed over the control protein column. All columns were washed with buffer containing 75 mM KCl, 10 mM Tris/HCl (pH 7.4), 2 mM $MgCl_2$, and 2 mM EGTA and then eluted with 250 or 500 mM KCl.

Purification of GST Fusion Proteins. The GST fusion proteins or GST alone was purified from bacterial lysate by affinity chromatography using glutathione Sepharose 4B (Amersham Pharmacia Biotech).

The glutathione Sepharose was equilibrated with the appropriate lysis buffer and the GST-NCAM cyt fragment-, the GST-PLCγ fragment-, or the GST-LANP-containing bacterial lysate was incubated with the Sepharose for 1 h at 4 °C. (For efficient coupling of GST-LANP, it is necessary to add 2−3% Triton X-100 to the bacterial lysate). Afterward the protein-coupled Sepharose was washed five times with PBS (GST-NCAM cyt fragments), with 50 mM Tris, pH 8.0 (GST-PLCγ), or with 50 mM Tris, 200 mM NaCl, 1 mM EDTA, and 1% Triton X-100 (GST-LANP).

GST Pull-Down Assay with GST-NCAM cyt Fragments. One hundred fifty microliters of GST-NCAM-fragment-coupled Sepharose was incubated with 3 mg of the cytosolic- or cytoskeleton-enriched protein fraction of rat brain (2 mg/mL) for 1 h at 4 °C, after equilibration of the Sepharose with the buffer intended for resolution of the protein fraction. The buffer contained an additional 75 mM KCl so that the final KCl concentration in the pull-down experiments was 150 mM. Afterward, the Sepharose was washed six times with 5 bed volumes of homogenization or cytoskeleton buffer (150 mM KCl). Bound protein and GST-NCAM fragment was removed from the matrix by boiling with 60 μL of 2× sample buffer. Fifteen microliter aliquots were analyzed by SDS−PAGE and Western blot for the presence of NCAM-fragment binding proteins.

GST Pull-Down Assays with GST-LANP or GST-PLCγ. GST Pull-Down Assay Using Membrane Protein-Enriched Fraction of Rat Brain. Seventy-five microliters of GST-protein-coupled Sepharose was equilibrated with solubilization buffer (10 mM Tris/HCl (pH 7.4), 2 mM EGTA, 2 mM $MgCl_2$, 75 mM KCl, protease inhibitor cocktail, PMSF (Sigma), and 0.5% Triton X-100 at pH 7.4) and incubated with 1 mg of the membrane protein-enriched fraction of rat brain (2 mg/mL) for 1 h at 4 °C. The Sepharose was then washed six times with 5 bed volumes of solubilization buffer. Bound proteins were eluted by boiling with 45 μL of 2× sample buffer. Twenty microliter aliquots were analyzed by SDS−PAGE and immunoblotting for the presence of NCAM.

GST Pull-Down Assay Using Overexpressed, Purified His-Tagged NCAM cyt Fusion Protein. Seventy-five microliters of GST-protein-coupled Sepharose was equilibrated with PBS containing 0.5% Triton X-100, then incubated for 1 h at 4 °C with 5 μg of purified His-tagged NCAM cyt protein or control protein (His-tag alone) dissolved in 400 μL of PBS/0.5% Triton X-100. After centrifugation, the Sepharose was washed five times with 1 mL of PBS/0.5% Triton X-100. Bound protein was removed by boiling with 45 μL of 2× sample buffer. Twenty microliter aliquots were analyzed by SDS−PAGE and immunoblotting for the presence of NCAM cyt.

Coimmunoprecipitation. Immunoprecipitations of NCAM were performed using protein G Sepharose (Fast Flow, Pharmacia). The Sepharose was washed four times with PBS. In the last washing step, the Sepharose was saturated with 0.1% ovalbumin in PBS for 20 min at 4 °C. Afterward, 100 μL of the Sepharose was incubated with 50 μg of specific antibody (mab H28 or mouse-antirabbit antibody) in PBS for 1 h at 4 °C. Unbound antibodies were then removed by washing the Sepharose two times with immunoprecipitation buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.65 mM PMSF, protease inhibitor, and 0.2% Triton X-100).

For preprecipitation, 100 μL of protein G Sepharose (without antibodies) was incubated with 5 mL of brain solubilizate of a 14 day old mouse containing 2 mg/mL protein for 30 min at 4 °C. After centrifugation (2 min,

2000*g*), the supernatants of solubilizates were used for the following precipitations.

For precipitation, 5 mL of supernatant from the preprecipitation (2 mg/mL) was added to 100 μL of antibody-coupled protein G Sepharose and incubated for at least 2 h at 4 °C on a rotator. After centrifugation and removal of supernatant, the Sepharose was washed six times with immunoprecipitation buffer before elution of bound proteins with 80 μL of 2× sample buffer. Fifteen microliter aliquots were analyzed by SDS−PAGE and immunoblotting for the presence of NCAM and coimmunoprecipitated proteins.

Analytical Procedures. Protein was determined in 96-well ELISA plates using 200 μL of bicinchonic acid protein reagent (Pierce) and a 50 μL sample. Plates were evaluated in a 96-well ELISA reader (Spectra) at 570 nm.

Preparation of Cytosolic- and Cytoskeleton-Enriched Protein Fractions of Rat Brain. Preparation was performed as described (*19*). In brief, brains of adult Wistar rats were homogenized in buffer containing 75 mM KCl, 10 mM Tris, 2 mM $MgCl_2$, 2 mM EGTA, and protease inhibitor cocktail, as well as PMSF (Sigma), at pH 7.4. The homogenate was centrifuged (30 000*g*, 1 h, 4 °C), and the supernatant represented enriched cytosolic proteins.

The remaining pellet was solubilized in buffer containing 75 mM KCl, 10 mM Tris, 2 mM $MgCl_2$, 2 mM EGTA, 0.5% Triton X-100, and protease inhibitor at pH 7.4 and stirred on ice for 1 h. Solubilizate was centrifuged (100 000*g*, 1 h, 4 °C). The pellet was once again homogenized in cytoskeleton buffer containing 75 mM KCl, 10 mM Tris, 0.2 mM CaCl, 0.2 mM ATP, and protease inhibitor cocktail at pH 7.4 and stirred on ice for another 1 h. The homogenate was again centrifuged (100 000*g*, 1 h, 4 °C), and the resulting supernatant was taken as the cytoskeleton-enriched protein fraction.

Immunoblotting. Samples were separated on SDS−polyacrylamide gels (BioRad) and transferred to nitrocellulose filters. The blots were blocked with 10% fat-free dry milk powder in PBS, incubated with the respective primary antibodies, washed with PBS, and incubated with the appropriate secondary antibodies, conjugated with peroxidase. After washing, proteins were detected by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions and visualized by exposing the blots to a Fuji imager system (LAS) for time periods between 10 and 1200 s.

In Situ Digestion with Trypsin and Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry. Following electrophoresis, proteins were stained with colloidal Coomassie brilliant blue (Pierce). The spots of interest were cut from the gel, cut into small pieces, destained with 50% (v/v) ethanol in water, washed extensively with water to remove ethanol, and dried in a vacuum centrifuge. Trypsin (Trypsin, Sequencing Grade, Sigma)-containing buffer (trypsin dissolved at 5 μg/mL in 100 mM Tris-HCI, pH 8.5) was added to the gel pieces. Protein digestion was performed overnight at 37 °C. Digestion was stopped by addition of 2.5% TFA.

Supernatant and gel pieces were separated by centrifugation. Peptides were extracted and purified from supernatant by absorption onto a stationary reversed-phase matrix in pipet tips (ZipTipC18, Millipore) according to the instructions of the manufacturer. After five washes with 0.1% TFA in water (v/v), bound peptides were eluted with 10 μL of saturated matrix-solution (α-cyano-4-hydroxycinnamic acid, Sigma) in 0.1% TFA (v/v) in 50% (v/v) acetonitrile/water. One microliter of each eluted sample was applied to the target and allowed to dry at room temperature. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out on a Bruker Biflex instrument (Bruker). Ionization was accomplished with a 337 nm beam from a nitrogen laser. Mass spectra were recorded in the positive ion mode using the reflector. The masses of peptides were determined using adrenocorticotropic hormone fragment 18-39 (Sigma) and angiotensin II (Sigma) as internal standards.

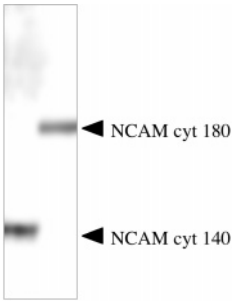


FIGURE 1: Expression of the cytosolic domains of NCAM 140 and NCAM 180 in *E. coli.* Immunoaffinity-purified NCAM cyt 140 and NCAM cyt 180 were separated by SDS−PAGE and visualized by Coomassie staining.

RESULTS

Identification of Novel Intracellular Binding Partners of NCAM. Recently, we identified novel intracellular binding partners of NCAM. Among them were several cytoskeleton proteins such as MAP 1A, α- and β-tubulin, β-actin, and α-actinin (*19*). In this study, we investigated whether other intracellular molecules bind to NCAM and might be involved in its various functions and signal transduction pathways. We approached this by the well-established method of ligand affinity chromatography and MALDI-TOF-MS. We expressed the cytoplasmic domains of NCAM 140, NCAM 180, and a control protein (empty vector-encoded protein) as His-tagged fusion proteins and purified them by immunoaffinity chromatography (NCAM cyt) or by Ni−NTA-affinity chromatography (control protein) as described (*19*). In Figure 1, purified cytoplasmic domains of NCAM 140 and NCAM 180 are presented in a Coomassie-stained SDS-gel. Proteins were immobilized on CNBr-activated Sepharose columns. Cytosolic- and cytoskeleton-enriched protein fractions of rat brain were passed over these columns. Each column set contained an empty precolumn blocked with ethanolamine, for removing components showing unspecific binding to the matrix. Bound proteins were eluted with increasing KCl concentrations and analyzed by SDS−PAGE (Figure 2A,B). Proteins eluted from the NCAM cyt 180 column marked with arrows in Figure 2A) were cut from 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 (see Table 1) by peptide mass fingerprinting.

To reconfirm the presence of these proteins in the eluates and to identify those proteins for which binding is isoform-

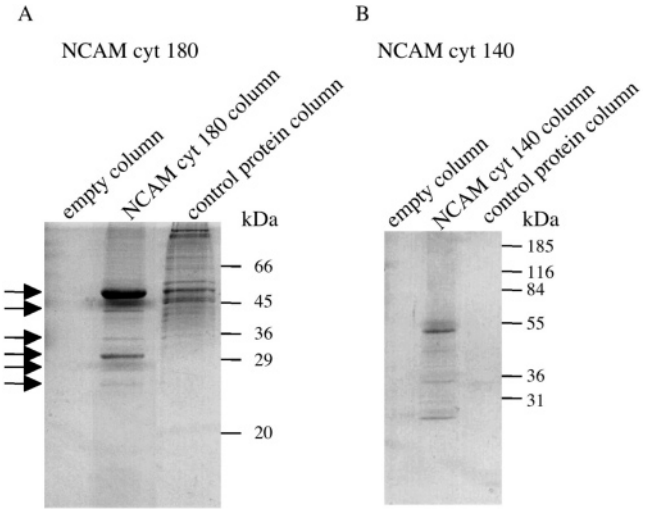


FIGURE 2: Ligand affinity chromatography of cytosolic-enriched protein fraction. Eluates of the empty column, the NCAM cyt 140 and NCAM cyt 180 columns, or the control protein column were separated by SDS−PAGE and stained with Coomassie Blue. Panel A shows eluates of the empty column/NCAM cyt 180 column set and the control protein column. Arrows indicate proteins in the NCAM cyt 180 column eluate that were excised from the gel and analyzed by MALDI-TOF-MS. Panel B shows eluates of the empty column/NCAM cyt 140 column set and the control protein column.

specific, we analyzed all the eluates of each affinity chromatography (empty column, NCAM cyt 140 column, or NCAM cyt 180 column and control protein column) by Western blotting. Furthermore, we tested the presence of three other proteins in the eluates: phospholipase Cγ (PLCγ), which is implicated in NCAM 140-mediated signal transduction and the serine/threonine phosphatases PP1 and PP2A, since LANP is known to be a regulator of these phosphatases (*23*, *24*). We identified syndapin and LANP in both NCAM cyt 140 and NCAM cyt 180 column eluates, whereas TOAD-64 was only present in the NCAM cyt 180 column eluate (Figure 3). PLCγ, PP1, and PP2A were also identified in the eluates of both NCAM cyt columns (Figure 3). None of these proteins could be detected in the eluates of the control protein or the empty column.

Mapping the Binding Domains of the Novel Intracellular Interacting Proteins to the Neural Cell Adhesion Molecule. To localize the binding of PLCγ, LANP, TOAD-64, syndapin, PP1, or PP2A to NCAM in more detail, we expressed five fragments of the intracellular domain of NCAM as GST fusion proteins, which comprise different parts of the whole cytoplasmic domain of rat NCAM 140 and NCAM 180 (Figure 4). All fragments were purified by affinity chromatography using glutathione Sepharose. Figure 5A shows all purified NCAM cyt fragments and GST on Ponceau Red-stained nitrocellulose. The Western blot analysis revealed that the monoclonal anti-NCAM antibody 5B8 binds only to NCAM cyt fragment 5, which represents the C-terminal part of NCAM 140 and NCAM 180 (Figure 5B). 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- and cytoskeleton-enriched protein fractions of rat brain. Figure 5C shows the results of all pull-down experiments. PLCγ, TOAD-64, syndapin, PP1, and PP2A interact with NCAM cyt fragment 1, which represents the membrane adjacent 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). Since we recently identified α- and β-tubulin as binding partners for NCAM 140 and NCAM 180 and the rho A binding kinase α (ROK α) as a binding partner for NCAM 180 (*19*), we performed additional pull-down experiments with these proteins. The results showed that α- and β-tubulin interact with the membrane-adjacent NCAM cyt fragment 1, whereas ROK α binds to NCAM cyt fragment 3, which is only present in NCAM 180.

Detailed Analysis of the Interaction of NCAM with PLCγ. To verify the interaction of PLCγ with NCAM in a more in vivo environment, we performed a coimmunoprecipitation with a fresh mouse brain preparation from a 14 day-old mouse and the mouse-NCAM-specific monoclonal antibody H28. As negative controls, a precipitation with a mouse-antirabbit antibody (MAR) and the mouse brain solubilizate was performed with protein G Sepharose. The resulting precipitates were separated by SDS−PAGE and analyzed after Western blotting with PLCγ- and NCAM-specific antibodies (Figure 6). Our results demonstrate that PLCγ was coimmunoprecipitated with NCAM from mouse brain.

For greater insight into the binding of PLCγ to NCAM, we performed GST-pull-down assays with GST-PLCγ fragments derived from human PLCγ. These fragments comprise different structural and functional domains of PLCγ: the PH-domain at the N-terminus of the protein, the N-terminal SH2-domain, the C-terminal SH2-domain, and both SH2-domains together in one construct. To confirm the interaction with NCAM, we used a membrane-enriched protein fraction of rat brain in the pull-down assays. The results of the PLCγ-pull-down assays are shown in Figure 7A. Both isoforms of NCAM could be pulled down with the PH-domain and the C-terminal SH2 domain of PLCγ, but not with the N-terminal SH2 domain. There was no unspecific binding of NCAM to GST.

In the next step, we investigated whether the interaction of the PLCγ domains with NCAM is direct or mediated by intermediate proteins. Therefore we performed the pull-down with purified recombinant NCAM cyt fusion proteins containing a histidine-tag (these were also used for the ligand affinity chromatography) (Figure 7B). As a negative control, a his-tagged fusion protein of the cytosolic domain of CEACAM short, another transmembrane protein of the immunoglobulin superfamily, was used. We demonstrated that the cytosolic domains of NCAM 140 and NCAM 180 directly interact with the PH-domain and the C-terminal SH2 domain of PLCγ, thereby reconfirming the earlier results. Both isoforms have a stronger affinity to the C-SH2 domain than to the PH-domain, as more NCAM-protein is detectable in the C-SH2-pull-down lane. A small amount of NCAM cyt 180 also interacts with the N-terminal SH2 domain. Little unspecific binding of NCAM cyt 180 to GST (representing 10% of the signal) was observed. There was no interaction between the cytosolic domain of CEACAM short and the PLCγ fragments (Figure 7B).

LANP Interacts Directly with NCAM 140 and NCAM 180. To determine whether LANP also directly associates with the cytosolic domains of NCAM 140 or NCAM 180, we performed a GST-LANP pull-down assay with the purified

Table 1: Summary of All Data Obtained by Peptide Mass Fingerprinting and Western Blot Analysis

protein	MW (kDa)	MALDI NCAM cyt 180	IB NCAM cyt 180 ligand affinity	IB NCAM cyt 140 ligand affinity	function
PLCγ	148	−	+	+	growth-factor-induced cell motility, growth and development
LANP	29	+	+	+	plays role in differentiation of cerebellar neurons, inhibitor of PP2A; stimulator of PP1
TOAD-64	63	+	+	−	plays role in axon growth, signal transduction
syndapin	51	+	+	+	plays role in clathrin-mediated endocytosis of synaptic vesicles; cytoskeletal dynamics
PP1	36	−	+	+	major mammalian serine/threonine protein phosphatase; plays role in synaptic plasticity; controls cell cycle and apoptosis
PP2A	36	−	+	+	major mammalian serine/threonine protein phosphatase; controls cell cycle and apoptosis

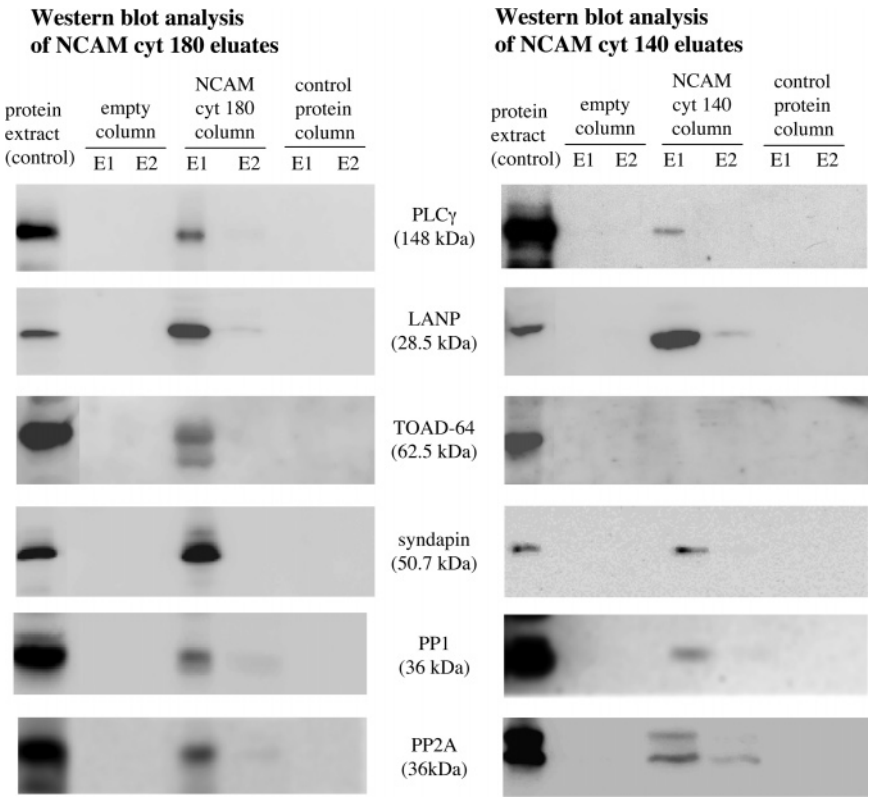


FIGURE 3: Western blot analysis of ligand affinity chromatography eluates. Antibodies directed to proteins identified by MALDI-TOF-MS (see Table 1) and to PLCγ, PP1, and PP2A were used in Western blot analysis. E1 represents elution with 250 mM KCl and E2 elution with 500 mM KCl. For control, 30 μg of the protein extract was used (control). The left-hand panel shows Western blot analysis of NCAM cyt 180 eluates, and the right-hand panel shows Western blot analysis of NCAM cyt 140 eluates.

recombinant cytoplasmic domains of NCAM. Figure 8A shows affinity purified GST-LANP and GST stained with Coomassie Blue. Five micrograms of purified NCAM cyt 140 or NCAM cyt 180 was incubated with GST-LANP- and GST-coupled Sepharose followed by elution of bound protein. As shown in Figure 8B, LANP bound to both NCAM 140 and NCAM 180.

DISCUSSION

Recently, we presented evidence that the cytoskeleton proteins α-actinin and α- and β-tubulin are associated with both NCAM 140 and NCAM 180, whereas the microtubuli-associated protein MAP 1A, β-actin, tropomyosin, and the rhoA binding kinase α preferentially bind to NCAM 180 (*19*).

In the current study, we identified several more interacting partners of the cytoplasmic domains of NCAM 140 and NCAM 180 by ligand affinity chromatography. These were cytoplasmic proteins, which could play a role in NCAM-mediated signal transduction.

The cytoplasmic domains of both transmembrane isoforms were expressed as histidine-tag fusion proteins, which were purified by immunoaffinity chromatography, then used for ligand affinity chromatography after coupling to CNBr-activated Sepharose.

By MALDI-TOF-MS and subsequent peptide mass fingerprinting, we identified LANP, TOAD-64, and syndapin in the eluates of the NCAM cyt 180 column. These results were confirmed by testing the same eluates in Western blot analysis, using the respective antibodies. Furthermore, we

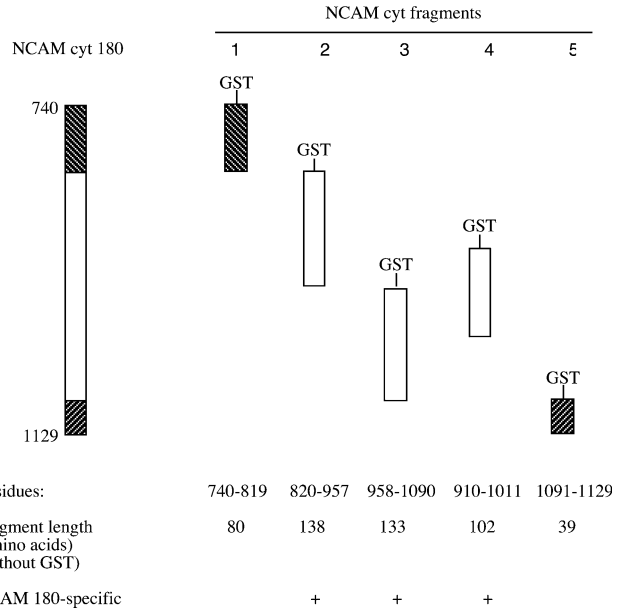


FIGURE 4: Scheme of the GST-NCAM cyt fragments. The rat NCAM cyt fragments consist of the following amino acid residues of rat NCAM 180: 1 (Δ740−819); 2 (Δ820−957); 3 (Δ958−1090); 4(Δ910−1011); 5(Δ1091−1129). Note that fragment 4 represents an overlapping sequence of fragments 2 and 3. The fragments 1 and 5 together represent the entire sequence of NCAM cyt 140.

could also identify LANP and syndapin, but not TOAD-64, in the eluates of the NCAM cyt 140 column. In addition, Western blot analysis revealed that PLCγ and the protein phosphatases PP1 and PP2A associate with both transmembrane isoforms of NCAM. In pull-down experiments using different fragments of the cytosolic domain of NCAM, we were able to identify specific NCAM sequences responsible for the binding of all these novel binding partners.

The binding of the leucine-rich acidic nuclear protein (LANP) to NCAM 140 and NCAM 180 was shown to be direct and is restricted to the last C-terminal 39 amino acids within the cytoplasmic domains of both NCAM isoforms, as demonstrated by GST-pull down experiments.

LANP, also known as pp32 (phosphoprotein with a molecular weight of 32 kDa) is a member of the leucine-rich protein family and was first isolated from rat cerebellum (*25*−*27*). The expression of LANP is up-regulated in the central nervous system during the early stage of postnatal development. The N-terminal domain of the protein contains many leucine-rich repeats, which are supposed to mediate protein−protein interactions. A cluster of acidic amino acids with a putative nuclear localization signal characterizes the C-terminal domain.

With respect to its temporal and spatial expression, as well as its structural characteristics, LANP is thought to play a role in signal transduction, leading to differentiation of cerebellar neurons. Recently Opal et al. showed that in undifferentiated neuronal cells LANP is predominantly present in the nucleus, but during neuritogenesis, it translocates into cytoplasm. 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 (*28*). Since LANP translocates from the nucleus into the cytoplasm during neuritogenesis, it is conceivable that it interacts

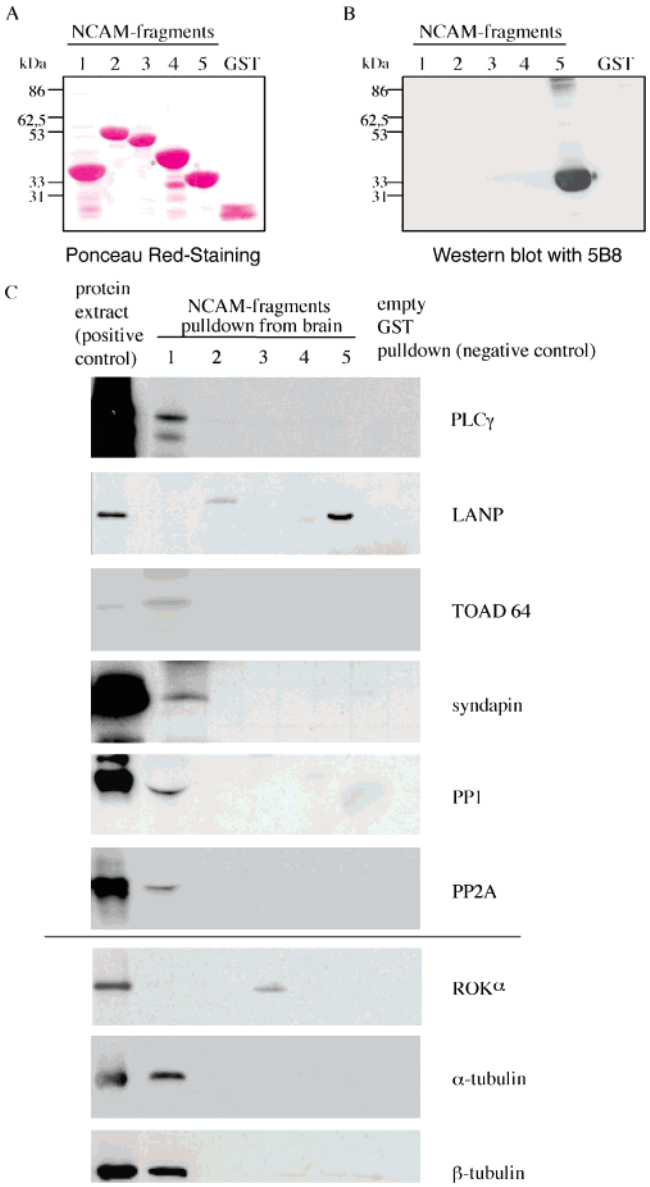


FIGURE 5: GST pull-down assay with GST-NCAM cyt fragments. Panel A shows purified GST-NCAM cyt fragments and GST. Affinity-purified GST-NCAM cyt fragments and GST were separated by SDS−PAGE, blotted to nitrocellulose, and visualized by Ponceau Red staining. Molecular weights of the NCAM cyt fragments and GST were as follows: 1, 35.7 kDa; 2, 40.5 kDa; 3, 39.8 kDa; 4, 36.6 kDa; 5, 31.2 kDa; GST, 27 kDa. Panel B shows Western blot analysis of the purified GST-NCAM cyt fragments using the monoclonal anti-NCAM antibody 5B8. Panel C shows pull-down of the novel binding partners with different NCAM cyt fragments. Protein extracts of rat brain were incubated with the GST-NCAM cyt fragment-coupled and GST-coupled Sepharose. Eluted proteins of the six pull-down probes were separated by SDS−PAGE and tested by immunoblotting for the presence of the binding partners using specific antibodies (note that the previously identified NCAM-binding proteins ROK α and α- and β-tubulin (19) were also included in the pull-down experiments with the NCAM fragments.

directly with membrane-bound NCAM in native cells. LANP may not only affect neuritogenesis by its interaction with MAP 1B, but may also be a novel signaling molecule in a signaling pathway mediated by NCAM.

We recently identified MAP 1A as a binding partner of NCAM 180. Since LANP has been demonstrated to interact not only with MAP 1B (*28*) but also with other members of

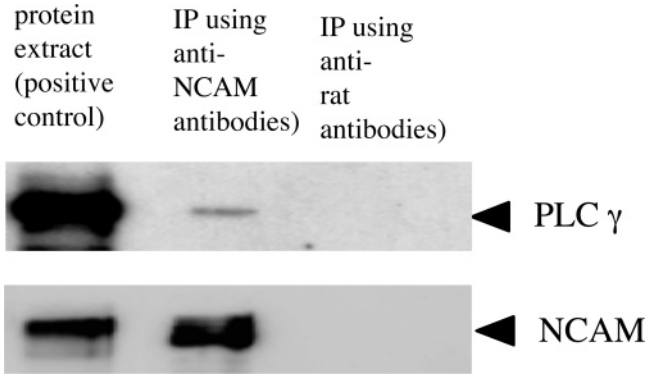


FIGURE 6: Coimmunoprecipitation of PLCγ with NCAM from mouse brain solubilizate. Brain of a 14 day old mouse was solubilized and used for coimmunoprecipitation. NCAM was precipitated with the monoclonal anti-mouse-NCAM antibody H28. As negative controls a precipitation with a mouse-antirabbit (MAR) antibody was performed, and the solubilizate was incubated with protein G Sepharose alone. Precipitates were subjected to SDS−PAGE and immunoblotted using anti-PLCγ or NCAM antibodies (IP = immunoprecipitation).

the microtubuli-associated protein family, such as tau, MAP 2, or MAP 4 (*29, 30*), it is also possible that LANP is a linker protein between NCAM 180 and MAP 1A. The sequence homology between MAP 1A and MAP 1B is very high (80%), especially within its light chains. Whether LANP interacts with MAP 1A remains to be investigated.

Interestingly, LANP has also been described as a heat-stable inhibitor of the protein phosphatase PP2A (*24*). It positively regulates the activation of PP1 (*23*). PP1 and PP2A, the two major mammalian serine/threonine phosphatases, have also been identified in this work as binding partners of NCAM 140 and NCAM 180. Nearly 100 mammalian proteins are known so far to bind directly or indirectly to the catalytic domain of PP1 or PP2A (*31*). The interaction of PP1 and PP2A with the cytoplasmic domains of NCAM indicates a possible dephosphorylation of serine and threonine residues of NCAM 140 and NCAM 180 by these phosphatases. The cytosolic domain of NCAM 140 contains five putative serine and six putative threonine phosphorylation sites, while NCAM 180 contains 28 putative serine and 21 putative threonine phosphorylation sites. Early studies demonstrated that several threonine and serine residues of NCAM 140 and NCAM 180 are phosphorylated by two kinases, casein kinase I (CK1) and glycogen synthase kinase 3 (GSK-3) (*17, 32*). Moreover, Mackie et al. showed that purified chicken NCAM phosphorylated by GSK-3 in vitro 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.

Besides phosphorylation of NCAM by CK I or GSK-3, NCAM might also be phosphorylated by the serine/threonine kinase ROK α (rho A binding kinase α), a kinase that we have recently identified as a binding partner of NCAM 180 (*19*). In this work, we were able to show that ROK α binds to the NCAM cyt fragment 3, which represents the C-terminal part of 133 amino acids within the NCAM 180-specific insert. This binding localization is consistent with

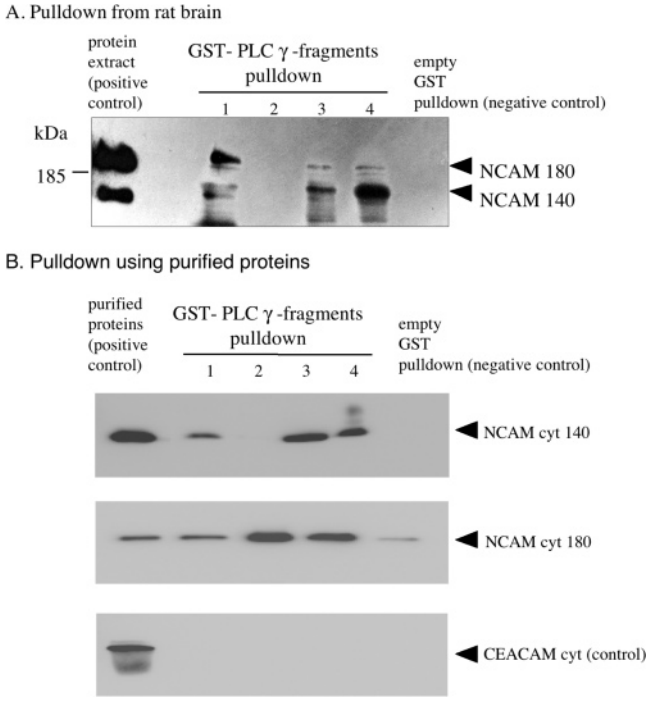


FIGURE 7: Panel A shows a pull-down assay of NCAM 140 and NCAM 180 from a membrane-enriched protein fraction of rat brain using GST-PLCγ fragments. A membrane-enriched protein fraction of rat brain was prepared and incubated with glutathione Sepharose coupled with different PLCγ-GST fragments. Twenty microliter aliquots of the pull-down probes and 4 μg of the membrane-enriched protein fraction were analyzed by SDS−PAGE and Western blotting using the monoclonal anti-NCAM antibody 5B8 (control, membrane-enriched protein fraction of rat brain; GST-PLCγ fragments, 1 GST-PLCγ-PH, 2 GST-PLCγ-N-SH2, 3 GST-PLCγ-C-SH2, 4 GST-PLCγ-N-SH2-C-SH2; GST-pd GST-pull-down). Panel B shows a pull-down assay of purified his-tag NCAM cyt 140 and NCAM cyt 180 with GST-PLCγ fragments. Five micrograms of purified his-tag NCAM cyt 140 or 180 or his-tag CEACAM short were incubated with GST-PLCγ fragment-coupled or GST-coupled Sepharose. Bound proteins were separated by SDS−PAGE and immunoblotted with anti-NCAM antibody 5B8 or anti-CEACAM antibody. As control, 340 ng of recombinant NCAM cyt 140 or NCAM cyt 180 or CEACAM cyt were separated by SDS−PAGE (GST-pd = GST-pull-down; IB = immunoblot).

our ligand affinity study, showing that ROK α interacts only with NCAM 180 (*19*). Within the ROK α binding sequence of the NCAM cyt fragment 3 of NCAM 180, there are nine predicted serine and six predicted threonine phosphorylation sites, which could be phosphorylated by ROK α.

Arimura et al. suggest that the phosphorylation of the collapsine response mediator protein 2 (CRMP-2) by ROK α is involved in the ROK α-induced growth cone collapse (*33*). Interestingly, CRMP-2 belongs, together with turned on after division-64 (TOAD-64), to the TUC (TOAD/Ulip/CRMP) family of proteins. Proteins of this family are all candidates for signaling in growth cones of neurons and are required for the growth cone collapsing activity of collapsin-1 (*34*). TOAD-64, which we have identified as an NCAM 180-specific interaction partner, is highly up-regulated during early postnatal neuronal development and has its highest expression rate during neurite outgrowth (*35*). TOAD-64 is a cytoplasmic protein but is noncovalently associated with membranes (*36*). The localization of NCAM in lamellipodia and filopodia of growth cones together with its association with membranes makes an interaction with NCAM possible.

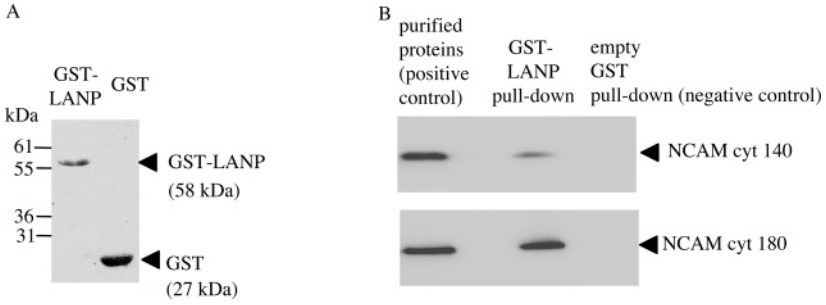


FIGURE 8: Pull-down assay of purified his-tag NCAM cyt 140 and NCAM cyt 180 with GST-LANP. Panel A shows purified GST-LANP and GST. Affinity-purified GST-LANP and GST were separated by SDS−PAGE and stained with Coomassie Blue. GST-LANP shows an apparent molecular mass of 56 kDa and has a theoretical molecular mass of 59 kDa. Panel B shows the pull-down assay of his-tag NCAM cyt 140 and NCAM cyt 180 with GST-LANP. Five micrograms of purified his-tag NCAM cyt 140 or 180 were incubated with GST-LANP or GST-control. Bound proteins were separated by SDS−PAGE and immunoblotted with anti-NCAM antibody 5B8. As a control for Western blotting, 340 ng of recombinant NCAM cyt 140 or 180 were used.

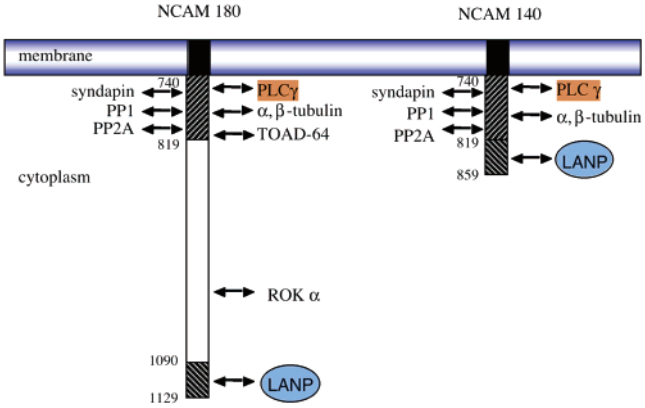
Although NCAM 140 is the active isoform for stimulating neurite outgrowth, TOAD-64 only associates with the cytoplasmic domain of NCAM 180. TOAD-64 binds to the first N-terminal NCAM cyt fragment 1, which is also present in NCAM 140, but TOAD-64 could not be identified in the eluate of the NCAM cyt 140 column in the ligand affinity chromatography. One explanation could be that the presence of the additional insert of 267 amino acids in NCAM cyt 180 and possibly in associated proteins alters the conformation of the membrane-adjacent domain, which then allows an interaction with TOAD-64.

Syndapin (synaptic dynamin-associated protein) was shown to interact with the N-terminal 80 amino acids of the cytoplasmic domain of NCAM 140 and NCAM 180. It is a 52 kDa protein highly enriched in brain (*37*) and contains one src homology domain 3 (SH3) at the C-terminus and two predicted coiled-coil stretches. Qualman and Kelly showed that syndapin I associates with the GTPase dynamin I and plays a role in clathrin-mediated endocytosis of synaptic vesicles (*38*). Many cell surface receptors are internalized by clathrin-mediated endocytosis, and these are thought to include NCAM. Minana et al. 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 (*39*). The interaction of syndapin with NCAM also indicates that NCAM might be internalized by clathrin-mediated endocytosis.

NCAM stimulates PLCγ indirectly via the FGF-receptor in response to extracellular NCAM−NCAM interaction (*11*). In the current work, we demonstrated that NCAM 140 and NCAM 180 interact directly with the PH-domain and the C-terminal SH2 domain of PLCγ. The interaction was confirmed in coimmunoprecipitation studies. Furthermore, the binding of PLCγ to NCAM involved only the first N-terminal 80 amino acids of the cytoplasmic domain of NCAM 140 and NCAM 180. However, our experiments suggest stronger binding of PLCγ to NCAM 140 than to NCAM 180.

SH2 domains bind to specific phosphotyrosine-containing motifs. The only cytoplasmic tyrosine residue of NCAM is present in the sequence DITCYFL proximal to the membrane. Distel et al. first demonstrated that this tyrosine residue could be phosphorylated in NCAM 180 by an unknown kinase (*18*). Perhaps the C-SH2 domain of PLCγ is able to bind this specific motif. However, a tyrosine phosphorylation of NCAM 140 has not yet been described. The pleckstrin homology (PH) domain is responsible for targeting PLCγ to the membrane, and it binds to phosphatidylinositol 3,4,5-triphosphate (PIP_3) (*40*). Therefore NCAM could also be responsible for recruiting PLCγ to the plasma membrane.

Scheme 1



PLCγ, PP1, PP2A, syndapin, TOAD-64, and α- and β-tubulin have all been found to bind to the first N-terminal NCAM cyt fragment (80 residues), and only PLCγ could be demonstrated to bind directly. It is not known whether the other six binding partners associate directly or indirectly with this fragment. It would be interesting to know whether these proteins (or which of these proteins) are able to bind at the same time to NCAM or whether they compete for the same binding sites. Not only the binding site but also the spatial distribution of one NCAM-interacting protein could prevent another one from binding to NCAM. However, LANP and ROK α have been shown to be the only binding partners of the C-terminal NCAM cyt fragment 5 (LANP) and the NCAM 180-specific cyt fragment 3 (ROK α), so these proteins are allowed to bind at the same time together with other interaction partners. All proteins identified as novel interaction partners of NCAM 140 and NCAM 180, together with their binding localization, are shown in Scheme 1.

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